PART I

INTRODUCTION

CHAPTER 1

Introduction to Toxicology

ERNEST HODGSON

Since the publication of the 3rd edition of this textbook (2004) major changes have been initiated in toxicology as the tools of molecular biology, genomics, proteomics, metabolomics, bioinformatics, and systems biology are increasingly brought to bear on the critical areas of mode of action, toxicity testing, and risk analysis. Chapter 2 provides information on new methodology and Part VIII—New Approaches in Toxicology is composed of two chapters of commentary on the current and expected impact of these new methods. While the traditional aspects and subdisciplines of toxicology, as outlined below, are still active and viable, during the next few years all are likely to be impacted and their development accelerated by these new approaches.

1.1 DEFINITION AND SCOPE

Toxicology can be defined as that branch of science that deals with poisons, and a poison can be defined as any substance that causes a harmful effect when administered, either by accident or by design, to a living organism. By convention, toxicology also includes the study of harmful effects caused by physical phenomena, such as radiation of various kinds, noise, and so on. In practice, however, many complications exist beyond these simple definitions, both in bringing more precise definition to the meaning of poison and to the measurement of toxic effects. Broader definitions of toxicology, such as "the study of the detection, occurrence, properties, effects, and regulation of toxic substances," although more descriptive, do not resolve the difficulties. Toxicity itself can rarely, if ever, be defined as a single molecular event, but is, rather, a cascade of events starting with exposure, proceeding through distribution and metabolism, and ending with interaction with cellular macromolecules (usually DNA or protein) and the expression of a toxic end point (Figure 1.1). This sequence may be mitigated by excretion and repair. It is to the complications, and to the science behind them and their resolution, that this textbook is dedicated, particular to the *how* and *why* certain substances cause disruptions in biologic systems that result in toxic effects. Taken together, these

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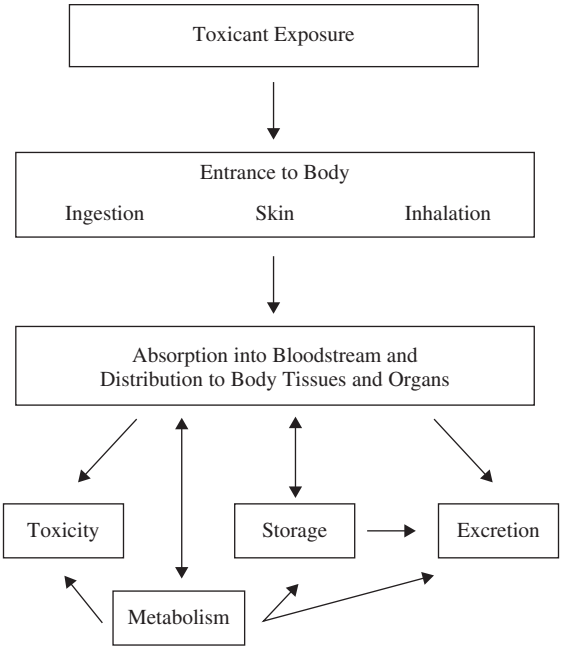

Figure 1.1 Fate and effect of toxicants in the body.

difficulties and their resolution circumscribe the perimeter of the science of toxicology.

The study of toxicology serves society in many ways, not only to protect humans and the environment from the deleterious effects of toxicants, but also to facilitate the development of more selective toxicants such as anticancer and other clinical drugs, pesticides, and so forth.

Poison is a quantitative concept, almost any substance being harmful at some doses but, at the same time, being without harmful effect at some lower dose. Between these two limits, there is a range of possible effects, from subtle long-term chronic toxicity to immediate lethality. Vinyl chloride may be taken as an example. It is a potent hepatotoxicant at high doses, a carcinogen with a long latent period at lower doses, and apparently without effect at very low doses. Clinical drugs are even more poignant examples because, although therapeutic and highly beneficial at some doses, they are not without deleterious side effects and may be lethal at higher doses. Aspirin (acetylsalicylic acid), for example, is a relatively safe drug at recommended doses and is taken by millions of people worldwide. At the same time, chronic use can cause deleterious effects on the gastric mucosa, and it is fatal at a dose of about 0.2–0.5 g/kg. Approximately 15% of reported accidental deaths from poisoning in children result from ingestion of salicylates, particularly aspirin.

The importance of dose is well illustrated by metals that are essential in the diet but are toxic at higher doses. Thus, iron, copper, magnesium, cobalt, manganese, and zinc can be present in the diet at too low a level (deficiency), at an appropriate level (maintenance), or at too high a level (toxic). The question of dose–response relationships is fundamental to toxicology (see Section 1.4).

The definition of a poison, or toxicant, also involves a qualitative biological aspect because a compound, toxic to one species or genetic strain, may be relatively harmless to another. For example, carbon tetrachloride, a potent hepatotoxicant in many species, is relatively harmless to the chicken. Certain strains of rabbit can eat *Belladonna* with impunity while others cannot. Compounds may be toxic under some circumstances but not others or, perhaps, toxic in combination with another compound but nontoxic alone. The methylenedioxyphenyl insecticide synergists, such as piperonyl butoxide, are of low toxicity to both insects and mammals when administered alone, but are, by virtue of their ability to inhibit xenobiotic-metabolizing enzymes, capable of causing dramatic increases in the toxicity of other compounds.

The measurement of toxicity is also complex. Toxicity may be acute or chronic, and may vary from one organ to another as well as with age, genetics, gender, diet, physiological condition, or the health status of the organism. As opposed to experimental animals, which are highly inbred, genetic variation is a most important factor in human toxicity since the human population is highly outbred and shows extensive genetic variation. Even the simplest measure of toxicity, the LD_{50} (lethal dose; the dose required to kill 50% of a population under stated conditions) is highly dependent on the extent to which the above variables are controlled. LD_{50} values, as a result, vary markedly from one laboratory to another.

Exposure of humans and other organisms to toxicants may result from many activities: intentional ingestion, occupational exposure, environmental exposure, as well as accidental and intentional (suicidal or homicidal) poisoning. The toxicity of a particular compound may vary with the portal of entry into the body, whether through the alimentary canal, the lungs, or the skin. Experimental methods of administration such as injection may also give highly variable results; thus, the toxicity from intravenous (IV), intraperitoneal (IP), intramuscular (IM), or subcutaneous (SC) injection of a given compound may be quite different. Thus, toxicity may vary as much as 10-fold with the route of administration. Following exposure, there are multiple possible routes of metabolism, both detoxifying and activating, and multiple possible toxic end points (Figure 1.1).

Attempts to define the scope of toxicology, including that which follows, must take into account that the various subdisciplines are not mutually exclusive and are frequently interdependent. Due to overlapping of mechanisms as well as use and chemical classes of toxicants, clear division into subjects of equal extent or importance is not possible.

Many specialized terms are used in the various subdisciplines of toxicology as illustrated in the *Dictionary of Toxicology*, 2nd edition (Hodgson et al., 1998). However, some terms are of particular importance to toxicology in general; these and some more recent terms are defined in the glossary to be found at the end of this volume.

Although B through F (following) include subdivisions that encompass essentially all of the many aspects of toxicology, there are two new approaches (A, following) that serve to integrate the discipline as a whole.

A. Integrative Approaches

1. *Bioinformatics*. In the narrow and original meaning, bioinformatics was the application of information technology to molecular biology. While this is still

the most important aspect of bioinformatics, it is increasingly applied to other fields of biology, including molecular and other aspects of toxicology. It is characterized by computationally intensive methodology and includes the design of large databases and the development of techniques for their manipulation, including data mining.

2. *Systems Biology*. Although systems biology has been defined in a number of ways, some involving quite simple approaches to limited problems, in the currently most commonly accepted sense, it is an integrative approach to biological structure and function that will be of increasing importance to biology in general and toxicology in particular. In large part, biology has been reductionist throughout its history, studying organs as components of organisms, cells as components of organs, enzymes, nucleic acids, and so on, as components of cells, with the goal of describing function at the molecular level. Systems biology, on the other hand, is holistic and has the objective of discerning interactions between components of biological systems and describing these interactions in rigorous mathematical models. Furthermore, the proponents of systems biology aim to integrate these models at higher and higher levels or organization in order to develop an integrated model of the entire organism.

   Clearly, systems biology is in its infancy; however, the ultimate value of having an integrative model that could clarify all of the effects, from the most proximate to the ultimate, of a toxicant on a living organism, will provide enormous benefits not only for fundamental studies but in such applied areas as human health risk assessment.

B. Modes of Toxic Action. This includes the consideration, at the fundamental level of organ, cell, and molecular function, of all events leading to toxicity *in vivo*: uptake, distribution, metabolism, mode of action, and excretion. The term mechanism of toxic action is now more generally used to describe an important molecular event in the cascade of events leading from exposure to toxicity, such as the inhibition of acetylcholinesterase in the toxicity of organophosphorus and carbamate insecticides. Important aspects include the following:

   1. *Biochemical and molecular toxicology* consider events at the biochemical and molecular levels, including enzymes that metabolize xenobiotics, generation of reactive intermediates, interaction of xenobiotics or their metabolites with macromolecules, gene expression in metabolism and modes of action, signaling pathways in toxic action, and so on.
   2. *Behavioral toxicology* deals with the effects of toxicants on animal and human behavior, which is the final integrated expression of nervous function in the intact animal. This involves both the peripheral and central nervous systems, as well as effects mediated by other organ systems, such as the endocrine glands.
   3. *Nutritional toxicology* deals with the effects of diet on the expression of toxicity and with the mechanisms of these effects.
   4. *Carcinogenesis* includes the chemical, biochemical, and molecular events that lead to the large number of effects on cell growth collectively known as cancer.

5. *Teratogenesis* includes the chemical, biochemical, and molecular events that lead to deleterious effects on development.
6. *Mutagenesis* is concerned with toxic effects on the genetic material and the inheritance of these effects.
7. *Organ toxicity* considers effects at the level of organ function (e.g., neurotoxicity, hepatotoxicity, and nephrotoxicity).

C. Measurement of Toxicants and Toxicity. These important aspects deal primarily with analytical chemistry, bioassay, and applied mathematics, and are designed to provide the methodology to answer certain critically important questions. Is the substance likely to be toxic? What is its chemical identity? How much of it is present? How can we assay its toxic effect, and what is the minimum level at which this toxic effect can be detected? A number of important fields are included:

1. *Analytical toxicology* is a branch of analytical chemistry concerned with the identification and assay of toxic chemicals and their metabolites in biological and environmental materials.
2. *Genomics.* The sometimes stated distinction that genomics deals with genomes while molecular biology deals with single genes is unrealistic and unnecessary; it is more appropriate to regard genomics as an aspect of molecular biology that deals not only with genomes and gene expression but also such important aspects as genetic polymorphisms, particularly single nucleotide polymorphisms (SNPs). Techniques, such as microarrays, are now available to examine simultaneously the expression of very large numbers of genes.
3. *Proteomics* deals with the protein complement of organisms, the entire complement being known as the proteome. Thus, while genomics is concerned with gene expression, proteomics examines the products of the expressed genes.
4. *Metabolomics* is the next step in the sequence from genomics through proteomics and is concerned with the profile of small molecules produced by the metabolic processes of an organism. Changes in the profile in response to chemical stress are of importance to both fundamental and applied toxicology.
5. *Toxicity testing* involves the use of living systems to estimate toxic effects. It covers the gamut from short-term tests for genotoxicity such as the Ames test and cell culture techniques to the use of intact animals for a variety of tests from acute toxicity to lifetime chronic toxicity. Although the term "bioassay" is used properly only to describe the use of a living organism to quantitate the amount of a particular toxicant present, it is frequently used to describe any *in vivo* toxicity test.
6. *Toxicologic pathology* is that branch of pathology that deals with the effects of toxic agents manifested as changes in subcellular, cellular, tissue, or organ morphology.
7. *Structure-activity* studies are concerned with the relationship between the chemical and physical properties of a chemical and toxicity and, particularly, the use of such relationships as predictors of toxicity.

8. *Biomathematics and statistics* relate to many areas of toxicology. They deal with data analysis, the determination of significance, and the formulation of risk estimates and predictive models.
9. *Epidemiology*, as it applies to toxicology, is of great importance as it deals with the relationship between chemical exposure and human disease in actual populations, rather than in experimental settings.

D. Applied Toxicology. This includes the various aspects of toxicology as they apply in the field or the development of new methodology or new selective toxicants for early application in the field setting.
   1. *Clinical toxicology* is the diagnosis and treatment of human poisoning.
   2. *Veterinary toxicology* is the diagnosis and treatment of poisoning in animals other than humans, particularly livestock and companion animals, but not excluding feral species. Other important concerns of veterinary toxicology are the possible transmission of toxins to the human population in meat, fish, milk, and other foodstuffs, and the care and ethical treatment of experimental animals.
   3. *Forensic toxicology* concerns the medicolegal aspects, including detection of poisons in clinical and other samples.
   4. *Environmental toxicology* is concerned with the movement of toxicants and their metabolites and degradation products in the environment and in food chains, and with the effect of such contaminants on individuals and, especially, populations. Because of the large number of industrial chemicals and possibilities for exposure, as well as the mosaic of overlapping laws that govern such exposure, this area of applied toxicology is well developed.
   5. *Industrial toxicology* is a specific area of environmental toxicology that deals with the work environment and constitutes a significant part of *industrial hygiene*.

E. Chemical Use Classes. This includes the toxicology aspects of the development of new chemicals for commercial use. In some of these use classes, toxicity, at least to some organisms, is a desirable trait; in others, it is an undesirable side effect. Use classes are not composed entirely of synthetic chemicals; many natural products are isolated and are used for commercial and other purposes and must be subjected to the same toxicity testing as that required for synthetic chemicals. Examples of such natural products include the insecticide, pyrethrin, the clinical drug, digitalis, and the drug of abuse, cocaine.
   1. *Agricultural chemicals* include many compounds, such as insecticides, herbicides, fungicides, and rodenticides, in which toxicity to the target organism is a desired quality whereas toxicity to "nontarget species" is to be avoided. Development of such selectively toxic chemicals is one of the applied roles of comparative toxicology.
   2. *Clinical drugs* are properly the province of pharmaceutical chemistry and pharmacology. However, toxic side effects and testing for them clearly fall within the science of toxicology.
   3. *Drugs of abuse* are chemicals taken for psychological or other effects and may cause dependence and toxicity. Many of these are illegal but some are of clinical significance when used correctly.

4. *Food additives* are of concern to toxicologists only when they are toxic or being tested for possible toxicity.
5. *Industrial chemicals* are so numerous that testing them for toxicity or controlling exposure to those known to be toxic is a large area of toxicological activity.
6. *Naturally occurring substances* include many phytotoxins, mycotoxins, minerals, and so on, all occurring in the environment. The recently expanded and now extensive use of herbal "remedies" and dietary supplements has become a cause of concern for toxicologists and regulators. Not only is their efficacy frequently dubious, but their potential toxicity is also largely unknown.
7. *Combustion products* are not properly a use class but are a large and important class of toxicants, generated primarily from fuels and other industrial chemicals.

F. Regulatory Toxicology. These aspects, concerned with the formulation of laws, and regulations authorized by laws, are intended to minimize the effect of toxic chemicals on human health and the environment.
   1. *Legal aspects* are the formulation of laws and regulations and their enforcement. In the United States, enforcement falls under such government agencies as the Environmental Protection Agency (EPA), the Food and Drug Administration (FDA) and the Occupational Safety and Health Administration (OSHA). Similar government agencies exist in many other countries.
   2. *Risk assessment* is the definition of risks, potential risks, and the risk–benefit equations necessary for the regulation of toxic substances. Risk assessment is logically followed by *risk communication* and *risk management.* Risk assessment, risk communication, and risk management are frequently referred to as *risk analysis*.

1.2 RELATIONSHIP TO OTHER SCIENCES

Toxicology is a highly eclectic science and human activity drawing from, and contributing to, a broad spectrum of other sciences and human activities. At one end of the spectrum are those sciences that contribute their methods and philosophical concepts to serve the needs of toxicologists, either in research or in the application of toxicology to human affairs. At the other end of the spectrum are those sciences to which toxicology contributes.

In the first group, chemistry, biochemistry, pathology, physiology, epidemiology, immunology, ecology, and biomathematics have long been important while molecular biology has, in the last two or three decades, contributed to dramatic advances in toxicology.

In the group of sciences to which toxicology contributes significantly are such aspects of medicine as forensic medicine, clinical toxicology, pharmacy, and pharmacology, public health, and industrial hygiene. Toxicology also contributes in an important way to veterinary medicine, and to such aspects of agriculture as the development and safe use of agricultural chemicals. The contributions of toxicology to environmental studies have become increasingly important in recent years.

Clearly, toxicology is preeminently an applied science, dedicated to the enhancement of the quality of life and the protection of the environment. It is also much more. Frequently, the perturbation of normal life processes by toxic chemicals enables us to learn more about the life processes themselves. The use of dinitrophenol and other uncoupling agents to study oxidative phosphorylation and the use of α-amanitin to study RNA polymerases are but two of many examples. The field of toxicology has expanded enormously in recent decades, both in numbers of toxicologists and in accumulated knowledge. This expansion has brought a change from a primarily descriptive science to one which utilizes an extensive range of methodology to study the mechanisms involved in toxic events.

1.3 A BRIEF HISTORY OF TOXICOLOGY

Much of the early history of toxicology has been lost, and in much that has survived, toxicology is of almost incidental importance in manuscripts dealing primarily with medicine. Some, however, deal more specifically with toxic action or with the use of poisons for judicial execution, suicide, or political assassination. Regardless of the paucity of the early record, and given the need for people to avoid toxic animals and plants, toxicology must be one of the oldest practical sciences.

The Egyptian papyrus, *Ebers*, dating from about 1500 BC, must rank as the earliest surviving pharmacopeia, and the surviving medical works of Hippocrates, Aristotle, and Theophrastus, published during the period 400–250 BC, all include some mention of poisons. The early Greek poet Nicander treats, in two poetic works, animal toxins (Therica) and antidotes to plant and animal toxins (Alexipharmica). The earliest surviving attempt to classify plants according to their toxic and therapeutic effects is that of Dioscorides, a Greek employed by the Roman emperor Nero about 50 AD.

There appear to have been few advances in either medicine or toxicology between the time of Galen (131–200 AD) and that of Paracelsus (1493–1541). It was the latter who, despite frequent confusion between fact and mysticism, laid the groundwork for the later development of modern toxicology by recognizing the importance of the dose–response relationship. His famous statement "All substances are poisons; there is none that is not a poison. The right dose differentiates a poison and a remedy" succinctly summarizes that concept. His belief in the value of experimentation was also a break with earlier tradition.

There were some important developments during the eighteenth century. Probably the best known is the publication of Ramazini's *Diseases of Workers* in 1700 which led to his recognition as the father of occupational medicine. The correlation between the occupation of chimney sweeps and scrotal cancer by Percival Pott in 1775 is almost as well-known although it was foreshadowed by Hill's correlation of nasal cancer and snuff use in 1761.

Orfila, a Spaniard working at the University of Paris in the early nineteenth century, is generally regarded as the father of modern toxicology. He clearly identified toxicology as a separate science and, in 1815, published the first book devoted exclusively to toxicology. An English translation in 1817 was entitled *A General System of Toxicology or, A Treatise on Poisons, Found in the Mineral, Vegetable*

and Animal Kingdoms, Considered in Their Relations with Physiology, Pathology and Medical Jurisprudence. Workers of the late nineteenth century who produced treatises on toxicology include Christian, Kobert, and Lewin. The recognition of the site of action of curare by Claude Bernard (1813–1878) began the modern study of the mechanisms of toxic action. Since then, advances have been numerous—too numerous to list in detail. They have increased our knowledge of the chemistry of poisons, the treatment of poisoning, the analysis of toxicants and toxicity, as well as modes of toxic action and detoxication processes, and specific molecular events in the poisoning process.

With the publication of her controversial book, *The Silent Spring*, in 1962, Rachel Carson became an important influence in initiating the modern era of environmental toxicology. Her book emphasized stopping the widespread, indiscriminate use of pesticides and other chemicals and advocated use patterns based on sound ecology. Although sometimes inaccurate and with arguments often based on frankly anecdotal evidence, her book is often credited as the catalyst leading to the establishment of the U.S. EPA and she is regarded by many as the mother of the environmental movement.

It is clear, however, that since the 1960s, toxicology has entered a phase of rapid development and has changed from a science that was largely descriptive to one in which the importance of mechanisms of toxic action is generally recognized. Since the 1970s, with increased emphasis on the use of the techniques of molecular biology, the pace of change has increased even further, and significant advances have been made in many areas, including chemical carcinogenesis and xenobiotic metabolism, among many others.

1.4 DOSE–RESPONSE RELATIONSHIPS

As mentioned previously, toxicity is a relative event that depends not only on the toxic properties of the chemical and the dose administered but also on individual and interspecific variation in the metabolic processing of the chemical. The first recognition of the relationship between the dose of a compound and the response elicited has been attributed to Paracelsus (see Section 1.3). It is noteworthy that his statement includes not only that all substances can be toxic at some dose, but that "the right dose differentiates a poison from a remedy," a concept that is the basis for pharmaceutical therapy.

A typical dose–response curve is shown in Figure 1.2, in which the percentage of organisms or systems responding to a chemical is plotted against the dose. For many chemicals and effects, there will be a dose below where no effect or response is observed. This is known as the *threshold dose*. This concept is of significance because it implies that a *no observed effect level* (NOEL) can be determined and that this value can be used to determine the safe intake for food additives and contaminants such as pesticides. Although this is generally accepted for most types of chemicals and toxic effects, for chemical carcinogens acting by a genotoxic mechanism, the shape of the curve is controversial, and for regulatory purposes, their effect is assumed to be a no-threshold phenomenon. Dose–response relationships are discussed in more detail in Chapter 10—Acute Toxicity and Chapter 20—Toxicity Testing.

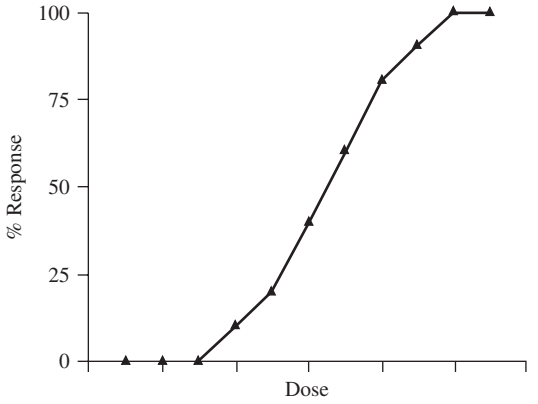

Figure 1.2 A typical dose–response curve.

1.5 SOURCES OF TOXIC COMPOUNDS

Given the enormous number of toxicants, it is difficult to classify them, either chemically, by function, or by mode of action since many of them would fall into several classes. Some are natural products, many are synthetic organic chemicals of use to society, while some are byproducts of industrial processes and waste disposal. It is useful, however, to categorize them according to the expected routes of exposure or according to their uses.

A. Exposure Classes. Exposure classes include toxicants in food, air, water, and soil as well as toxicants characteristic of domestic and occupational settings. Toxicant use classes are described in detail in Chapter 3.
B. Use Classes. Use classes include drugs of abuse, therapeutic drugs, agricultural chemicals, food additives and contaminants, metals, solvents, combustion products, cosmetics, and toxins. Some of these, such as combustion products, are the products of use processes rather than being use classes. All of these groups of chemicals are discussed in detail in Chapter 4.

1.6 MOVEMENT OF TOXICANTS IN THE ENVIRONMENT

Chemicals released into the environment rarely remain in the form, or at the location, of release. For example, agricultural chemicals used as sprays may drift from the point of application as air contaminants or enter run-off water as water contaminants. Many of these chemicals are susceptible to fungal or bacterial degradation and are rapidly detoxified, frequently being broken down to products that can enter the carbon, nitrogen, and oxygen cycles. Other agricultural chemicals, particularly halogenated organic compounds, are recalcitrant to a greater or lesser degree to metabolism by microorganisms and persist in soil and water as contaminants; they may enter biologic food chains and move to higher trophic levels or persist in processed crops as food contaminants. This same scenario is applicable to

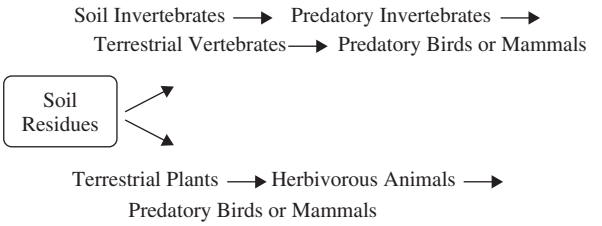

Figure 1.3 Examples of simplified food chains.

any toxicant released into the environment either for a specific use or as a result of industrial processes, combustion, and so on. Chemicals released into the environment are also susceptible to chemical degradation, a process often stimulated by ultraviolet light.

Although most transport between inanimate phases of the environment results in wider dissemination, but, at the same time, dilution of the toxicant in question, transfer between living creatures may result in increased concentration or bioaccumulation. Lipid-soluble toxicants are readily taken up by organisms following exposure in air, water, or soil. Unless rapidly metabolized, they persist in the tissues long enough to be transferred to the next trophic level. At each level, the lipophilic toxicant tends to be retained while the bulk of the food is digested, utilized, and excreted, thus increasing the toxicant concentration. At some point in the chain, the toxicant can become deleterious, particularly if the organism at that level is more susceptible than those at the level preceding it. Thus, the eggshell thinning in certain raptorial birds was almost certainly due to the uptake of DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane) and DDE (1,1-dichloro-2,2-bis(4-chlorophenyl) ethane) and their particular susceptibility to this type of toxicity. Simplified food chains are shown in Figure 1.3.

It is clear that such transport can occur through both aquatic and terrestrial food chains, although in the former, higher members of the chains, such as fish, can accumulate large amounts of toxicants directly from the medium. This accumulation occurs because of the large area of gill filaments, their intimate contact with the water, and the high flow rate of water over them. Given these characteristics and a toxicant with a high partition coefficient between lipid membranes and water, considerable uptake is inevitable.

These and all other environmental aspects of toxicology are discussed in Part VII.

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SAMPLE QUESTIONS

1. Briefly define the following terms:
   a. Toxicology
   b. Poison
   c. Genomics
   d. Proteomics
   e. Metabolomics

2. Toxicity has been described as a cascade of events initiated by exposure to a harmful chemical. Name the principal steps in this cascade.

3. Name and define three important chemical use classes.

CHAPTER 2

Introduction to Biochemical and Molecular Methods in Toxicology

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2.1 INTRODUCTION

This chapter is not designed to summarize biochemical methods long used in toxicology such as colorimetric and radiometric methods for the investigation of xenobiotic metabolism, either *in vivo* or *in vitro*, but rather to give a brief summary of the methods of molecular, biochemical, and cellular biology that have become, more recently, of critical importance in toxicological research. Areas of methodology that have achieved prominence since the publication of the 3rd edition of this textbook (Hodgson, 2004) include proteomics (Section 2.5, below), metabolomics (Section 2.6, below), and bioinformatics (Section 2.7, below). The current chapter owes much to Chapters 2 through 8 of *Molecular and Biochemical Toxicology* (Smart and Hodgson, 4th edition, 2008; see Bibliography), and the reader is referred to these chapters for additional information.

2.2 CELL CULTURE TECHNIQUES

While scientists have had the ability to culture many unicellular organisms for some time, recent advances in the culture of cells from multicellular organisms have played a pivotal role in recent advances in toxicology. Cells can be isolated and either maintained in a viable state for enough time to conduct informative experiments or, in some cases, can be propagated in culture. The advantages of cultured cells are that they can provide living systems for the investigation of toxicity that are simplified relative to the intact organism or they can be used as replacements for whole animal toxicity testing if the toxic end point can be validated. Human cells play an important role in the extrapolation of toxic effects, discovered in experimental animals, to humans. Cultured cells, either from humans or other mammals, are utilized in many of the molecular methods mentioned below. There

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are, however, limitations in the use of cellular methods. It has not been possible to culture many cell types, and of those that have been cultured, the loss of differentiated cell function is a common problem. Extrapolation of findings to the intact animal is often problematical, and the use of undefined media constituents such as serum, often essential for cell viability, may have unwanted or undefined effects on cell function and toxicant bioavailability.

Studies have been carried out on cells isolated from tissues and maintained in suspension culture or on cells that have formed monolayers.

2.2.1 Suspension Cell Culture

Circulating blood cells or cells easily obtained by lavage such as peritoneal and alveolar macrophages can normally survive in suspension culture when provided with a suitable nutrient medium. Cells from organized solid organs or tissues must be separated from the tissue and, if possible, separated into cell types, before being suspended in such a medium.

Cell association within organs depends on protein complex formation, which in turn is Ca^{2+}-dependent. Consequently, dissociation media generally contain a proteolytic enzyme and the Ca^{2+} chelator EDTA (ethylenediaminetetraacetic acid). There are a number of methods available to separate cell types from the mixture of dispersed cells, the commonest being centrifugation without a density gradient, wherein cells are separated by size, or centrifugation through a density gradient wherein cells are separated on the basis of their buoyant density.

Cells in suspension may be maintained for a limited period of time in defined media or for longer periods in nutrient, but less well-defined, media. In either case, these cultures are often used for studies of xenobiotic metabolism.

2.2.2 Monolayer Cell Culture

Proliferation of most cells in culture requires attachment to a substrate and occurs until limited by cell-to-cell contact, resulting in the formation of a cellular monolayer. The substrate provided for attachment is usually polystyrene modified to carry a charge. The medium for continued maintenance and growth contains salts and glucose, usually with a bicarbonate buffer. Because of the bicarbonate buffering system, these cultures are maintained in a 5–10% CO_2 atmosphere in a temperature and humidity controlled incubator. Many cells require serum for optimal growth, inducing considerable variability into the experimental system. Since the factors provided by serum are numerous and complex, defined serum substitutes are not always successful. The factors provided by serum include proteins such as growth factors, insulin, and transferrin (to provide available iron), small organic molecules such as ethanolamine, and pyruvate and inorganic ions, such as selenium.

2.2.3 Indicators of Toxicity in Cultured Cells

Routine observation of cultured cells is usually carried out by phase contrast microscopy, utilizing the inverted phase contrast microscope. More recently, more detailed observations have become possible utilizing fluorescent tags and inverted fluorescent microscopes. Fluorescent tags currently in use permit the assessment of oxidant

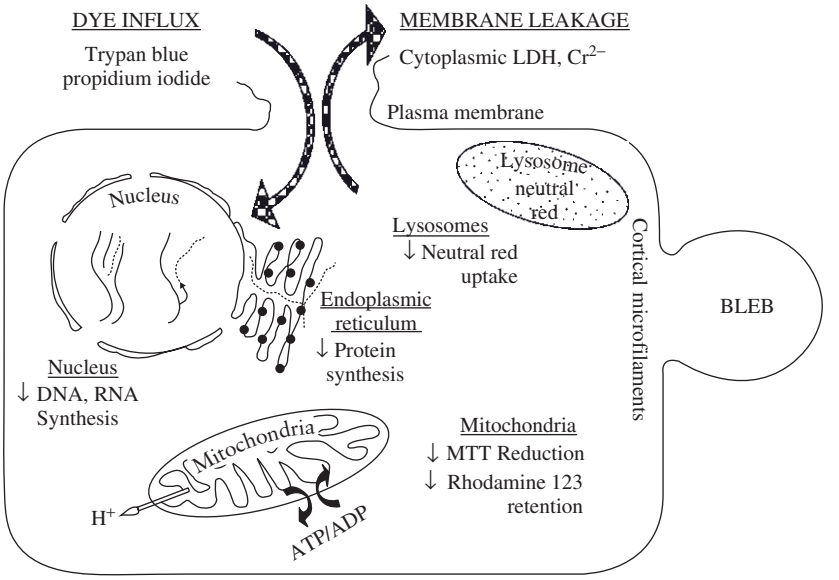

Figure 2.1 Idealized diagram of a cell to illustrate parameters often used to measure cytotoxicity and the corresponding affected subcellular organelle. From *A Textbook of Modern Toxicology*, 3rd ed., ed. E. Hodgson. New York: Wiley, 2004.

status and mitochondrial function as well as the intracellular concentration of sulfhydryl groups, Ca^{2+}, H^+, Na^+, and K^+.

Toxicity to cultured cells may be the result either of inadequacies in the culture or the toxicity effects of the chemical being investigated. Short-term toxicity is usually evaluated by examination of end points that indicate effects on cellular organelles such as leakage of cell constituents into the medium, uptake of dyes into the cell, and the formation of surface "blebs." This is illustrated in Figure 2.1.

Longer-term assessments of cell toxicity are highly dependent upon the relevant toxic end point. They may include measurement of growth competence, apoptosis, and/or necrosis, incorporation of radioactive precursors into essential cellular constituents such as RNA, DNA, and protein and specialized cellular functions. Some examples of the use of cultured cell lines in the study of toxicity effects are shown in Table 2.1.

2.2.4 Use of Stem Cells

Although the use of human stem cells is still controversial, their use in biomedical research is increasing. At the same time, stem cells from surrogate animals have long been used in biomedical, including toxicological, research. Probably the best example is the use of cultured mouse embryonic stem cells for generation of "knockout" mice. Such mice have been widely used in the study of xenobiotic-metabolizing enzymes (XMEs), nuclear receptors, and the like.

The value of cultured stem cells in toxicology lies in their ability to provide a continuous source of cells that can be manipulated to provide a desired mature cell

TABLE 2.1 Application of Human Cell Lines Retaining Differentiated Properties for the Study of Toxic Effects and a Comparison with Primary Human Hepatocytes

Cell Type	Source	Differentiated Cell Type	Toxicant	Measured End Point
Cell lines				
SK-N-SH	Human neuroblastoma	Neuron	Anesthetic N_2O	Depressed cholinergic Ca^{2+} signaling
HepG2	Human hepatoblastoma	Hepatocyte	Cyclophosphamide (antineoplastic)	Cytochrome P450 (CYP)-dependent genotoxicity
			Rifampicin (PXR ligand)	Inhibition of bile acid synthesis
Caco-2	Human colon adenocarcinoma	Intestinal epithelial cell	Arsenic	Transepithelial leakiness
Primary hepatocyte			Fipronil, fipronil sulfoxide	Adenylate kinase release, Induction of caspase 3/7 Induction of CYP isoforms
			Deltamethrin (pyrethroid)	Adenylate kinase release, Induction of caspase 3/7 Induction of CYP isoforms
			Permethrin (pyrethroid)	Adenylate kinase release, Induction of caspase 3/7 Induction of CYP isoforms
			DEET (N,N-diethyl-meta-toluamide) (repellent)	Adenylate kinase release, Induction of caspase 3/7 Induction of CYP isoforms
			Chlorpyrifos (OP)	Adenylate kinase release, Induction of caspase 3/7 Induction of CYP isoforms

PXR, pregnane X receptor; OP, organophosphorus.

type. This could alleviate the use of surrogate animals in toxicity testing and provide metabolically competent human cell types.

2.2.5 Cell Culture Models as "Alternative" Toxicity Tests

Due primarily to the fact that the cell represents an excellent intermediate level of biological organization between the intact organism and the cellular organelle or enzyme/receptor levels, the primary use of cell culture models to date has been in mechanistic studies of chemical toxicity. At present, however, much effort is being placed on development of cell culture models as replacements for surrogate animals in toxicity. This arises not only from ethical concerns over animal use but also for economy of time and expense. Further, the use of human-derived cell lines may be an advantage in studies related to human health assessment.

Although difficulties are often encountered, particularly in agreement between the cell culture method and *in vivo* results as well as quantitative relationships between toxicants of related chemical structure or mode of toxic action, it appears that cell culture methods will be useful as early screens in tiered protocols for product safety testing.

Another emerging application of cell culture toxicity testing techniques is the development of cell lines engineered for a particular function, often for high-throughput screening protocols. An excellent example is in area of testing for endocrine disruptors and the recent mandate that chemicals in commerce be tested for endocrine disrupting activity. This involves the development of cell lines engineered to contain a vector with a reporter gene whose expression is responsive to activation of a cotransfected steroid hormone receptor. A similar approach is being adopted for the detection of dioxin-like compounds through their interaction with the aryl hydrocarbon receptor (Ah receptor).

2.3 MOLECULAR TECHNIQUES

Recombinant DNA techniques, including molecular cloning, have provided recent dramatic advances in many areas of both fundamental and applied biology, toxicology not excepted. Responses to toxicants often involve changes in gene expression and the microarray techniques enable the simultaneous examination of the global level of expression of thousands of genes in a single experiment. The completion of the human genome project now permits toxic effects in humans to be investigated and will facilitate extrapolation from experimental animals. The human genome will also provide the essential genetic background information for studies of polymorphisms in xenobiotic-metabolizing and other enzymes. Such polymorphisms have already been shown to be very important in individual sensitivity to clinical drugs and in the definition of populations and/or individuals at increased risk from particular toxicants. Identification of carcinogen-induced mutations, particularly in oncogenes and tumor-suppressor genes, are important in chemical carcinogenesis. The ability to develop "knock-out" and "knock-in" animals that lack a particular gene or express an altered gene in place of the wild-type gene, respectively, as well as knockdown of specific genes in cell culture are proving important in toxicological studies. Polymerase chain reaction (PCR) is an extremely versatile technique that

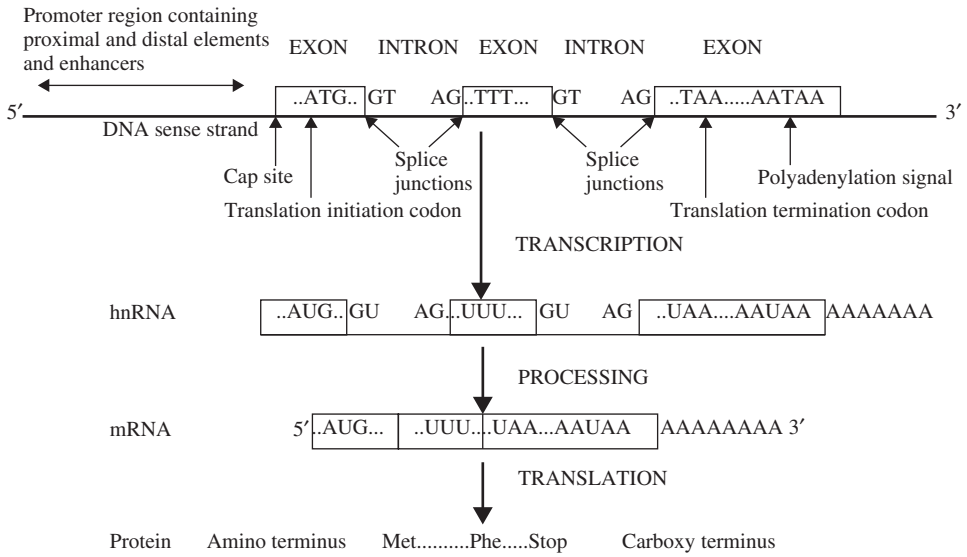

Figure 2.2 Transcription, mRNA processing, and translation. DNA sense strand is designated by bold lines, hnRNA and mRNA by thinner lines. Exons are shown as rectangles and introns as the intervening spaces between exons. From *A Textbook of Modern Toxicology*, 3rd ed., ed. E. Hodgson. New York: Wiley, 2004.

can be used for many applications including gene cloning, gene mutagenesis, and quantitative gene expression analysis.

Gene structure and any of the processes involved in DNA expression, including transcription, mRNA processing, and translation and protein synthesis (Figure 2.2), can all be examined by molecular techniques. In toxicology, this may include toxic effects on these processes or the role of the processes in the mechanism of toxic action.

2.3.1 Molecular Cloning

The basic principle of molecular cloning is the insertion of a DNA segment into a suitable vector. The vector is an autonomously replicating DNA molecule, and the inserted DNA segment may be as large as a gene or as small as a few nucleotides. The vector containing the DNA is inserted into a cell such as a bacterium, where it can be replicated many times and either the DNA or the expressed protein subsequently isolated (Figure 2.3).

2.3.2 cDNA and Genomic Libraries

cDNA or genomic libraries are collections of DNA fragments incorporated into a recombinant vector and transformed into an appropriate host cell. In the case of cDNA libraries, the cDNAs complementary to all of the mRNAs in the tissue or cell sample are synthesized in a procedure using reverse transcriptase, before incorporation into the vector. With genomic DNA libraries the genomic DNA is digested,

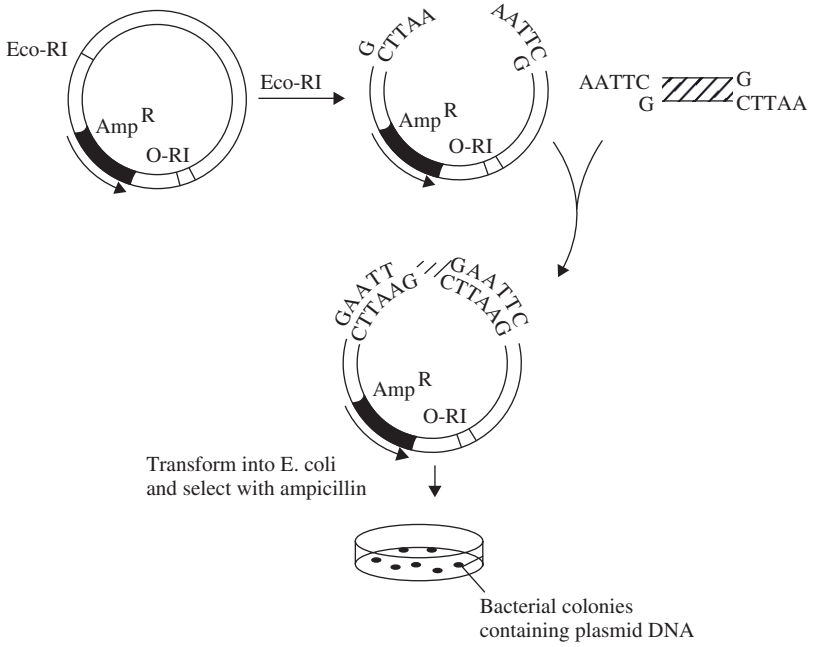

Figure 2.3 Molecular cloning using a plasmid vector. From *A Textbook of Modern Toxicology*, 3rd ed., ed. E. Hodgson. New York: Wiley, 2004.

before cloning into the vector, with a restriction enzyme to produce an overlapping set of DNA fragments of some 12–20 kb.

These libraries have been used in many screening procedures, including gene identification and gene regulation. Today, with availability of genomic information/annotation for numerous species including mouse, rat, and human, direct bioinformatic analysis of such information allows for PCR approaches for the cloning of genes, promoter regions, and mRNA (cDNA). In fact, most applications that used cDNA and genomic libraries have been superceded by other methods, particularly those based on PCR.

2.3.3 Northern and Southern Blot Analysis

Northern analysis is usually used to identify and quantitate specific mRNAs in a sample. Southern analysis is used to determine whether or not a gene of interest is present as well as its copy number. Other uses for Southern analysis include identifying restriction fragment length polymorphisms and changes in heterozygosity.

In both Southern and Northern analyses, restriction digested DNA fragments or RNA, respectively, are separated by size when electrophoresed on agarose gel. The separated molecules are transferred, by electroblotting or capillary blotting, onto a nylon or nitrocellulose membrane. The immobilized RNA or DNA is reacted with a radiolabeled, chemiluminescent, or fluorescent probe that is complementary to the DNA/RNA of interest, unbound probe is washed off and the membrane exposed,

in the case of radioactive probes, to radioautographic film to visualize the sample of interest.

2.3.4 PCR

PCR is a powerful technique that can, starting with amounts of DNA as small as those found in single cells, amplify the DNA until large amounts are available for many different kinds of research. Twenty to forty cycles of PCR can provide up to 10^5 times the original DNA sample.

It is necessary to know the flanking sequence of the DNA of interest in order to construct appropriate primers. These primers are complementary to the sequence at each end of the DNA sequence to be amplified. The DNA is incubated in a thermal cycler with thermostable DNA polymerase, all four deoxyribonucleotide triphosphates (dNTP), and the primers. The incubation temperature is raised to separate the DNA strands, lowered to permit annealing of the primers to the complementary regions of the DNA, and then raised to permit the polymerase to synthesize DNA. This cycle is then repeated up to 40 times. The PCR technique has been used for many types of toxicological investigation including uncovering polymorphisms in XMEs, cloning genes for functional studies as well as promoter regions of genes for gene regulation studies.

2.3.5 Evaluation of Gene Expression, Regulation, and Function

The methods used for the evaluation of regulation of gene expression are too numerous to be described in detail here. They include Northern analysis to determine levels of a particular mRNA, nuclear run-on to determine whether an increase in mRNA is due to an increase in the rate of transcription, and promoter deletion analysis to identify specific elements in the promoter region responsible for the control of expression, and the electrophoretic mobility shift assay (EMSA) is used to measure binding of a transcription factor to its specific DNA consensus sequence. High-throughput reporter gene assays are currently used to examine molecular pathways altered by toxicants. These assays employ specific regulatory promoter elements that respond to specific types of stressors/inputs; for example, estrogenic agents, reactive oxygen stress, and dioxin-related agents are engineered upstream of a reporter gene (i.e., luciferase), and cell lines containing these constructs can be treated with the toxicant of interest and reporter output quantified.

Of much current interest is the use of microarrays that permit the study of the expression of hundreds to thousands of genes at the same time. Microarrays are based on the principle that any gene being expressed at any point in time is giving rise to a specific, corresponding mRNA. The microarray itself consists of spots of DNA (c. 200 μ) bound to a suitable matrix. The mRNAs in the biological sample in question bind to the corresponding DNA and can be visualized by techniques involving dyes. Given the complexity of the data obtained (often thousands of genes are evaluated on a single microarray), special techniques have been developed for array scanning, data extraction, and statistical analysis. A typical microarray experiment is illustrated in Figure 2.4.

Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) is commonly used to amplify and quantitate mRNAs of interest. In fact, this technique is

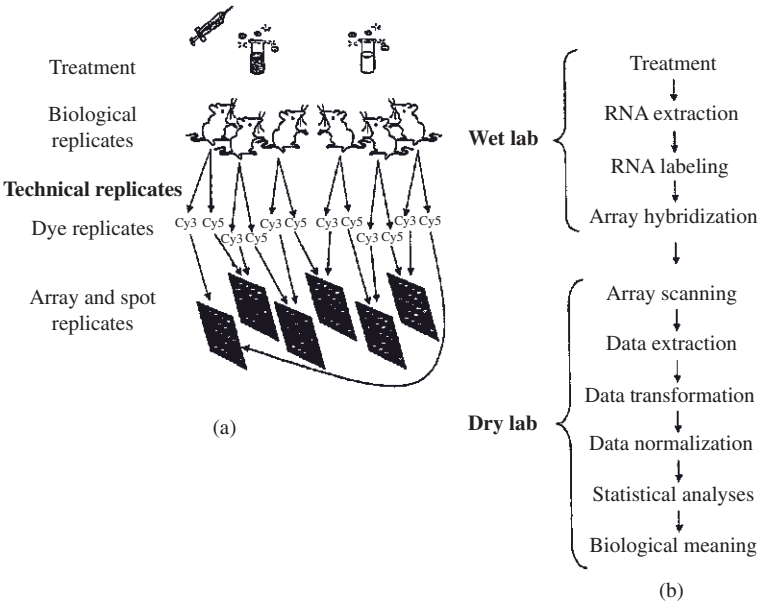

Figure 2.4 Schematic of a microarray experiment. (a) Experimental design incorporating both biological and technical replication. There are three treated mice and three control mice, providing biological replication. RNA from each mouse is labeled with each dye and hybridized more than once, providing technical replication. (b) Outline of the wet and dry laboratory steps involved in a microarray experiment. From *Molecular and Biochemical Toxicology*, 4th ed., eds. R.C. Smart and E. Hodgson. Hoboken, NJ: Wiley, 2008.

replacing the Northern technique described above as the preferred technique to measure changes in gene expression and the mRNA level. Gene function in cultured cells can be investigated by the forced expression of the gene product in a suitable expression system or through the use of small interfering RNAs (siRNAs), where the expression of the gene of interest can be knocked down in cultured cells. Gene function can also be studied *in vivo* through the creation of transgenic mice which overexpress the gene of interest or knock-out mice in which the gene in question has been functionally deleted or knock-in mice where an altered gene (i.e., serine is replaced by alanine to study the role of posttranslational modifications involving phosphorylation) is expressed in place of the wild-type gene.

A general, but more detailed and specific, account of these methods may be found in Smart (2008) and Oleksiak (2008) (see Bibliography).

2.4 IMMUNOCHEMICAL TECHNIQUES

Most of the recently developed methods for the detection, characterization, and quantitation of proteins (Leblanc, 2008) are immunoassays based on the fact that proteins are antigens, compounds that can be recognized by an antibody. It is also true that, by combining small molecules (haptens) with a larger carrier molecule such as a protein, these methods can be extended to small molecules of interest

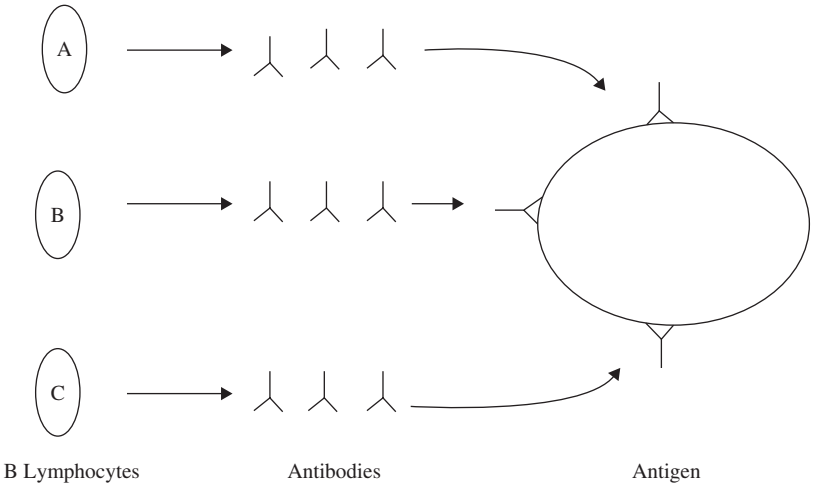

Figure 2.5 The generation of antibodies of several clonal origins (polyclonal antibodies) with antibodies from each clonal origin (monoclonal antibodies A, B, and C) recognizing a distinct epitope on the antigen. From *A Textbook of Modern Toxicology*, 3rd ed., ed. E. Hodgson. New York: Wiley, 2004.

since antibodies can be produced that recognize epitopes (specific sites on the antigen recognized by the antibody) that include the hapten.

The antibodies used may be polyclonal or monoclonal, each with characteristics fitting them for use in particular immunochemical methods. Injection of a mammal with a foreign protein (immunogen) gives rise to an immune reaction that includes the generation of antibodies from B lymphocytes. Each B lymphocyte gives rise to only a single antibody type that recognizes a single epitope on the antigen. However, since these antibodies are derived from many different B lymphocytes, the mixture of antibodies can recognize and bind to many different epitopes on the antigen. This mixture of antibodies can be isolated from the serum of the treated animal and is known, collectively, as *polyclonal antibodies*. However, if individual B lymphocytes from a treated animal can be isolated and cultured, because they are of a single clonal origin, they will produce a specific *monoclonal antibody* that recognizes only a single epitope on the antigen (Figure 2.5). Because of the multiple sites for binding, polyclonal antibodies are highly reactive. They are also relatively easy to produce. Monoclonal antibodies, although more difficult to produce are, on the other hand, more specific. The advantages and disadvantages of each must be considered to determine which is the antibody of choice for a particular application. The most important immunochemical methods include the following:

Immunolocalization is a technique for identifying the presence of a protein within the cell, its relative abundance, and its subcellular localization. After suitable preparation of the cells, they are treated with an antibody (the primary antibody) which binds to the protein of interest. An antibody that binds to the primary antibody (the secondary antibody) is then allowed to bind and form an antigen–primary antibody–secondary antibody complex. The detection system generally consists of

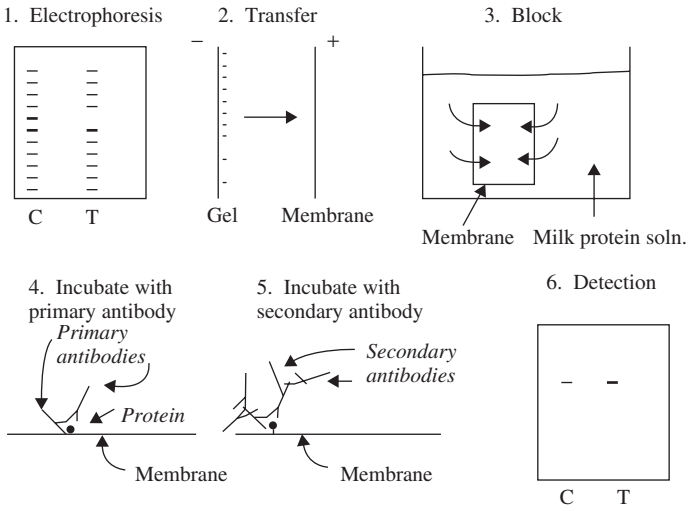

Figure 2.6 Diagrammatic representation of the use of immunoblotting to assess relative levels of a P450 protein following treatment of rats with a polychlorinated biphenyl (PCB). C, hepatic microsomal proteins from a control, untreated rat; T, hepatic microsomal proteins from a rat treated with PCBs. From *A Textbook of Modern Toxicology*, 3rd ed., ed. E. Hodgson. New York: Wiley, 2004.

the formation of a colored insoluble product of an enzymatic reaction, the enzyme, such as alkaline phosphatase or horseradish peroxidase, being covalently linked to the secondary antibody.

Immunoaffinity purification involves the use of antibodies, bound to an insoluble matrix, for chromatography. The advantage of this method is that it is highly specific, often permitting purification in a single step. *Immunoprecipitation* is a variant of immunoaffinity purification and is a means to remove a protein from a complex mixture in a highly specific manner.

Western blotting is a widely used technique in which antibodies are used to detect proteins following electrophoresis, generally sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis that permits the separation of proteins on the basis of their molecular weights (Figure 2.6). Western blotting can be used to determine the presence and relative amount of a particular protein in a biological sample as well as its molecular weight.

Radioimmunoassay (RIA) is a very sensitive method used to measure minute quantities of an antigen. Since this method is most often used to measure drugs, toxicants, and other xenobiotics, the antigen used to produce the antibody is the small molecule (hapten) linked covalently to a protein. Among the techniques used in the actual measurement is the antigen capture method, in which the competition between radiolabeled antigen and the unlabeled antigen in the sample is the most common.

Depending upon the design of the method, *enzyme-linked immunosorbent assay* (ELISA) can be used to measure either antigens or antibodies in mixtures by using enzymatic-mediated detection of the corresponding immobilized immune complex. Even though this method has proven most useful for the rapid estimation of

antibodies or antigens in complex biological mixtures, it has also been used for the quantitation of small molecules in a manner analogous to RIAs.

Inhibitory antibodies are frequently used in studies of xenobiotic metabolism, usually to estimate the contribution of particular enzymes in multienzyme mixtures. An important example is the use of antibodies to estimate the contribution of individual cytochrome P450 (CYP) isoforms to the overall metabolism of a xenobiotic in microsomal preparations.

2.5 PROTEOMICS

The proteome is defined as the protein complement present in the biological unit (e.g., cell, organ, organism) and represents that portion of the genome currently being expressed. Proteomics is represented by broad, inclusive techniques to separate, identify, and study the structure of the proteins of the proteome. Separation is usually by two-dimensional polyacrylamide gel electrophoresis and identification by a number of variants of mass spectrometry. Details are available in Merrick (2008).

2.6 METABOLOMICS

Genomics has the goal of determining, through analysis of mRNA, which genes are being expressed. Proteomics (Deighton, 2008) has the goal of determining whether expression of mRNA results in protein synthesis, while metabolomics has the goal of determining whether the expressed proteins are metabolically active. Metabolomics is, therefore, the identification and quantification of all of the metabolites in a biological system at some point in time. It is important to remember that the metabolites in question are the products of the normal endogenous metabolism of the cell, organ, or organism and not the metabolic products of toxicants or other xenobiotics although in the latter case, the techniques of metabolomics can be invaluable.

Given the large number, chemical diversity, and concentration range of the entire metabolome, of necessity, a number of techniques are needed to obtain the complete picture needed. Initially, an unbiased extraction technique must be selected or developed. Since no single extraction technique is likely to extract all metabolites, several techniques are usually employed. Metabolite identification depends on two sensitive techniques, mass spectrometry and nuclear magnetic resonance spectroscopy.

2.7 BIOINFORMATICS

In the narrow and original meaning, bioinformatics was the application of information technology to molecular biology. While this is still the most important aspect of bioinformatics, it is increasingly applied to other fields of biology, including molecular and other aspects of toxicology. It is characterized by computationally intensive methodology and includes the design of large databases and the development of techniques for their manipulation, including data mining.

2.8 SUMMARY AND CONCLUSIONS

Rapid and dramatic progress in development of new techniques based on molecular biology and analytical chemistry are stimulating new approaches and progress in many fields of toxicology. This progress is reflected not only in advances in the understanding of the fundamental mechanisms of toxicity but also in new approaches to toxicity testing. Both of these aspects have important implications for human health and for human health risk assessment. Further rapid progress is expected in the immediate future.

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SAMPLE QUESTIONS

1. Briefly discuss the importance of cultured cell techniques in toxicology.

2. Define the following terms:
   a. Northern and Southern blot analysis;
   b. Polymerase chain reaction;
   c. Microarray.

3. Define the following terms:
   a. Western blot;
   b. Polyclonal and monoclonal antibodies;
   c. Immunoaffinity purification and immunoprecipitation.