PART VI

APPLIED TOXICOLOGY

CHAPTER 20

Toxicity Testing

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20.1 INTRODUCTION

The purpose of this chapter is not to describe all available tests for chemical toxicity but rather to summarize those that are, or can be, currently required by regulatory agencies. Although not without difficulties, either in execution or injustification, the tests described have been validated and used extensively. Tests for endocrine disruption have been under development and validation for several years and will probably be required in the near future (see Chapter 29).

As a consequence of the large number of chemicals to be tested under a number of federal statutes and the extensive backlog of untested chemicals, it will be necessary to develop high throughput, rapid assays that can handle many chemicals simultaneously. From both cost and animals welfare considerations, it will not be possible to use whole animal assays, and assays based on human cell lines will doubtless be used. Testing systems based on quantitative structure–activity relationships, using engineered human cell lines and the techniques of genomics, proteomics, metabolomics, and systems biology are currently being developed (see Chapters 28 and 29). However, at least for some considerable time, these new systems will be used to establish priorities rather than make final regulatory decisions, and the tests described in this chapter will still be used.

Although current testing for toxicity, usually for the purposes of human health risk assessment, might be expected to be one of the more routine aspects of toxicology, it is actually one of the more controversial. Among the many areas of controversy are the use of animals for testing and the welfare of the animals; extrapolation of animal data to humans; extrapolation from high-dose to low-dose effects; and the increasing cost and complexity of testing protocols relative to the benefits expected.

Most testing can be subdivided into *in vivo* tests for acute, subchronic, or chronic effects and *in vitro* tests for genotoxicity or cell transformation, although other tests are used and are described in this chapter.

A Textbook of Modern Toxicology, *Fourth Edition*. Edited by Ernest Hodgson

TABLE 20.1 Some Agencies and Statutes Involved in Regulation of Toxic Chemicals in the United States

Food and Drug Administration (FDA)
Food, Drug and Cosmetic Act
Labor Department
Occupational Safety and Health Act
Consumer Products Safety Commission
Environmental Protection Agency (EPA)
Federal Insecticide, Fungicide, and Rodenticide Act
Clean Air Act
Federal Water Pollution Control Act
Safe Drinking Water Act
Toxic Substances Control Act
Resource Conservation and Recovery Act
State governments
Various state and local laws
Enforcement of certain aspects of federal law delegated to states

Toxicity assessment is the determination of the potential of any substance to act as a poison, the conditions under which this potential will be realized, and the characterization of its action. *Risk assessment*, however, is a quantitative assessment of the probability of deleterious effects under given exposure conditions. Both are involved in the regulation of toxic chemicals. *Regulation* is the control, by statute, of the manufacture, transportation, sale, or disposal of chemicals deemed to be toxic after testing and according to criteria laid down in the law in question.

Testing in the United States is carried out by many groups: industrial, governmental, academic, and others. Regulation, however, is carried out by a narrow range of governmental agencies, each charged with the formulation of regulations under a particular law or laws and with the administration of those regulations. The principal regulatory agencies for the United States are shown in Table 20.1. Other industrialized countries have counterpart laws and agencies for the regulation of toxic chemicals.

Although the objective of most toxicity testing is the elimination of potential risks to humans, most of the testing is carried out on experimental animals. This is necessary because current knowledge of quantitative structure–activity relationships (QSAR) does not permit accurate extrapolation to new compounds. Human data are difficult to obtain experimentally for ethical reasons, but are necessary for such deleterious effects as irritation, nausea, allergies, odor evaluation, and some higher nervous system functions. Some insight may be obtained in certain cases from occupational exposure data, although this often tends to be irregular in time and not clearly defined as to the composition of the toxicant or the exposure levels. Clearly, any experiments involving humans must be carried out under carefully defined conditions after other testing is complete.

Although extrapolation from experimental animals to humans presents problems due to a variety of reasons, including differences in metabolic pathways, dermal

TABLE 20.2 A Summary of Toxicity Tests and Related End Points

I Chemical and physical properties
For the chemical in question, probable contaminants from synthesis as well as intermediates and waste products from the synthetic process.

II Exposure and environmental fate
- a. Degradation studies—hydrolysis, photodegradation, etc.
- b. Degradation in soil, water, under various conditions
- c. Mobility and dissipation in soil water and air
- d. Accumulation in plants, aquatic animals, wild terrestrial animals, food plants and animals, etc.

III *In vivo* tests
- a. Acute
  1. LD_{50} and LC_{50}—oral, dermal, or inhaled
  2. Eye irritation
  3. Dermal irritation
  4. Dermal sensitization
- b. Subchronic
  1. 90-day feeding
  2. 30- to 90-day dermal or inhalation exposure
- c. Chronic/Reproduction
  1. Chronic feeding (including oncogenicity tests)
  2. Teratogenicity
  3. Reproduction (multigeneration)
- d. Special tests
  1. Neurotoxicity
  2. Potentiation
  3. Metabolism
  4. Pharmacodynamics
  5. Behavior

IV *In vitro* tests
- a. Mutagenicity—prokaryote (Ames test)
- b. Mutagenicity—eukaryote (*Drosophila*, mouse, etc.)
- c. Chromosome aberration (*Drosophila*, sister chromatid exchange, etc.)

V Effects on wildlife
Selected species of wild mammals, birds, fish, and invertebrates: acute toxicity, accumulation, and reproduction in laboratory simulated field conditions.

penetration, mode of action, and others, experimental animals present numerous advantages in testing procedures. These advantages include the possibility of clearly defined genetic constitution and their amenity to controlled exposure, controlled duration of exposure, and the possibility of detailed examination of all tissues following necropsy.

Although not all tests are required for all potentially toxic chemicals, any of the tests shown in Table 20.2 may be required. The particular set of tests required depends on the predicted or actual use of the chemical, the predicted or actual route of exposure, and the chemical and physical properties of the chemical.

20.2 EXPERIMENTAL ADMINISTRATION OF TOXICANTS

20.2.1 Introduction

Regardless of the chemical tested and whether the test is for acute or chronic toxicity, all *in vivo* testing requires the reproducible administration of a known dose of the chemical under test that is generally related to the expected route of humans exposure. The nature and degree of the toxic effect can be affected by the route of administration (Table 20.3). This may be related to differences at the portals of entry or to effects on pharmacokinetic processes. In the latter case, one route (e.g., intravenous) may give rise to a concentration high enough to saturate some rate-limiting process, whereas another (e.g., subcutaneous [SC]) may distribute the dose over a longer time and avoid such saturation. Another key question is that of appropriate controls. To identify effects of handling and other stresses as well as the effects of the solvents or other carriers, it is usually better to compare treated animals with both solvent-treated and untreated or possibly sham-treated controls.

20.2.2 Routes of Administration

Oral Oral administration is often referred to as administration per os (PO). Compounds can be administered mixed in the diet, dissolved in drinking water, by gastric gavage, by controlled-release capsules, or by gelatin capsules. In the first two cases, either a measured amount can be provided or access can be *ad libitum* (available 24 h per day), with the dose estimated from consumption measurements. For certain tests pair-feeding of controls should be considered; that is, controls are permitted only the amount of food consumed by treated animals, and, in any case, it is essential to consider possible nutritional effects caused by reduction of food intake due to distasteful or repellent test materials. In the case of gastric gavage, the test material is administered through a stomach tube or gavage needle; if a

TABLE 20.3 Variation in Toxicity by Route of Exposure

Chemical	Species/Gender	Route	LD50 (mg/kg)
N-methyl-*N* -(1-naphthyl) fluoroacetamide[a]	Mouse/M	Oral	371
		Dermal	402
		Subcutaneous	250
	Rat/M	Oral	115
		Dermal	300
		Subcutaneous	78
Chlordane[b]	Rat/M	Oral	335
		Dermal	840
Endrin[b]	Rat/M	Oral	18
		Dermal	18

[a] Data from Hashimoto, Y., et al. *Toxicol. Appl. Pharmacol.* **12**:536–547, 1968.
[b] Data from Allen, J. R., et al. *Pharmacol. Ther.* **7**:513–547, 1979.

solvent is necessary for preparation of dosing solutions or suspensions, the vehicle is administered also to control animals.

Dermal Dermal administration is required for estimation of toxicity of chemicals that may be absorbed through the skin, as well as for estimation of skin irritation and photosensitization. Compounds are applied, either directly or in a suitable solvent, to the skin of experimental animals after hair has been removed by clipping. Often dry materials are mixed with water to make a thick paste that can be applied in a manner to assure adequate contact with the skin. Frequently, the animals must be restrained to prevent licking and hence oral uptake of the material. Solvent and restraint controls should be considered when stress is involved. Skin irritancy tests may be conducted on either animals or humans, using volunteer test panels for human tests.

Inhalation The respiratory system is an important portal of entry and, for evaluation purposes, animals must be exposed to atmospheres containing potential toxicants. The generation and control of the physical characteristics of such contaminated atmospheres is technically complex and expensive in practice. The alternative—direct instillation into the lung through the trachea—presents problems of reproducibility as well as stress, and for these reasons is generally unsatisfactory.

Inhalation toxicity studies are conducted in inhalation chambers. The complete system contains an apparatus for the generation of aerosol particles, dusts, or gas mixtures of defined composition and particle size, a chamber for the exposure of experimental animals, and a sampling apparatus for the determination of the actual concentration within the chamber. All these devices present technical problems that are difficult to resolve. For rat studies, a particle size of 4μ is usually targeted.

Animals are normally exposed for a fixed number of hours each day and a fixed number of days each week. Exposure may be nose only, in which the nose of the animal is inserted into the chamber through an airtight ring, or whole body, in which animals are placed inside the chamber. In the latter case, variations due to unequal distribution in the test atmosphere are minimized by rotation of the position of the cages in the chamber during subsequent exposures. Whole body exposure results are usually less satisfactory due to test material accumulation on the fur of the animals and subsequent ingestion during grooming. Figure 20.1 shows a typical inhalation system and supporting equipment.

Injection Except in the case of certain pharmaceuticals and drugs of abuse, injection (parenteral administration) does not correspond to any of the expected modes of exposure. It may be useful, however, in studies of mechanism or in QSAR studies in order to bypass absorption and/or permit rapid action. Methods of injection include intravenous (IV), intramuscular (IM), intraperitoneal (IP), and SC. Infusion of test materials over an extended period is also possible. Again, both solvent controls and untreated controls are necessary for proper interpretation of the results.

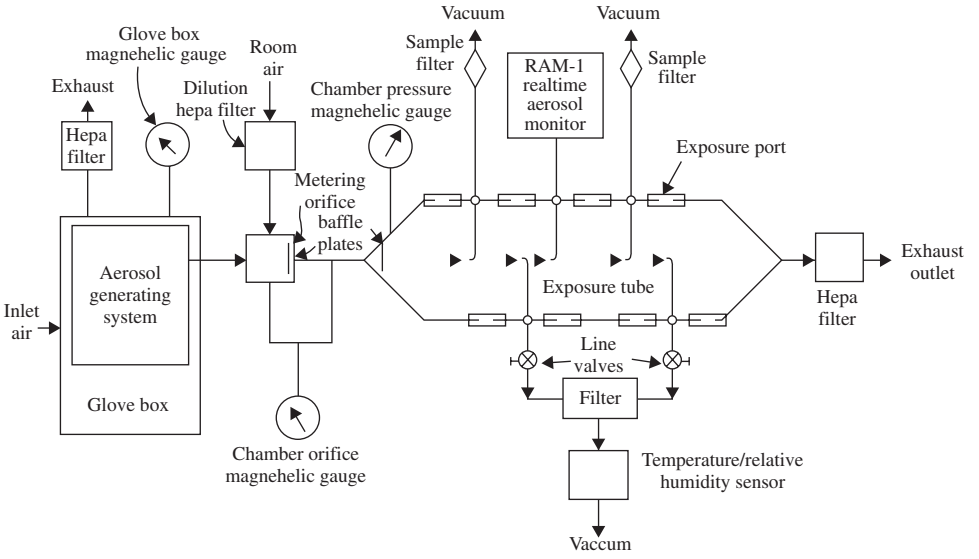

Figure 20.1 An inhalation exposure system. Modified from Adkins et al. *Am. Int. Hyg. Assoc. J.* **41**:494, 1980.

20.3 CHEMICAL AND PHYSICAL PROPERTIES

Although the determination of chemical and physical properties of known or potential toxicants does not constitute a test for toxicity, it is an essential preliminary for such tests.

The information obtained can be used as follows: Structure activity comparisons with other known toxicants may indicate the most probable hazards. These comparisons may also aid in identification in subsequent poisoning episodes. Determination of stability to light, heat, freezing, and oxidizing or reducing agents, may enable preliminary estimates of persistence in the environment as well as indicate the most likely breakdown products that may also require testing for toxicity. Establishing such properties as the lipid solubility or octanol/water partition coefficient may enable preliminary estimates of rate of uptake and persistence in living organisms. Vapor pressure may indicate whether the respiratory system is a probable route of entry. Acquiring knowledge of the chemical and physical properties is needed to develop analytical methods for the measurement of the compound and its degradation products. If the chemical is being produced for commercial use, similar information is needed on intermediates in the synthesis or by products of the process because both are possible contaminants in the final product.

20.4 EXPOSURE AND ENVIRONMENTAL FATE

Data on exposure and environmental fate are needed, not to determine toxicity, but to provide information that may be useful in the prediction of possible exposure in the event that the chemical is toxic. Primarily useful for chemicals released

into the environment such as pesticides, these tests include the rate of breakdown under aerobic and anaerobic conditions in soils of various types, the rates of leaching into surface water from soils of various types, or the rate of movement toward groundwater. The effect of physical factors on degradation through photolysis and hydrolysis studies and the identification of the product formed can indicate the rate of loss of the hazardous chemical or the possible formation of hazardous degradation products. Tests for accumulation in plants and animals and movement within the ecosystem are considered in Section 20.7.

20.5 *IN VIVO* TESTS

Traditionally, the basis for the determination of toxicity has been administration of the test compound, *in vivo*, to one or more species of experimental animal, followed by examination for clinical signs of toxicity and/or mortality in acute tests. In addition, pathological examination for tissue abnormalities is also performed, especially in tests of longer duration. The results of these tests are then used, by a variety of extrapolation techniques, to estimate hazard to humans. These tests are summarized in the remainder of this section. While these tests offer many advantages and are widely used, they suffer from a number of disadvantages: they require the use of experimental animals, the numbers of which are often deemed unnecessary by both animal rights and animal welfare advocates; they are extremely expensive to conduct; and they are time-consuming. As a result, they have been supplemented by many specialized *in vitro* tests, some of which are summarized in Section 20.6, and research is ongoing to further develop tests with fewer disadvantages.

20.5.1 Acute Toxicity

Acute toxicity test methods measure the adverse effects that occur within a short time of administration of a single dose of a test substance. This testing is performed principally in rodents and is usually done early in the development of a new chemical or product to provide information on its potential toxicity. This information is used to protect individuals who are working with the new material and to develop safe handling procedures for transport and disposal. The information gained also serves as the basis for hazard classification and labeling of chemicals in commerce. Acute toxicity data can help identify the mode of toxic action of a substance and may provide information on doses associated with target-organ toxicity and lethality that can be used in setting dose levels for repeated-dose studies. This information may also be extrapolated for use in the diagnosis and treatment of toxic reactions in humans. The results from acute toxicity tests can also provide information for comparison of toxicity and dose–response among members of chemical classes and help in the selection of candidate materials for further work.

The results of acute toxicity tests also have a wide variety of regulatory applications. These include determination of the need for childproof packaging, determination of reentry intervals after pesticide application, establishment of the requirement and basis for training workers in chemical use, determination of requirements for protective equipment and clothing, and decision making about general registration of pesticides or their restriction for use by certified applicators. Acute oral toxicity

may be used in risk assessments of chemicals for humans and nontarget environmental organisms.

The various national and international regulatory authorities have used different hazard classification systems in the past. In light of the importance of hazard classification, the Organisation for Economic Co-operation and Development (OECD) recently harmonized criteria for hazard classification for global use. For example, the five harmonized categories for acute oral toxicity (in mg/kg body weight) are: 0–5, 5–50, 50–300, 300–2000, and 2000–5000.

Acute Oral Testing Traditionally, acute oral toxicity testing focused on determining the dose that kills half the animals (i.e., the median lethal dose or LD_{50}), the timing of lethality following acute chemical exposure, as well as observing the onset, nature, severity, and reversibility of toxicity. The LD_{50} concept was developed by Trevan in 1927. Original testing methods were designed to characterize the dose–response curve by using several animals (usually at least 5/gender) at each of several test doses. Data from a minimum of three doses is required. The LD_{50} values are presented as estimated doses (mg/kg) with confidence limits. The simplest method for the determination of the LD_{50} is a graphic one and is based on the assumption that the effect is a quantal one (all or none), that the percentage responding in an experimental group is dose-related, and that the cumulative effect follows a normal distribution. Data from a typical example, its analysis and implications, are discussed in Chapter 10, Acute Toxicity.

As a result of much recent controversy, the LD50 test has been the subject of considerable regulatory attention and recent changes in requirements have been promulgated. These changes are intended to obtain more information but, at the same time, use fewer animals.

Criticism of the LD_{50} Test The criticisms of the test include:

- Used uncritically, it is an expression of lethality only, not reflecting other acute effects.
- It requires large numbers of experimental animals to obtain statistically acceptable values. Moreover, the results of LD_{50} tests are known to vary with species, strain, gender, age, and so on (Table 20.4); thus, the values are seldom closely similar from one laboratory to another, in spite of the numbers used.
- Because, for regulatory purposes, the most important information needed concerns chronic toxicity, little useful information is derived from the LD_{50} test. The small amount of information that is acquired could be acquired as well from an approximation requiring only a small number of animals.
- Extrapolation to humans is difficult.

TABLE 20.4 Factors Causing Variation in LD_{50} Values

Species	Health	Temperature
Strain	Nutrition	Time of day
Age	Gut contents	Season
Weight	Route of administration	Human error
Gender	Housing	

Support of the LD_{50} Test Continued use of the test has been advocated, however, on the grounds that it is of use in the following ways:

- Properly conducted, acute toxicity tests yield not only the LD_{50}, but also information on other acute effects such as cause of death, time of death, symptomatology, nonlethal acute effects, organs affected, and reversibility of nonlethal effects.
- Information concerning mode of action and metabolic detoxication can be inferred form the slope of the mortality curve.
- The results can form the basis for the design of subsequent subchronic studies.
- The test is useful as a first approximation of hazards to workers.
- The test is rapidly completed.

For the previously listed reasons, there has been a concerted effort in recent years to modify the concept of acute toxicity testing as it is embodied in the regulations of many countries and to substitute more meaningful methods that use fewer experimental animals. The article by Zbinden and Flury-Roversi is an excellent summary of the factors affecting LD_{50} determinations, the advantages and disadvantages of requiring such tests, and the nature and value of the information derived. It concludes that the acute toxicity test (single-dose toxicity) is still of considerable importance for the assessment of risk posed by new chemical substances, and for a better control of natural and synthetic agents in the human environment. It is not permissible, however, to regard a routine determination of the LD_{50} in various animal species as a valid substitute for an acute toxicity study.

Current Status Recently, attention has been focused on developing alternatives to the classical LD_{50} test to reduce the number of animals used or refine procedures to make exposures less stressful to animals. OECD adopted several alternative methods for determining acute oral toxicity: a limit test for materials with anticipated low toxicity, a fixed-dose procedure, an acute toxic class method, and an up-and-down procedure. The fixed-dose procedure and the acute toxic class method estimate the LD_{50} within a dose range for use in classification and labeling. The up-and-down procedure generates point estimates and confidence intervals of the LD_{50} and therefore, may be useful in a wider set of applications.

The fixed-dose procedure (Guideline OECD 420) aims to identify the appropriate hazard class for new chemicals; it does not provide a point estimate of the LD_{50}. This method calls for testing animals sequentially at one of four doses: 5, 50, 300, or 2000 mg/kg body weight. The test begins with a sighting study in which animals are tested, one at a time, at doses selected from the set doses. Once clear signs of toxicity appear, additional animals (females or the more sensitive sex) are dosed at that level for a total of five animals. Subsequent groups of animals may receive doses at higher or lower levels, if necessary, depending on the outcome of the previous group. Decision criteria based on the number of animals surviving or showing evident toxicity provide for classification decisions.

The acute toxic class method (Guideline OECD 423) aims to identify the appropriate hazard and labeling classification and provides a range for lethality rather than a point estimate of the LD_{50}. Groups of three animals (females or the more

sensitive sex) receive one of four or five doses: 5, 50, 300, 2000 and, if necessary, 5000 mg/kg body weight. Depending on the survival or mortality of the first group of animals, three or more animals may receive the same or a higher or lower dose. The number of animals that survive or die determines the classification decisions.

The up-and-down procedure (Guideline OECD 425) employs sequential dosing, using only a single animal at each step, the dosage depending on whether the previously dosed animal lives or dies. The test provides a point estimate of lethality and confidence intervals, and can be used to evaluate lethality up to 5000 mg/kg. The main test incorporates elements of range finding and uses a flexible stopping point. A sequential limit test uses up to five animals. Default dose spacing is 3.2 times the previous dose. The starting dose should be slightly below the estimated LD_{50}. If no information is available to estimate the LD_{50}, the starting dose is 175 mg/kg. A computer program was developed by the U.S. Environmental Protection Agency (U.S. EPA) to simplify both the experimental phase of the test and the calculation of the LD_{50} and confidence intervals.

For all three guidelines, selection of a starting dose close to the actual LD_{50} should decrease the number of animals necessary, reduce study duration, and decrease the amount of test substance needed. Therefore, it is desirable that all available information on the test substance be made available to the testing laboratory for consideration prior to conducting the study. Such information includes the identity and chemical structure of the substance, its physicochemical properties, the results of any other toxicity test tests on the substance, toxicological data on structurally related substances, the anticipated uses of the substance, or cytotoxicity data on the substance. This information will aid the testing laboratory in selecting the most appropriate test to satisfy regulatory requirements and in choosing the starting dose.

As with the traditional acute oral toxicity methods, the alternative tests involve the administration of a single-bolus dose of a test substance to fasted healthy young adult rodents by oral gavage, observation for morbidity/mortality for up to 14 days after dosing, with recording of body weight (weekly) and clinical signs (daily), and a necropsy at study termination. At the time of dosing, each animal should be between 8 and 12 weeks old and its weight should fall in an interval within ±20% of the mean weight of all previously dosed animals taken on their day of dosing. Observation of the postdosing effects on each animal should be for at least 48 h or until it is clear whether the dosed animals will survive. However, depending on the characteristics of the test material, investigators can vary this time between dosing, so long as the interval is sufficient. Only when the results are clear can a decision be made about whether an additional dose is necessary, and if so, whether to dose the next animal or group of animals at the same, higher, or lower dose. The information from every animal, even those that die after the initial observation period, is used in the final determination of the test outcome.

These newer methods call for testing to be done in a single gender to reduce variability in the test population. This reduction in variability in turn minimizes the number of animals needed. Normally, females are used. Although there is usually little difference in sensitivity between males and females, in those cases where there are observable differences, females are most commonly the more sensitive gender. Normally, animal suppliers have an excess of female rats because many researchers order only male rats to avoid physiological changes associated with estrus cycling

in females; therefore, preferential use of female animals for acute testing should not result in excess male animals.

Eye Irritation Because of the prospect of permanent blindness, ocular toxicity has long been a subject of both interest and concern. Although all regions of the eye are subject to systemic toxicity, usually chronic but sometimes acute, the tests of concern in this section are tests for irritancy of compounds applied topically to the eye. The tests used are all variations of the Draize test, and the preferred experimental animal is the albino rabbit.

The test consists of placing the material to be tested directly into the conjunctival sac of one eye, with the other eye serving as the control. The lids are held together for a few seconds, and the material is left in the eye for at least 24 h. After that time, it may be rinsed out, but in any case, the eye is examined and graded after 1, 2, and 3 days. Grading is subjective and is based on the appearance of the cornea, particularly as regards opacity; the iris, as regards both appearance and reaction to light; the conjunctiva, as regards redness and effects on blood vessels; and the eyelids, as regards swelling. Fluorescein dye may be used to assist visual examination because the dye is more readily absorbed by damaged tissues, which then fluoresce when the eye is illuminated. Each end point in the evaluation is scored on a numerical scale and chemicals are compared on this basis. In addition to the "no-rinse" test, some protocols also investigate the effect of rinsing the eye 1 min after exposure to determine if this reduces the potential for irritation. In addition, eyes may be graded for up to 21 days after administration of an irritating test material to evaluate recovery.

The eye irritation test is probably the most criticized by advocates of animal rights and animal welfare, primarily on the grounds that it is inhumane. It has also been criticized on narrower scientific grounds in that both concentration and volumes used are unrealistically high, and that the results, because of high variability and the greater sensitivity of the rabbit eye, may not be applicable to humans. It is clear, however, that because of great significance of visual impairment, tests for ocular toxicity will continue.

Attempts to solve the dilemma have taken two forms: to find substitute *in vitro* tests and to modify the Draize test so that it becomes not only more humane but also more predictive for humans. Substitute tests consist of attempts to use cultured cells or eyes from slaughtered food animals, but neither method is yet acceptable as a routine test. Modifications consist primarily of using fewer animals. Usually, one animal is tested first and, if the material is severely irritating, no further eye testing is conducted. EPA has reduced the required number of animals from six to three. In addition, eye irritation should never be carried out on materials with a pH of less than 2 or more than 10 as these materials can be assumed to be potential eye irritants.

Dermal Irritation and Sensitization These are tests for dermal irritation caused by topical application of chemicals and fall into four general categories: primary irritation, cutaneous sensitization, phototoxicity, and photosensitization. Because many foreign chemicals come into direct contact with the skin, including cosmetics, detergents, bleaches, and many others, these tests are considered essential to the proper regulation of such products. Less commonly, dermal effects may be caused by systemic toxicants.

In the typical primary irritation test, the backs of albino rabbits are clipped free of hair and an area of about 5 cm^2 on each rabbit is used in the test. This area is then treated with either 0.5 mL or 0.5 g of the compound to be tested and then covered with a gauze pad. The entire trunk of the rabbit is wrapped to prevent ingestion. After 4–24 h, the tape and gauze are removed, the treated areas are evaluated for erythematous lesions (redness of the skin produced by congestion of the capillaries) and edematous lesions (accumulation of excess fluid in SC tissue), each of which is expressed on a numerical scale. After an additional 24–48 h, the treated areas are again evaluated.

Skin sensitization tests are designed to test the ability of chemicals to affect the immune system in such a way that a subsequent contact causes a more severe reaction than the first contact. The latter may be elicited at a much lower concentration and in areas beyond the area of initial contact. The antigen involved is presumed to be formed by the binding of the chemical to body proteins, the ligand–protein complex then being recognized as a foreign protein to which antibodies can be formed. Subsequent exposure may then give rise to an allergic reaction. Skin sensitization tests generally follow protocols that are modifications of the Buchler (dermal inductions) method or the Magnesson and Kligman (intradermal inductions) method. The test animal commonly used in skin sensitization tests is the guinea pig; animals are treated with the test compound in a suitable vehicle, with the vehicle alone, or with a positive control such as 2, 4-dinitrochlorobenzene (a relatively strong sensitizer) or cinnamaldehyde (a relatively weak sensitizer) in the same vehicle. During the induction phase, the animals are treated for each of 3 days evenly spaced during a 2-week period. This is followed by a 2-week rest period followed by the challenge phase of the test. This consists of a 24-h topical treatment carried out as described for primary skin irritation tests. The lesions are scored on the basis of severity and the number of animals responding (incidence). If there is a greater skin reaction in the animals given induction doses compared to those given the test material for the first time, the compound is considered to be a dermal sensitizer.

Other test methods include those in which the induction phase is conducted by intradermal injection together with Freund's adjuvant (a chemical mixture that enhances the antigenic response) and the challenge by dermal application, or tests in which both induction and challenge doses are topical but the former is accompanied by intradermal injections of Freund's adjuvant. It is important that compounds that cause primary skin irritation be tested for skin sensitization at concentrations low enough that the two effects are not confused.

Phototoxicity tests are designed to evaluate the combined dermal effects of light (primarily ultra violet [UV] light) and the chemical in question. Tests have been developed for both phototoxicity and photoallergy. In both cases, the light energy is believed to cause a transient excitation of the toxicant molecule, which, on returning to the lower energy state, generates a reactive, free-radical intermediate. In phototoxicity, these organic radicals act directly on the cells to cause lesions, whereas in photoallergy, they bind to body proteins. These modified proteins then stimulate the immune system to produce antibodies, because the modifications cause them to be recognized as foreign or "nonself" proteins. These tests are basically modifications of the tests for primary irritation and sensitization except that, following

application of the test chemical, the treated area is irradiated with UV light. The differences between the animals treated and irradiated and those treated and not irradiated is a measure of the phototoxic effect.

Safety Pharmacology Studies Safety pharmacology studies investigate the potential undesirable pharmacodynamic effects of a test article on physiological functions in relationship to exposure. These tests are typically conducted as part of the development of new drugs. The objectives of safety pharmacology studies are threefold. First, to identify undesirable effects of a test article which may have relevance to its use in humans. Second, to evaluate a test article for possible effects observed in toxicology or clinical studies. And third, to investigate the mechanism underlying any undesirable effects of the test article. Safety pharmacology consists of a core battery of studies with follow-up studies as indicated by preliminary findings. The core studies are designed to target vital organ systems, particularly the central nervous system, cardiovascular system, and pulmonary system. These studies are typically conducted using small numbers of rats and dogs. In the study for pulmonary function, end points measured are respiratory rate, minute volume, and tidal volume. In the cardiovascular telemetry study, end points include heart rate, blood pressure, and electrocardiogram evaluation. In telemetry studies, a radio transmitter is implanted in all animals to permit continuous monitoring for 24 h pretest and 24 h after dosing. A cardiopulmonary study can also be conducted in which respiratory rate, minute volume, tidal volume, blood pressure, heart rate, electrocardiogram, and body temperature are monitored in restrained animals for typically 2 h after dosing.

20.5.2 Subchronic Tests

Subchronic tests examine toxicity caused by repeated dosing over an extended period, but not one that that constitutes a significant portion of the normal life span of the species tested. A 28- or 90-day oral study in the rat or dog would be typical of this type of study, as would a 21- to 28-day dermal application study or a 28- to 90-day inhalation study. Such tests provide information on essentially all types of chronic toxicity other than carcinogenicity and are usually believed essential for establishing the dose regimens for prolonged chronic studies. They are frequently used as the basis for the determination of the no observable effect level (NOEL). This value is often defined as the highest dose level at which no deleterious or abnormal effect can be measured, and is used in risk assessments. Subchronic tests are also useful in providing information on target organs and on the potential of the test chemical to accumulate in the organism.

Ninety-Day Tests Chemicals are usually tested by administration in the diet, less commonly in the drinking water, and only when absolutely necessary, by gavage, because the last process involves much handling and subsequent stress. Numerous experimental variables must be controlled and biologic variables must be evaluated. In addition, the number of end points that can be measured is large and, as a consequence, record keeping and data analysis must be carefully planned. If all is done with care, much may be learned from such tests.

Experimental (Nonbiologic) Variables Several environmental variables may affect toxicity evaluations, some directly and others by their effects on animal health. Major deviations from the optimum temperature and humidity for the species in question can cause stress reactions. Stress can also be caused by housing more than one species of experimental animal in the same room. Many toxic or metabolic effects show diurnal variations that are related to photoperiod. Cage design and the nature of the bedding have also been shown to affect the toxic response. Thus, the optimum housing conditions are clean rooms, each containing a single species, with the temperature, humidity, and photoperiod being constant and optimized for the species in question. Cages should be the optimum design for the species, bedding should be inert (not cause enzyme induction or other metabolic effect), and cages should not be overcrowded, with individual caging whenever possible.

Dose selection, preparation, and administration are all important variables. Subchronic studies are usually conducted using three (less often, four) dose levels. The highest should produce obvious toxicity but not high mortality and the lowest no toxicity (NOEL), whereas the intermediate dose should give effects clearly intermediate between these two extremes. Although the dose can be extrapolated from acute tests, such extrapolation is difficult, particularly in the case of compounds that accumulate in the body; and frequently, a 14-day range finding study is made. Although the route of administration should ideally mimic the expected route of exposure in humans, in practice, the chemical is usually administered *ad libitum* in the diet, because this is, on average, most appropriate. Diets containing known amounts of the test material are presented to the animals. Measurement of food consumption is recommended to provide an estimate of the test material consumed. In cases in which a highly accurate measurement of dose is an important factor in the experimental design, the animals may be treated by gavage or by capsules containing the test material.

To avoid effects from nonspecific variations on the diet, enough feed from the same batch should be obtained for the entire study. Part is set aside for the controls, and the remainder is mixed with the test chemical at the various dose levels. Care should be taken to store all food in such a way that not only does the test chemical remain stable, but the nutritional value is also maintained. The identity and concentration of the test chemical should be checked periodically by chemical analysis. Treated diets may be prepared at set intervals, such as weekly, depending upon the stability of the test material in the diet.

Subchronic studies are usually conducted with 10–20 males and 10–20 females of a rodent species at each dose level and 4–8 of each gender of a larger species, such as the dog, at each dose level. Animals should be drawn from a larger group and assigned to control or treatment groups by a random process, but the larger group should not vary so much that the mean weights and ages of the subgroups vary significantly at the beginning of the experiment.

Biologic Variables Subchronic studies should be conducted on two species, ideally a rodent, and a nonrodent. Ideally, the species chosen should be those with the greatest pharmacokinetic and metabolic similarity to humans; for the compound in question, this information is seldom available. In practice, the most common rodent used is the rat, and the most common nonrodent used is the dog. It has long been held that inbred rodent strains should be used to reduce variability. This and the

search for strains that were sensitive to chemical carcinogenesis but did not have an unacceptably high spontaneous tumor rate led to widespread use of the F344 rat and B6C3F1 mouse. Other researchers believe that an outbred strain such as the Sprague Dawley rat is more robust and prefer to use them.

Although ideally the age should be matched to the expected exposure period in terms of the stage of human development, this is not often done. Young adult or adolescent animals that are still growing are preferred in almost all cases, and both sexes are routinely used.

Good animal care is critical at all times because toxicity has been shown to vary with diet, disease, and environmental factors. Animals should be quarantined for some time before being admitted to the test area, their diet should be optimum for the test species, and the facility should be kept clean at all times. Regular inspection by a veterinarian is essential, and any animal showing unusual symptoms not related to the treatment (e.g., in controls or in low-dose but not high-dose animals) should be removed from the test and autopsied.

Results Although the information required from subchronic tests varies somewhat from one regulatory agency to another, the requirements are basically similar (Table 20.5).

For explanatory purposes, the data obtained from these tests can be described as two types: that which can be obtained from living animals during the course of the test and that which is obtained from animals sacrificed either during or at the end of the test period. Many of the tests performed on living animals can be carried out first before the test period begins to provide a baseline for comparison to subsequent measurement. A satellite group of treated animals can be added to the test for evaluation of "recovery." For these animals, the treated food would be removed at the end of the test period, and they would be returned to the control diet for 21–28 days while the various end points are followed. This is done to establish whether any effects noted are reversible. Autopsies should be performed on all animals found dead or moribund during the course of the test. The following is a list of end points that may be measured during a 90-day oral toxicity study.

A. In-Life Tests. Interim tests are carried out at intervals before the study, to establish baselines at intervals during the study, and at the end of the study.
    1. Appearance—Mortality and morbidity as well as the condition of the skin, fur, mucous membranes, and orifices should be checked at least daily. Presence of palpable masses or external lesions should be noted.
    2. Eyes—Ophthalmologic examination of both cornea and retina should be carried out at the beginning and at the end of the study.
    3. *Food consumption*
    4. *Body weight*
    5. *Behavioral abnormalities*
    6. *Respiration rate*
    7. ECG—Particularly with the larger animals
    8. EEG—particularly with the larger animals.

TABLE 20.5 Summary of Subchronic Test Guidelines by Regulatory Agency

Character of Tests	EPA Pesticide Assessment Guidelines	FDA "Red Book"	FDA IND/NDA Pharmacology Review Guidelines	OECD	EPA Health Effects Guidelines	NTP
Purpose	Pesticide registration support	Food and color additives; safety assessment	IND/NDR pharmacology review guidelines	Assessment and evaluation of toxic characteristics	Select chronic dose levels	Predict dose range for chronic study
	No observed effect level	No observed adverse effects, no effect level	Characterize pharmacology, toxicology, pharmacokinetics, and metabolism of drugs for precautionary clinical decisions	Select chronic dose levels. Use information and permissible human exposure	Establish safety criteria for human exposure, no observed effect level	
Species	Rat, dog, mouse	Rat, dog	Rat, mouse, other rodents, dog, monkey, other non-rodents	Rat, dog	Rat, dog	Fischer 344 rats, B6CF1 mice
Doses	3 dose levels	3 dose levels	3 dose levels	3 dose levels	3 dose levels	5 dose levels
End points	Clinical signs	Clinical signs				
	Ophthalmology	Ophthalmology	Ophthalmology	Ophthalmology	Ophthalmology	Ophthalmology
	Hematology	Hematology	Hematology	Hematology	Hematology	Hematology
	Clinical chemistry	Clinical chemistry	Clinical chemistry	Clinical chemistry	Clinical chemistry	Clinical chemistry
	Histopathology	Histopathology	Histopathology	Histopathology	Histology	Weight loss Histopathology
	Target organs	Target organs	Target organs Behavioral and pharmacological effects	Target organs	Target organs	Target organs

Source: National Toxicology Program, Washington, DC: Department of Health and Human Services. Report of the NTP Ad hoc Panel on Chemical Carcinogenesis Testing and Evaluation.

EPA, Environmental Protection Agency; FDA, Food and Drug Administration; IND/NDA, investigative new drug/new drug assessment; OECD, Organization for Economic Cooperation and Development; NTP, National Toxicology Program.

9. Hematology—Assessment should be made prior to chemical administration (pretest) and at least prior to termination. Hemoglobin, hematocrit, RBC, WBC, differential counts, platelets, reticulocytes, and clotting parameters should be assessed.
10. Blood Chemistry—Should be done pretest and at least prior to termination. Electrolytes and electrolyte balance; acid–base balance; glucose; urea nitrogen; serum lipids; serum proteins (albumin-globulin ratio); enzymes indicative of organ damage such as transaminases and phosphatases; also, plasma and RBC cholinesterase levels should be measured. Toxicant and metabolite levels should be assessed as needed.
11. Urinalysis—Should be done pretest and at least prior to termination. Microscopic appearance (sediment, cells, stones, etc.), pH, specific gravity, chemical analysis for reducing sugars, proteins, ketones, bilirubin etc., as well as toxicant and metabolite levels should all be assessed.
12. Fecal Analysis—Occult blood, fluid content, and toxicant and metabolite levels should be assessed.

B. Termination Tests. Because the number of tissues that may be sampled is large (Table 20.6) and the number of microscopic methods is also large, it is necessary to consider all previous results before carrying out the pathological examination. For example, clinical tests or blood chemistry analyses may implicate a particular target organ that can then be examined in greater detail. All control and high dose animals are examined in detail. If lesions are found, the next lowest dose group is examined for these lesions, and this method continues until a no effect group is reached.

Because pathology is largely a descriptive science with a complex terminology that varies form one practitioner to another, it is critical that the terminology be defined at the beginning of the study and that the same pathologist examine the slides form both treated and control animals. Pathologists are not in agreement on the necessity or the wisdom of coding slides so that the assessor is not aware of the treatment given the animal from which a particular slide

TABLE 20.6 Tissues and Organs to be Examined Histologically in Chronic and Subchronic Toxicity Tests

Adrenals	Larynx	Salivary gland
Bone and bone marrow	Liver	Sciatic nerve
Brain	Lungs and bronchi	Seminal vesicles
Cartilage	Lymph nodes	Skin
Cecum	Mammary glands	Spinal cord
Colon	Mandibular lymph node	Spleen
Duodenum	Mesenteric lymph node	Stomach
Esophagus	Nasal cavity	Testes
Eyes	Ovaries	Thigh muscle
Gallbladder	Parathyroids	Thymus
Ileum	Pituitary	Urinary bladder
Jejunum	Prostate	Uterus
Kidneys	Rectum	

is derived. Such coding, however, eliminates unintentional bias, a hazard in a procedure that depends on subjective evaluation. Other items of utmost importance are quality control, slide identification, and data recording. Many tissues may be examined; consequently, an even larger number of tissue blocks must be prepared. Because each of these may yield many slides to be stained, comparable quality of staining and the accurate correlation of a particular slide with its parent block, tissue, and animal is critical.

1. *Necropsy*—This must be conducted with care to avoid postmortem damage to the specimens. Tissues are removed, weighed, and examined closely for gross lesions, masses, etc. Tissues are then fixed in buffered formalin for subsequent histologic examination.
2. *Histology*—The tissues listed in Table 20.6 plus any lesions, masses, or abnormal tissues are embedded, sectioned, and strained for light microscopy. Parafin embedding and staining with hemotoxylin and eosin are the preferred routine methods, but special stains may be used for particular tissues or for a more specific examination of certain lesions. Electron microscopy may also be used for more specific examination of lesions or cellular changes after their initial localization by more routine methods.

Repeated Dose Dermal Tests Twenty-one- to twenty-eight-day dermal tests are particularly important when the expected route of human exposure is by contact with the skin, as is the case with many industrial chemicals or pesticides. Compounds to be tested are usually applied daily to clipped areas on the back of the animal, either undiluted or in a suitable vehicle. In the latter case, if a vehicle is used, it is also applied to the controls. Selection of a suitable solvent is difficult because many affect the skin, causing either drying or irritation, whereas others may markedly affect the rate of penetration of the test chemical. Corn oil, ethanol, or carboxymethyl cellulose are preferred to dimethyl sulfoxide (DMSO) or acetone. It should also be considered that some of the test chemical may be ingested as a result of grooming by the animal, although this can be controlled to some extent by use of restraining collars and/or wrapping.

The criteria for environment, dose selection, species selection, and so on, are not greatly different to the criteria used for 90-day feeding tests, although the list of end points to be examined is often shorter (e.g., fewer organs may be examined). It is necessary, however, to pay close attention to the skin at the point of application because local effects may be as important as systemic ones.

Twenty-Eight to Ninety-Day Inhalation Tests Inhalation studies are indicated whenever the route of exposure is expected to be through the lungs. Animals are commonly exposed for 6–8 h each day, 5 days each week, in chambers of the type previously discussed. Even in those cases in which the animals are maintained in the inhalation chambers during nonexposure hours, food is always removed during exposure. In spite of this, exposure tends to be in part dermal and, due to grooming of the fur, in part oral. Environmental and biologic parameters are the same as for other subchronic tests, as are the routine end points to be measured before, during, and after the test period. Particular attention must be paid, however, to effects on

the tissues of the nasal cavity and the lungs, because these are the areas of maximum exposure.

If the test material is particulate, consideration must be given to the particle size and its inhalation potential. Particles of 4 μ in size are considered to be inhalable; larger particles will be cleared from the respiratory tract by ciliary action and subsequently swallowed (oral exposure) or expelled by sneezing or expectoration.

20.5.3 Chronic Tests

Chronic tests are those conducted over a significant part of the life span of the test animal. The duration of a chronic study is generally 1 year or more. Typically, rat and dog are the preferred species; for carcinogenicity studies, rats and mice are used.

Chronic Toxicity and Carcinogenicity Descriptions of tests for both chronic toxicity and carcinogenicity are included here because the design is similar—so similar in fact that they can be combined into one test. Chronic toxicity tests are designed to discover any of numerous toxic effects and to define safety margins to be used in the regulation of chemicals. As with subchronic tests, two species are usually used, one of which is either a rat or a mouse strain, in which case the tests are run for 2 years or 1.5–2.0 years, respectively. Data is gathered after 1 year to determine chronic effects without potential confounding effects of aging. Data are gathered after 1.5 years (mouse) or 2 years (rat) to determine carcinogenic potential. The non-rodent species used may be the dog, a nonhuman primate, or, rarely, a small carnivore such as the ferret. Chronic toxicity tests may involve administration in the food, in the drinking water, by capsule, or by inhalation, the first being the most common. Gavage is rarely used. The dose used is the maximum tolerated dose (MTD) and usually two lower doses, perhaps 0.25 MTD and 0.125 MTD with the lowest dose being a predicted no effect level.

MTD The MTD has been defined for testing purposes by the U.S. EPA as:

> the highest dose that causes no more than a 10% weight decrement, as compared to the appropriate control groups; and does not produce mortality, clinical signs of toxicity, or pathologic lesions (other than those that may be related to a neoplastic response) that would be predicted to shorten the animals' natural life span.

This dose is determined by extrapolation from subchronic studies.

The requirements for animal facilities, housing, and environmental conditions are as described for subchronic studies. Special attention must be paid to diet formulation because it is impractical to formulate all of the diets for a 2-year study form a single batch. In general, semisynthetic diets of specified components should be formulated regularly and analyzed before use for test material content.

The end points used in these studies are those described for the subchronic study: appearance, ophthalmology, food consumption, body weight, clinical signs, behavioral signs, hematology, blood chemistry, urinalysis, organ weights, and pathology. Some animals may be killed at fixed intervals during the test (e.g., 6, 12, or 18 months) for histologic examination. Particular attention is paid to any organs or tests that showed compound-related changes in the subchronic tests.

Carcinogenicity tests have many requirements in common (physical facilities, diets, etc.) with both chronic and subchronic toxicity tests as previously described. Because of the numbers and time required, these tests are usually carried out using rats and/or mice, but in some cases, a non-rodent species may also be used. The chemical under test may be administered in the food, in the drinking water, by dermal application, by gavage or by inhalation, the first two methods being the most common. Because the oncongenic potency of chemicals varies through extreme limits, the purity of the test chemical is of great concern. A 1% contaminant need only be 100 times as potent as the test chemical to have an equivalent effect, and differences of this magnitude and greater are not unheard of.

Dosing is carried out over the major part of the life span for rodents, beginning at or shortly after weaning. The highest dose used is the MTD. The principal end point is tumor incidence as determined by histologic examination. The statistical problem of distinguishing between spontaneous tumor occurrence in the controls and chemical-related tumor incidence in the treated animals is great; for that reason, large numbers of animals are used. A typical test involves 50 or more rats or mice of each gender in each treatment group. Some animals are necropsied at intermediate stages of the test (e.g., at 12 months), as are all animals found dead or moribund. All surviving animals are necropsied at the end of the test. Tissues to be examined are listed in Table 20.6, with particular attention being paid to abnormal masses and lesions.

Reproductive Toxicity and Teratogenicity The aim of developmental and reproductive testing is to examine the potential for a compound to interfere with the ability of an organism to reproduce. This includes testing to assess reproductive risk to mature adults as well as the developing individual at various stages of life, from conception to sexual maturity. Traditionally, animal studies have been conducted in three "segments": (I) in adults, treatment during a premating period and optionally continuation for the female through implantation or lactation; (II) in pregnant animals treatment during the major period of organogenesis; and (III) treatment of pregnant/lactating animals from the completion of organogenesis through lactation (peri- and postnatal study). Although guidelines addressing treatment regimens have been rather similar throughout the world, required end points measured in adults and developing organisms have varied. International harmonization of guidelines has shown a need for flexibility in testing for reproductive and developmental toxicity, and toxicologists are now often challenged to design unique studies to examine potential effects on all the parameters considered in the classical segment I, II, and III studies. In adults, these include development of mature egg and sperm, fertilization, implantation, delivery of offspring (parturition), and lactation. In the developing organism, these include early embryonic development, major organ formation, fetal development and growth, postnatal growth including behavioral assessments, and attainment of full reproductive function. These evaluations are usually best carried out in several separate studies.

Some Definitions in Reproductive Biology At this point, some discussion of reproductive biology is helpful in the understanding of study designs to evaluate reproductive and developmental toxicity. Tests to assess general reproductive

performance and fertility are generally conducted using rats. In the rat, multiple eggs are ovulated from mature follicles in the ovary. The follicle that an egg leaves behind develops into glandular tissue known as a corpus luteum. The corpora lutea secrete progesterone, a hormone needed to maintain pregnancy (unlike humans in which progesterone is secreted by the placenta). Corpora lutea are visible as blister-like protuberances on the ovary. A count of the corpora lutea in the ovary allows one to determine the maximum number of potential offspring for that pregnancy. Fertilized eggs develop into zygotes that may attach to the wall of the uterus (implantation). The discrete areas of implantation may be observed and counted upon examination of the uterus at C-section. Calculation of pre- and postimplantation loss are important end points in a reproductive toxicity study. Preimplantation loss is the death of a fertilized ova prior to implantation in the uterine wall. Postimplantation loss (i.e., resorption and/or fetal death) is the death of the conceptus after implantation in the uterine wall and prior to parturition. Postimplantation loss can be broken down into early and late resorptions and fetal death. A late resorption has discernable features such as limbs, eyes, and nose, whereas an early resorption has none of these features.

Single and Multiple Generation Tests Fertility and general reproductive performance can be evaluated in single and multiple generation tests. These tests are usually conducted using rats. Fertility is defined as the ability to produce a pregnancy while the ability to produce live offspring is known as fecundity. An abbreviated protocol for a single generation test is shown in Figure 20.2.

In typical tests, 25 males per dose group are treated for 70 days prior to mating and 25 females per dose group are treated for 14 days premating. The number of animals is chosen to yield at least 20 pregnant females per dose group including controls. The treatment durations are selected to coincide with critical times during which spermatogenesis and ovulation occur. It takes approximately 70 days in the rat for spermatogonial cells to become mature sperm capable of fertilization. In the female rat, the estrus cycle length is 4–5 days and a 14-day dosing period is considered sufficient time to detect potential effects on hormonal or other systems which may effect ovulation. In some study designs, both males and females are treated for 70 days premating. Treatment of the females is continued through pregnancy (21 days) and until the pups are weaned. Pups are usually 21 days of age. The test compound is administered at three dose levels either in the feed, in drinking water, or by gavage. The high dose is chosen to cause some, but not excessive, maternal toxicity (e.g., an approximate 10% decrease in body weight gain, or effects on target organs). Low doses are generally expected to be no-effect levels.

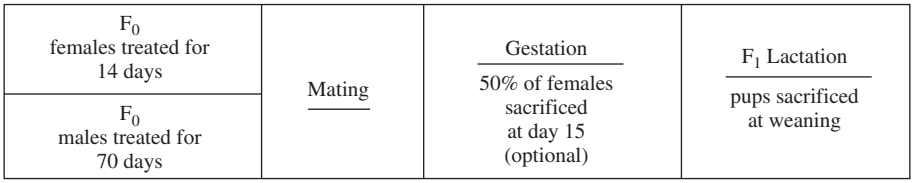

Figure 20.2 Abbreviated protocol for a one generation reproductive toxicity test.

After the premating period, the rats are placed in cohabitation, with one male and one female caged together. Mating is confirmed by the appearance of spermatozoa in a daily vaginal smear. Day 1 of gestation is the day insemination is confirmed. The females bear and nurse their pups. After birth, the pups are counted, weighed, and examined for external abnormalities. The litters are frequently culled to a constant number (usually 8–10) after 4 days. At weaning, the pups are killed and autopsied for gross and internal abnormalities. In a multigeneration study, approximately 25 of each gender/group are saved to produce the next generation. Brother-sister pairings are avoided. Treatment is continuous throughout the test, which can be carried out for two, sometimes, three generations. An abbreviated protocol for a multiple generation test can be seen in Figure 20.3. Note that the parental generation is known as the F_0 generation and the offspring are known as the F_1's and F_2's. In some studies, parents produce two litters, for example the F_1A and F_1B litters.

Because both males and females are treated in this type of study design, it is not possible to distinguish between maternal and paternal effects in the reproductive

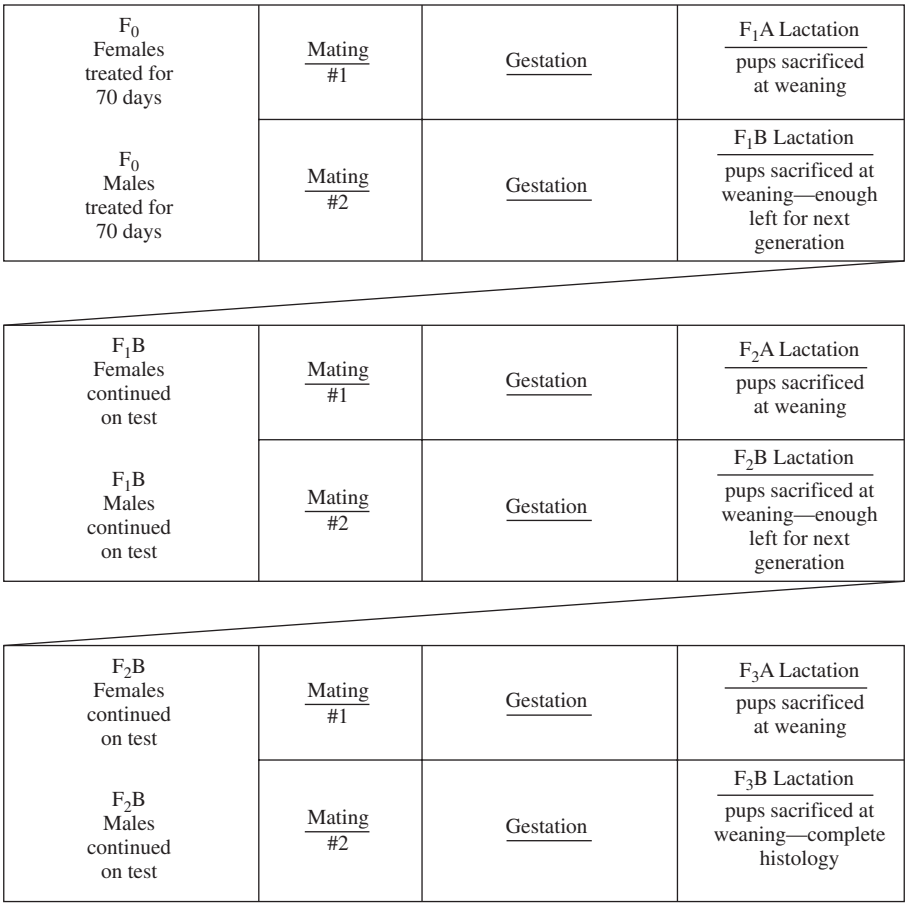

Figure 20.3 Abbreviated protocol for a multigeneration reproductive toxicity test.

performance. To permit this separation, it is necessary to treat additional animal to the stage of mating and then breed them to untreated members of the opposite sex. Similarly, if effects are seen postnatally, it may not be possible to distinguish between effects mediated *in utero* or mediated by lactation. This distinction can be made by "cross-fostering" the offspring of treated females to untreated females and vice versa.

The end points observed in these types of tests, depending on study design, are as follows:

1. Fertility index, the number of pregnancies relative to the number of matings
2. The number of live births, relative to the number of total births
3. Preimplantation death, or number of corpora lutea in the ovaries relative to the number of implantation sites
4. Postimplantation death, or the number of resorption sites in the uterus relative to the number of implantation sites
5. Duration of gestation
6. Effects on male or female reproductive systems
7. Litter size and condition, gross morphology of pups at birth, gender and ano-genital distance
8. Survival of pups
9. Weight gain and performance of pups
10. Time of occurance of developmental landmarks, for example, eye opening, tooth eruption, vaginal opening in females, preputial separation in males
11. Morphological abnormalities in weanlings

Results from single and multiple generation tests provide important information for assessment of test materials that may perturb a variety of systems including the endocrine system. A number of variations of the single and multiple generation tests exist. For example, a number of weanlings may be left to develop and be tested later for behavioral and/or physiological defects (e.g., developmental neurotoxicity testing).

Teratology is the study of abnormal fetal development. For an agent to be labeled a teratogen, it must significantly increase the occurence of adverse structural or functional abnormalities in offspring after its administration to the female during pregnancy or directly to the developing organism. In teratology testing, exposure to the test chemical may be from implantation to parturition, although it has also been restricted to the period of major organogenesis, the most sensitive period for inducing structural malformations. Observations may be extended throughout life, but usually, they are made immediately prior to birth after a C-section. The end points observed are mainly morphologic (structural changes and malformations), although embryo-fetal mortality is also used as an end point. Figure 20.4 shows an outline of a typical teratology study.

Teratology studies are carried out in two species, a rodent species (usually the rat) and in another species such as the rabbit (rarely in the dog or primate). Enough females should be used so that, given normal fertility for the strain, there are 20 pregnant females in each dosage group. Traditionally, the timing of compound

Teratology

Untreated females Untreated males	Mating	Gestation Pregnant females treated on days 6–15. Pups and dams sacrificed day 20.

Perinatal/Postnatal

Untreated females Untreated males	Mating	Gestation Pregnant females treated on days 15–21	Lactation Females treated to weaning. Pups and dams sacrificed at weaning.

Figure 20.4 Abbreviated protocol for a teratology test and for a perinatal/postnatal toxicity test.

administration has been such that the dam is exposed during the period of major organogenesis, that is, days 6 through 15 of gestation in the rat or mouse and days 6 through 18 for the rabbit. Newer study designs call for dosing until C-section. Day 1 is the day spermatozoa appear in the vagina in the case of rats, or the day of mating in the rabbit.

The test chemical is typically administered directly into the stomach by gavage which is a requirement of the EPA and some other regulatory agencies. This method of dosing allows a precise calculation of the amount of test material received by the animal. Studies typically have three dose levels and a control group that receives the vehicle used for test material delivery. The high dose level is chosen to be one at which some maternal toxicity is known to occur, but never one that would cause more than 10% mortality. The low dose should be one at which no maternal toxicity is apparent, and the intermediate dose(s) should be chosen as a predicted low effect level.

The test is terminated by performing a C-section on the day before normal delivery is expected. The uterus is examined for implantation and resorption sites and for live and dead fetuses and the ovaries are examined for corpora lutea. In rodent studies, half of the fetuses are examined for soft tissue malformations, and the remaining are examined for skeletal malformations. In nonrodents, all fetuses are examined for both soft tissue and skeletal malformations. The various end points that may be examined include maternal toxicity, embryo-fetal toxicity, external malformations, and soft tissue and skeletal malformations.

Careful evaluation of maternal toxicity is necessary in assessing the validity of the high-dose level and the possibility that maternal toxicity is involved in subsequent events. The parameters evaluated include body weight, food consumption, clinical signs, and necropsy data such as organ weights. Because exposure starts after implantation, conception and implantation rates should be the same in controls and all treatment levels. If not, the test is suspect, with a possible error in the timing of the dose or use of animals from a source unsuitable for this type of testing.

Embryo-fetal toxicity is determined from the number of dead fetuses and resorption sites relative to the number of implantation sites. In addition to the possibility

of lethal malformations, such toxicity can be due to maternal toxicity, stress, or direct toxicity to the embryo or fetus that is not related to developmental malformations. Fetal weight and fetal size may also be a measure of toxicity but should not be confused with the variations seen as a result of differences in the number of pups per litter. Smaller litters tend to have larger pups while larger litters have smaller pups.

Anomalies may be regarded as either variations that may not adversely affect the fetus and not have a fetal outcome, or as malformations that are considered to have adverse effects on the fetus. For some findings, there is disagreement as to which class it belongs, such as the number of ribs in the rabbit which inherently has a large amount of variability. Common external anomalities are listed in Table 20.7

TABLE 20.7 External Malformations Commonly Seen in Teratogenicity Tests

Brain, cranium, spinal cord
Encephalocele—protrusion of brain through an opening of the skull.
Cerebrum is well formed and covered by transparent connective tissue.
Exencephaly—lack of skull with disorganized outward growth of the brain.
Microcephaly—small head on normal sized body.
Hydrocephaly—marked enlargement of the ventricles of the cerebrum.
Craniorachischisis—exposed brain and spinal cord.
Spina bifida—Nonfusion of spinal processes. Usually ectoderm covering is missing and spinal cord is evident.
Nose
Enlarged naris—enlarged nasal cavities
Single naris—a single naris, usually median
Eye
Microphthalmia—small eye
Anophthalmia—lack of eye
Open eye—no apparent eyelid, eye is open
Ear
Anotia—absence of the external ear
Microtia—small ear
Jaw
Micrognathia—small lower jaw
Agnathia—absence of lower jaw
Aglossia—absence of tongue
Astomia—lack of mouth opening
Bifid tongue—forked tongue
Cleft lip—either unilateral or bilateral cleft of upper lip
Palate
Cleft palate—a cleft or separation of the median portion of the palate
Limbs
Clubfoot—foot that has grown in a twisted manner, resulting in an abnormal shape or position. It is possible to have a malposition of the whole limb.
Micromelia—abnormal shortness of the limb.
Hemimelia—absence of any of the long bones, resulting in a shortened limb.
Phomelia—absence of all of the long bones of a limb, the limb is attached directly to the body.

and are determined by examination of fetuses at C-section. Visceral anomalities are determined by examination of fetuses after fixation using either the dissection method of Staples or by the hand-sectioning method of Wilson. Common visceral findings are listed in Table 20.8. Fetal skeletons are examined after first fixing the fetus and then staining the bone with Alizarin Red. Numerous skeletal variations occur in controls and may not have an adverse effect on the fetus (Table 20.9).

TABLE 20.8 Some Common Visceral Anomalies Seen in Teratogenicity Tests

Intestines
Umbilical hernia—protrusion of the intestines into the umbilical cord
Ectopic intestines—extrusion of the intestines outside the body wall
Heart
Dextrocardia—rotation of the heart axis to the right
Enlarged heart—either the atrium or the ventricle may be enlarged
Lung
Enlarged lung—all lobes are usually enlarged
Small lung—all lobes are usually small. Lung may appear immature
Uterus/testes
Undescended testes—testes are located anterior to the bladder instead of lateral; may be bilateral or unilateral
Agenesis of testes—one or both testes may be missing
Agenesis of uterus—one or both horns of the uterus may be missing
Kidney
Hydronephrosis—fluid-filled kidney, often grossly enlarged; may be accompanied by a hydroureter (enlarged, fluid-filled ureter)
Fused—kidneys fused, appearing as one misshapen kidney with two ureters
Agenesis—one or both kidneys missing
Misshapen—small, enlarged (usually internally), or odd-shaped kidneys

TABLE 20.9 Skeletal Abnormalities Commonly Seen in Teratogenicity Tests

Digits
Polydactyly—presence of extra digits, in mouse six or more, instead of five
Syndactyly—fusion of two or more digits
Oligodactyly—absence of one or more digits
Brachydactyly—smallness of one or more digits
Ribs
Wavy—ribs may be any aberrant shape
Extra—may have extra ribs on either side
Fused—may be fused anywhere along the length of the rib
Branched—single base and branched
Tail
Short—short tail, usually lack of vertebrae
Missing—absence of tail
Corkscrew—corkscrew-shaped tail

Their frequency of occurrence may, however, be dose related and should be evaluated.

Almost all chemically induced malformations have been observed in control animals, and most malformations are known to be produced by more than one cause. Thus, it is obvious that great care is necessary in the interpretation of teratology studies. For an agent to be classified as a development toxicant or teratogen, it must produce adverse effects on the conceptus at exposure levels that do not induce toxicity in the mother. Signs of maternal toxicity include reduction in weight gain, changes in eating patterns, hypo or hyperactivity, neurotoxic signs, and organ weight changes. Adverse effects on development under these conditions may be secondary to stress on the maternal system. Findings in the fetus, at dose levels that produce maternal toxicity cannot be easily separated from the maternal toxicity. Compounds can be deliberately administered at maternally toxic dose levels to determine the threshold for adverse effects on the offspring. In such cases, conclusions can be qualified to indicate that adverse effects on the offspring were found at maternally toxic dose levels and may not be indicative of selective or unique developmental toxicity.

Effect of Chemicals in Late Pregnancy and Lactation (Perinatal and Postnatal Effects) These tests are usually carried out on rats, and 20 pregnant females per dosage group are treated during the final third of gestation and through lactation to weaning (day 15 of pregnancy through day 21 postpartum) (Figure 20.4). The duration of gestation, parturition problems, and the number and size of pups in the naturally delivered litter are observed, as is the growth performance or the offspring. Variations of this test are the inclusion of groups treated only to parturition and only postpartum in order to separate prenatal and postnatal effects. Cross-fostering of pups to untreated dams may also be used to the same end. Behavioral testing of the pups has been suggested, and this and other physiological testing are to be recommended.

20.5.4 Special Tests

This general heading is used to include brief assessments of tests that are not always required but that may be required in particular cases or have been suggested as useful adjuncts to current testing protocols.

Neurotoxicity The nervous system is complex, both structurally and functionally, and toxicants can affect one or more units of this system in selective fashion. It is necessary, therefore, to devise tests, or sequences of tests that measure not only changes in overall function but that also indicate which basic unit is affected and how the toxicant interacts with its target. This is complicated by the fact that the nervous system has a considerable functional reserve, and specific observable damage may not affect overall function until it becomes even more extensive. Types of damage to the nervous system are classified in various ways but include neuronal toxicity, axonopathy, toxic interruption of impulse transmission, myelinopathy, and synaptic alterations in transmitter release or receptor function. Signs of neuropathy are frequently revealed by the acute, subchronic, chronic, and other tests that are required by regulatory agencies. Neurotoxicity is of great significance in toxicology,

however, and tests have been devised to supplement those routinely required. These include acute and subchronic neurotoxicity studies as well as developmental neurotoxicity studies.

Behavioral and Pharmacological Tests Behavioral and pharmacological tests involve the observation of clinical signs and behavior. These include signs of changes in awareness, mood, motor activity, central nervous system excitation, posture, motor incoordination, muscle tone, reflexes, and autonomic functions. If these tests so indicate, more specialized tests can be carried out that evaluate spontaneous motor activity, conditioned avoidance responses, operant conditioning, as well as tests for motor incoordination such as the inclined plane or rotarod tests.

Tests for specific classes of chemicals include the measurement of transmitter stimulated adenyl cyclase and Na/K-ATPase for chemicals that affect receptor function or cholinesterase inhibition for organophosphates or carbamates. Electrophysiological techniques may detect chemicals such as DDT or pyrethroids, which affect impulse transmission.

Acute and Subchronic Neurotoxicity Tests Acute and Subchronic Neurotoxicity Tests may be designed to assess a wide range of effects including CNS stimulation or depression, reflex perturbation, peripheral nerve damage, cognitive effects on learning and memory, motor activity effects, and neuropathology. These tests are conducted using rats or sometimes mice.

In the acute neurotoxicity study, approximately 10–15 animals per sex per dose group are administered a single gavage (bolus) dose of the test material. There are usually three dose groups and a control. Behavioral assessments are made on the day of dosing, and at 1 and 2 weeks post dose. The assessments include tests on motor activity, a functional observation battery (FOB), and neuropathology (at termination). The FOB screens for sensorimotor, neuromuscular, autonomic, and general physiological effects of a test compound. Table 20.10 depicts component tests of the FOB. These functional tests have the advantage over biochemical

TABLE 20.10 Example of Behavioral Procedures Included in a Functional Observation Battery

Home-Cage and Open-Field	Manipulative	Physiological
Arousal	Ease of removal	Body temperature
Gait	Ease of handling	Body weight
Posture	Touch response	
Vocalizations	Righting response	
Piloerection	Hindlimb foot splay	
Lacrimation	Forelimb grip strength	
Salivation	Hindlimb grip strength	
Urination/defecation	Finger-snap response	
Grooming behavior	Catalepsy	
Rearing	Palpebral closure	
Abnormal movements	Pupil function	
Tremors, convulsions		

measures that they permit repeated evaluation of individual animals over time to determine the onset, progression, duration, and reversibility of neurotoxic effects. Motor activity is also measured over time and can be evaluated by a variety of devices. One such device that has been frequently used is the figure 8 maze which consists of a series of interconnected alleys converging on a central open area and covered with transparent acrylic plastic. Motor activity is detected by photobeams, and an activity count is registered each time a photobeam is interrupted by the animal. Motor activity sessions are generally 60 min in length and each session is divided into 5- to 10-min reporting intervals (epochs). "Habituation" is an end point evaluated in the motor activity test and this is defined as a decrement in activity during the test session. Activity is expected to decrease toward the end of the test as the animal's exploratory activity normally lessens as the time in the maze increases. Neuropathological examinations are the same as those described below for the subchronic neurotoxicity test.

Before the acute neurotoxicity study is conducted, it is necessary to conduct a preliminary test to determine the time of peak effect after dosing of the test material. Preliminary tests may evaluate a selected group of end points in the FOB or other sensitive end points if known for a particular test material. Results of this preliminary test will determine the time when observations are performed on the day of dosing in the acute neurotoxicity study.

In the subchronic neurotoxicity study, end points measured are similar to those measured in the acute neurotoxicity study. However, the duration of dosing is 90 days and exposure to the test material is usually via the diet. As for the acute neurotoxicity study, these studies consist of three test groups and a control group. The FOB and motor activity tests are conducted at selected intervals such as weeks 5, 9, and 13, as well as pretest. At test termination, at least six animals per group are perfused via the heart with fixative to ensure optimal fixation of nervous tissues for histopathology examination. Nervous tissues examined include brain, spinal cord (various segments), and selected nerves such as the optic, sciatic, tibial, and sural nerves.

For all behavioral tests, it is important that the person making the actual observations is unaware of the treatment group for each animal ("blind" to dose group assignment). In addition, laboratories that conduct neurotoxicity studies for regulatory agencies must demonstrate that their methods are validated. Therefore, these laboratories must conduct positive control studies using known neurotoxicants and provide this information to regulators as necessary. Also, since it is not feasible for one person to perform the observations on all animals on all test occasions, laboratories must maintain evidence of interobserver reliability (agreement) for individuals who are involved with performing the FOBs.

Developmental Neurotoxicity Testing Developmental neurotoxicity testing is a separate component of developmental toxicology that focuses on potential behavioral or morphological modifications resulting from exposures to toxicants during early development. These studies track the outcome of such exposures through the postnatal period and into early adulthood. In a developmental neurotoxicity study, at least 20 pregnant female rats for each of three treatment groups plus a control are administered test material from gestation day (GD) 6 through weaning on lactation day 21. FOBs are conducted on the maternal animals at selected intervals such

as GD 6, GD 17, lactation day 11, and lactation day 21. Evaluations include observations in the home cage, during handling and outside the home cage in an open field. Body weights and food consumption are also monitored in the maternal animals. After birth, the offspring are counted, weighed, and gender is determined. On postnatal day (PND) 4, litters are culled to eight pups per litter. Following culling, at least 10 pups of each gender/group are assigned to one of the following tests: learning and memory, motor activity, or acoustic startle. Additional pups are assigned for neuropathology and brain weight evaluations on PND 11 and PND 70 (10/sex/group). FOBs are performed on the offspring at selected intervals such as PND 11, 17, 21, 35, and 60/70. Indicators of physical development such as preputial separation (male sexual maturation) and vaginal patency (female sexual maturation) are evaluated for all offspring as well as body weight and food consumption. Learning and memory can be evaluated with a variety of tests. Frequently, a water maze is used where the rat learns to swim through a series of alleys to find a platform it can use to climb out of the water. The time it takes for the animal to swim through the maze to the platform (trial latency) and the number of mistakes made are some of the end points evaluated in this test. The trials are conducted over a series of days and the assay provides an index of the development of both working memory (with-in day performance) as well as reference memory (between-day performance). The startle test measures the animal's response to a burst of loud noise and also how quickly it becomes habituated to 10 pulses of startle-eliciting tones in five blocks. For the startle test, special chambers lined with sound-attenuating and vibration-absorbing material are used. These chambers can measure the force exerted on a platform on which the animal stands during the test procedure. The startle test is conducted at two time points such as PND 22 and 60. When the offspring are approximately 70 days old, the test is terminated. Selected animals are perfused with fixative for neuropathology assessments. In addition to detailed microscopic evaluation of at least five different sections of the brain, simple morphometric analysis of the cerebrum, hippocampus, and cerebellum are conducted.

Delayed Neuropathy (organophosphate-induced delayed neuropathy [OPIDN]) The delayed neurotoxic potential of certain organophosphates such as tri-*o*-cresyl phosphate (TOCP) is usually evaluated by observation of clinical signs (paralysis of leg muscles in hens) or pathology (degeneration of the motor nerves in hens), but a biochemical test involving the ratio between the ability to inhibit cholinesterase and the ability to inhibit an enzyme that has been referred to as the neurotoxic esterase (NTE) has been suggested. The preferred test organism is the mature hen, because the clinical signs are similar to those in humans and such symptoms cannot be readily elicited in the common laboratory rodents.

Potentiation Potentiation and synergism represent interactions between toxicants that are potential sources of hazard because neither humans nor other species are usually exposed to one chemical at a time. The enormous number of possible combinations of chemicals makes routine screening for all such effects impossible.

One of the classic cases is the potentiation of the insecticide malathion by another insecticide, EPN, the LD_{50} of the mixture being dramatically lower than that of either compound alone. This potentiation can also be seen between malathion and certain contaminants that are formed during synthesis, such as isomalathion. For

this reason, quality control during manufacture is essential. This example of potentiation involves inhibition, by EPN or isomalathion, of the carboxylesterase responsible for the detoxication of malathion in mammals.

It is practical to test for potentiation only when there has been some preliminary indication that it might occur or when either or both compounds belong to chemical classes previously known to cause potentiation. Such a test can be conducted by comparing the LD_{50}, or any other appropriate toxic end point, of a mixture of equitoxic doses of the chemicals in question with the same end point measured with the two chemicals administered alone.

In the case of synergism, in which one of the compounds is relatively nontoxic when given alone, the toxicity of the toxic compound can be measured when administered alone or after a relatively large dose of the nontoxic compound.

Toxicokinetics and Metabolism Routine toxicity testing without regard to the mechanisms involved is likely to be wasteful of time and of human, animal, and financial resources. A knowledge of toxicokinetics and metabolism can give valuable insights and provide for testing that is both more efficient and more informative. Such knowledge provides the necessary background to make the most appropriate selection of test animal species and of dose levels, and the most appropriate method for extrapolating from animal studies to the assessment of human hazard. Moreover, they may provide information on possible reactive intermediates as well as information on induction or inhibition of the enzymes of xenobiotic metabolism, the latter being critical to an assessment of possible interaction.

The nature of metabolic reactions and their variations between species is detailed in Chapters 7 and 8, with some aspects of toxicokinetics in Chapter 5. The methods used for the measurement of toxicants and their metabolites are detailed in Chapter 24. The present section is concerned with the general principles, use, and need for metabolic and toxicokinetics studies in toxicity testing.

Toxicokinetics studies are designed to measure the amount and rate of the absorption, distribution, metabolism, and excretion of a toxicant. These data are used to construct predictive mathematical models so that the distribution and excretion of other doses can be simulated. Such studies are usually carried out using radiolabeled compounds to facilitate measurement and total recovery of the administered dose. This can be done entirely *in vivo* by measuring levels in blood, expired air, feces, and urine; these procedures can be done relatively noninvasively and continuously in the same animal. Tissue levels can be measured by sequential sacrifice and analysis of organ levels. It is important to measure not only the compound administered but also its metabolites, because simple radioactivity counting does not differentiate among them.

The metabolic study, considered separately, consists of treatment of the animal with the radiolabeled compound followed by chemical analysis of all metabolites formed *in vivo* and excreted via the lungs, kidneys, or bile. Although reactive intermediates are unlikely to be isolated, the chemical structure of the end products may provide vital clues to the nature of the intermediates involved in their formation. The use of tissue homogenates, subcellular fractions, and purified enzymes may serve to clarify events occurring during metabolic sequences leading to the end products.

Information of importance in test animal selection is the similarity in toxicodynamics and metabolism to that of humans. Although all of the necessary information may not be available for humans, it can often be inferred with reference to metabolism and excretion of related compounds, but it is clearly ill advised to use an animal that differs from most others in the toxicokinetics or metabolism of the compound in question or that differs from humans in the nature of the end products. Dose selection is influenced by knowledge of whether a particular dose saturates a physiological process such as excretion or whether it is likely to accumulate in a particular tissue because these factors are likely to become increasingly important the longer a chronic study continues.

Behavior Although the primary emphasis in toxicity testing has long been the estimation of morphologic changes, much recent interest has focused on more fundamental evaluations. One such aspect has been the evaluation of chemical effects on behavior.

The categories of methods used in behavioral toxicology fall into two principal classes, stimulus-oriented behavior, and internally generated behavior. The former includes two types of conditioned behavior: operant conditioning, in which animals are trained to perform a task in order to obtain a reward or to avoid a punishment, and classical conditioning, in which an animal learns to associate a conditioning stimulus with a reflex action. Stimulus-oriented behavior also involves unconditioned responses in which the animal's response to a particular stimulus is recorded.

Internally generated behavior includes observation of animal behavior in response to various experimental situations, and includes exploratory behavior, circadian activity, social behavior, and so on. The performance of animals treated with a particular chemical is compared with that of untreated controls as a measure of the effect of the chemical.

Many of the variables associated with other types of testing must also be controlled in behavioral tests: sex, age, species, environment, diet, and animal husbandry. Behavior may vary with all of these. Norton describes a series of four tests that may form an appropriate series inasmuch as they represent four different types of behavior; the series should therefore reflect different types of nervous system activity. They are as follows:

1. Passive avoidance. This test involves the use of a shuttle box, in which animals can move between a light side and a dark side. After an acclimatization period, in which the animal can move freely between the two sides, it receives a mild electric shock while in the dark (preferred) side. During subsequent trials, the time spent in the "safe side" is recorded.
2. Auditory startle. This test involves the response (movement) to a sound stimulus either without, or preceeded by, a light-flash stimulus.
3. Residential maze. Movements of animals in a residential maze are automatically recorded during both light and dark photoperiods.
4. Walking patterns. Gait is measured in walking animals, including such characteristics as the length and width of stride and the angles formed by the placement of the feet.

Problems associated with behavioral toxicology include the functional reserve and adaptability of the nervous system. Frequently, behavior is maintained in spite of clearly observable injury. Other problems are the statistical ones associated with multiple tests, multiple measurements, and the inherently large variability in behavior.

The use of human subjects occupationally exposed to chemicals is often attempted, but such tests are complicated by the subjective nature of the end points (e.g. dizziness).

Covalent Binding Toxicity has been associated with covalent binding in a number of ways. Organ-specific toxicants administered *in vivo* bind covalently to macromolecules, usually at a higher level in the target tissues that in nontarget tissues. Examples include acetaminophen in the liver, carbon tetrachloride in the liver, *p*-aminophenol in the kidney, and ipomeanol in the lung. Similarly, many carcinogens are known to give rise to DNA adducts. In general, covalent binding occurs as a result of metabolism of the toxicant to highly reactive intermediates, usually, but not always, by cytochrome P450. Because these intermediates are highly reactive electrophiles, they bind to many nucleophilic sites on DNA, RNA, or protein molecules, not just the site of toxic action. Thus, measurement of covalent binding may be a measure of toxic potential rather than a specific measurement, related directly to a mechanism of action. The occurrence of covalent binding at the same time as toxicity is so common an occurrence, however, that a measurement of covalent binding of a chemical may be regarded as an excellent although perhaps not infallible indication of potential for toxicity.

The measurement of DNA adducts is an indirect indication of genotoxic (carcinogenic) potential, and DNA adducts in the urine are an indication, obtained by a noninvasive technique, of recent exposure. Protein adducts give an integrated measure of exposure because they accumulate over the life span of the protein and, at the same time, indicate possible organ toxicity.

Tissue protein adducts are usually demonstrated in experimental animals following injection of radiolabeled chemicals and, after a period to time, the organs are removed, homogenized and, by rigorous extraction, all the noncovalently bound material is removed. Extraction methods include lipid solvents, acids and bases, concentrated urea solutions, and solubilization and precipitation of the proteins. They tend to underestimate the extent of covalent binding because even covalent bonds may be broken by the rigorous procedures used. Newer methods involving dialysis against detergents and separation of adducted proteins will probably prove more appropriate.

Blood proteins, such as hemoglobin, may be used in tests of human exposure because blood is readily and safely accessible. For example, the exposure of mice to ethylene oxide or dimethylnitrosamine was estimated by measuring alkylated residues in hemoglobin. The method was subsequently extended to people exposed occupationally to ethylene oxide by measuring *N*-3-(2-hydroxyethyl) histidine residues in hemoglobin. Similarly, methyl cysteine residues in hemoglobin can be used as a measure of methylation.

DNA–RNA adducts can also be measured in various ways, including rigorous extraction, separation, and precipitation following administration of labeled

compounds *in vivo*, or use of antibodies raised to chemically modified DNA or RNA.

Although many compounds of different chemical classes have been shown to bind covalently when activated by microsomal preparations *in vitro* (e.g., aflatoxin, ipomeanol, stilbene, vinyl chloride), these observations have not been developed into routine testing procedures. Such procedures could be useful in predicting toxic potential.

Immunotoxicity Immunotoxicology comprises two distinct types of toxic effects: the involvement of the immune system in mediating the toxic effect of a chemical and the toxic effects of chemicals on the immune system. The former is shown, for example, in tests for cutaneous sensitization, whereas the latter is shown in impairment of the ability to resist infection.

Tests for immunotoxicity are not required by all regulatory agencies, but it is an area of great interest, both in the fundamental mechanisms of immune function and in the design of tests to measure impairment of immune function. Both of these aspects are discussed in detail in Chapter 19.

20.6 *IN VITRO* AND OTHER SHORT-TERM TESTS

20.6.1 Introduction

The toxicity tests that follow are tests conducted largely *in vitro* with isolated cell systems. Some are short-term tests carried out *in vivo* or are combinations of *in vivo* and *in vitro* systems. The latter are included because of similarities in approach, mechanism, or intent. In general, these tests measure effects on the genome or cell transformation; their importance lies in the relationship between such effects and the mechanism of chemical carcinogenesis. Mutagenicity of cells in the germ line is itself an expression of toxicity, however, and the mutant genes can be inherited and expressed in the next or subsequent generations.

The theory that the initiating step of chemical carcinogenesis is a somatic mutation is well recognized, and considerable evidence shows that mutagenic potential is correlated with carcinogenic potential. Thus, the intent of much of this type of testing is to provide early warning of carcinogenic potential without the delay involved in conducting lifetime chronic feeding studies in experimental animals. In spite of the numerous tests that have been devised, regulatory agencies have not yet seen fit to substitute any of them, or any combination of them, for chronic feeding studies. Instead, they have been added as additional testing requirements. One function of such tests should be to identify those compounds with the greatest potential for toxicity and enable the amount of chronic testing to be reduced to more manageable proportions.

20.6.2 Prokaryote Mutagenicity

Ames Test The Ames test, developed by Bruce Ames and his coworkers of the University of California, Berkeley, depends on the ability of mutagenic chemicals to bring about reverse mutations in *Salmonella typhimurium* strains that have

defects in the histidine biosynthesis pathway. These strains will not grow in the absence of histidine but can be caused to mutate back to the wild type, which can synthesize histidine and hence can grow in its absence. The postmitochondrial supernatant (*S*-9 fraction), obtained from homogenates of livers of rats previously treated with polychlorinated biphenyls (PCBs) in order to induce certain cytochrome P450 isoforms, is also included in order to provide the activating enzymes involved in the production of the potent electrophiles often involved in the toxicity of chemicals to animals.

Bacterial tester strains have been developed that can test for either base-pair (e.g., strain TA-1531) or frameshift (e.g., strains TA-1537, TA-1538) mutations. Other, more sensitive strains such as TA-98 and TA-100 are also used, although they may be less specific with regard to the type of mutation caused.

In brief, the test is carried out (Figure 20.5) by mixing a suspension of bacterial cells with molten top agar. This also contains cofactors, *S*-9 fraction, and the material to be tested. The mixture is poured onto Petri plates containing hardened minimal agar. The number of bacteria that revert and acquire the wild-type ability to grow in the absence of histidine can be estimated by counting the colonies that develop on incubation. To provide a valid test, a number of concentrations are tested, and positive controls with known mutagens are included along with negative controls that lack only the test compound. The entire test is replicated often enough to satisfy appropriate statistical tests for significance. Parallel tests without the *S*-9 fraction may help distinguish between chemicals with intrinsic mutagenic potential and those that require metabolic activation.

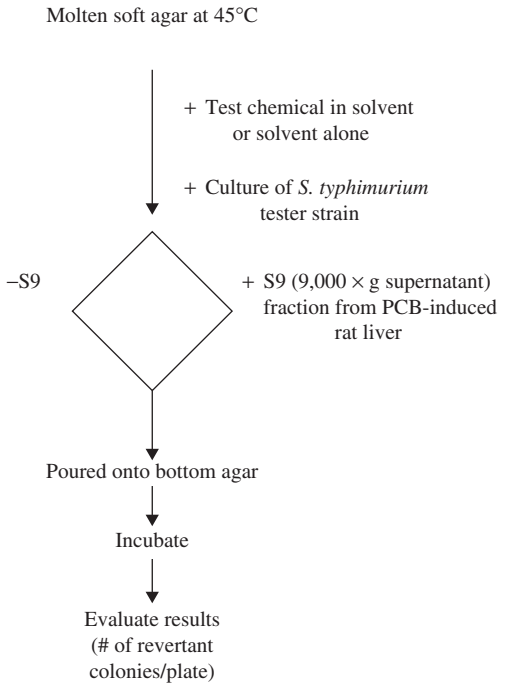

Figure 20.5 Protocol for the Ames test for mutagenicity.

The question of correlation between mutagenicity and carcinogenicity is crucial in any consideration of the utility of this or similar tests. In general, this appears to be high, although a small proportion of both false positives and false negatives occurs. For example, certain base analogs and inorganics such as manganese are not carcinogens but are mutagens in the Ames test, whereas diethylstilbestrol (DES) is a carcinogen but not a bacterial mutagen (see Chapter 11 for additional detail).

Related Tests Related tests include tests based on reverse mutations, as in the Ames test, as well as tests based on forward mutations. Examples include:

1. Reverse mutations in *Escherichia coli.* This test is similar to the Ames test and depends on reversion of tryptophane mutants, which cannot synthesize this amino acid, to the wild type, which can. The *S*-9 fraction from the liver of induced rats can also be used as an activating system in this test. Other *E. coli* reverse mutation tests utilize nicotinic acid and arginine mutants.
2. Forward mutations in *S. typhimurium.* One such assay, dependent on the appearance of a mutation conferring resistance to 8-azaguanine in a histidine revertant strain, has been developed and is said to be as sensitive as the reverse-mutation tests.
3. Forward mutations in *E. coli.* These mutations depend on mutation of galactose non-fermenting *E. coli* to galactose fermenting *E. coli* or the change from 5-methyltryptophane to 5-methyltryptophane resistance.
4. DNA repair. Polymerase-deficient, and thus DNA repair-deficient, *E. coli* has provided the basis for a test that depends on the fact that the growth of a deficient strain is inhibited more by a DNA-damaging agent than is that of a repair-competent strain. The recombinant assay using *Bacillus subtilis* is conducted in much the same way, because recombinant deficient strains are more sensitive to DNA-damaging agents.

20.6.3 Eukaryote Mutagenicity

Mammalian Cell Mutation The development of cell culture techniques that permit both survival and replication have led to many advances in cell biology, including the use of certain of these cell lines for detection of mutagens. Although such cells, if derived from mammals, would seem ideal for testing for toxicity toward mammals, there are several problems. Primary cells, which generally resemble those of the tissue of origin, are difficult to culture and have poor cloning ability. Because of these difficulties, certain established cell lines are usually used. These cells, such as Chinese hamster ovary cells and mouse lymphoma cells, clone readily and do not become senescent with passage through many cell generations. Unfortunately, they have little metabolic activity toward xenobiotics and thus do not readily activate toxicants. Moreover, they usually show chromosome changes, such as aneuploidy (i.e., more or fewer than the usual diploid number of chromosomes).

The characteristics usually involved in these assays are resistance to 8-azaguanine or 6-thioguanine (the hypoxanthine guanine phosphoribosyl transferase or HGPRT

locus), resistance to bromodeoxyuridine or trifluorothymidine (the thymidine kinase or TK locus) or resistance to ouabain (the OU or Na/K-ATPase locus). HGPRT is responsible for incorporation of purines from the medium into the nucleic acid synthesis pathway. Its loss prevents uptake of normal purines and also of toxic purines such as 8-azaguanine, which would kill the cell. Thus, mutation at this locus confers resistance to these toxic purine analogs. Similarly, TK permits pyrimidine transport, and its loss prevents uptake of toxic pyrimidine analogs and confers resistance to them. In the absence of HGPRT or TK, the cells can grow by *de novo* synthesis of purines and pyrimidines. Ouabain kills cells by combining with the Na/K-ATPase. Mutation at the OU locus alters the ouabain-binding site in a way that prevents inhibition and thus confers resistance.

A typical test system is the analysis of the TK locus in mouse lymphoma cells for mutations that confer resistance to bromodeoxyuracil. The tests are conducted with and without the *S*-9 fraction from induced rat liver because the lymphoma cells have little activating ability. Both positive and negative controls are included, and the parameter measured is the number of cells formed that are capable of forming colonies in the presence of bromodeoxyuridine.

***Drosophila* Sex-Linked Recessive Lethal Test** The advantages of *Drosophila* tests are that they involve an intact eukaryotic organism with all of its interrelated organ systems and activation mechanisms but, at the same time, are fast, relatively easy to perform, and do not involve mammals as test animals. The most obvious disadvantages are that the hormonal and immune systems of insects are significantly different from those of mammals and that the nature, specificity, and inducibility of the cytochrome P450s are not as well understood in insects as they are in mammals.

In a typical test, males that are 2 days postpuparium and that were raised from eggs laid within a short time period (usually 24 h) are treated with the test compound in water to which sucrose has been added to increase palatability. Males from a strain carrying a gene for yellow body on the X chromosome are used. Preliminary tests determine that the number of offspring of the survivors of the treatment doses (usually 0.25 LD_{50} and 0.5 LD_{50}) are adequate for future crosses. Appropriate controls, including a solvent control (with emulsifier if one was necessary to prepare the test solution), and a positive control, such as ethyl methanesulfonate, are routinely included with each test. Individual crosses of each surviving treated male with a series of three females are made on a 0- to 2-, 3- to 5-, and 6- to 8-day schedule. The progeny of each female is reared separately, and the males and females of the F_1 generation are mated in brother-sister matings. If there are no males with yellow bodies in a particular set of progeny, it should be assumed that a lethal mutation was present on the treated X chromosomes. A comparison of the F_2 progeny derived from females inseminated by males at different times after treatment allows a distinction to be made between effects on spermatozoa, spermatids, and spermatocytes.

In the Basc (Muller-5) test shown in Figure 20.6, the strain used for the females in the F_1 cross is a multiple-marked strain that carries a dominant gene for bar eyes and recessive genes for apricot eyes and a reduction of bristles on the thorax (scute gene). (Basc is an acronym for bar, apricot, and scute.).

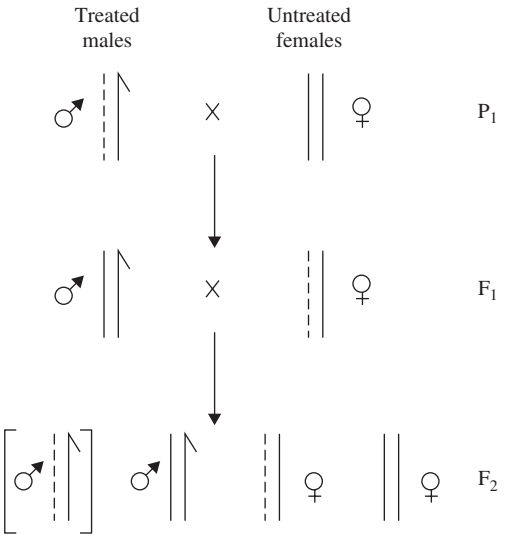

Figure 20.6 The Basc (Muller-5) mating scheme. Dashed lines represent the treated X chromosome of males. Brackets indicate males with yellow bodies, which would be absent if a lethal mutation occurred on the X chromosome of the treated male.

Related Tests Many tests related to the two types of eukaryote-mutation tests are discussed in Mammalian Cell Mutation section and in *Drosophila* Sex-Linked Recessive Lethal Test section, and many of them are simply variations of the tests described. Two distinct classes are worthy of mention: the first uses yeasts as the test organisms, and the second is the spot test for mutations in mice.

One group of tests using yeasts includes tests for gene mutations and strains that can be used to detect forward mutations, in genes that code for enzymes in the purine biosynthetic pathway: other strains can be used to detect reversions. Yeasts can also be used to test for recombinant events such as reciprocal mitotic recombination (mitotic crossing over) and nonreciprocal mitotic recombination. *Saccharomyces cerevisiae* is the preferred organism in almost all these tests. Although they possess cytochrome P450s capable of metabolizing xenobiotics, their specificity and sensitivity are limited as compared with those of mammals, and an *S*-9 fraction is often included, as in the Ames test, to enhance activation.

The gene mutation test systems in mice include the specific locus test, in which wild-type treated males are crossed with females carrying recessive mutations for visible phenotypic effects. The F_1 progeny have the same phenotype as the wild-type parent unless a mutation, corresponding to a recessive mutant marker, has occurred. Such tests are accurate, and the spontaneous (background) mutation rate is very low, making them sound tests which are predictive for other mammals. Unfortunately, the large number of animals required has prevented extensive use. Similar tests involving the activity and electrophoretic mobility of various enzymes in the blood or other tissues in the F_1 progeny from treated males and untreated females have been developed. In the previously mentioned tests, as with many others, sequential mating of males with different females can provide information about the stage of sperm development at which the mutational event occurred.

20.6.4 DNA Damage and Repair

Many of the end points for tests described in this chapter, including gene mutation, chromosome damage, and oncogenicity, develop as a consequence of damage to or chemical modification of DNA. Most of these tests, however, also involve metabolic events that occur both prior to and subsequent to the modification of DNA. Some tests, however, use events at the DNA level as end points. One of these, the unscheduled synthesis of DNA in mammalian cells, is described in some detail; the others are summarized briefly.

Unscheduled DNA Synthesis in Mammalian Cells The principle of this test is that it measures the repair that follows DNA damage and is thus a reflection of the damage itself. It depends on the autoradiographic measurement of the incorporation of tritiated thymidine into the nuclei of cells previously treated with the test chemical.

The preferred cells are usually primary hepatocytes in cultures derived from adult male rats, the cells of which are dispersed and allowed to attach themselves to glass coverslips. From this point on, the test is carried out on the attached cells. Both positive controls with agents known to stimulate unscheduled DNA synthesis, such as the carcinogen aflatoxin B1 or 2-acetylaminofluorene, and negative controls, which are processed through all procedures except exposure to the test compound, are performed routinely with every test. Cells are exposed by replacing the medium for a short time with one containing the test chemical. The dose levels are determined by a preliminary cell viability test (trypan blue exclusion test) and consist of several concentrations that span the range from no apparent loss of viability to almost complete loss of viability. Following exposure, the medium is removed and the cells are washed by several changes of fresh medium and finally placed in a medium containing tritiated thymidine. The cells are fixed and dried, and the coverslip with the cells attached is coated with photographic emulsion. After a suitable exposure period (usually several weeks), the emulsion is developed and the cells are stained with hemotoxylin and eosin. The number of grains in the nuclear region is corrected by subtracting nonnuclear grains, and the net grain count in the nuclear area is compared between treated and untreated cells.

This test has several advantages in that primary liver cells have considerable activation capacity and the test measures an event at the DNA level. It does not, however, distinguish between error-free repair and error-prone repair, the latter being itself a mutagenic process. Thus, it cannot distinguish between events that might lead to toxic sequelae and those that do not. A modification of this test measures *in vivo* unscheduled DNA synthesis. In this modification, animals are first treated *in vivo*, and primary hepatocytes are then prepared and treated as already described.

Related Tests Tests for the measurement of binding of the test material to DNA have already been discussed under covalent binding. Another method of assessing DNA damage is the estimation of DNA breakage following exposure to the test chemical; the DNA-strand length is estimated by using alkaline elution or sucrose density gradient centrifugation. This has been done with a number of cell lines and with freshly prepared hepatocytes, in the latter case following

treatment either *in vivo* or *in vitro*. It may be regarded as promising but not yet fully validated. The polymerase-deficient *E. coli* tests as well as recombinant tests using yeasts are also related to DNA repair.

20.6.5 Chromosome Aberrations

Tests for chromosome aberrations involve the estimation of effects on extended regions of whole chromosomes rather than on single or small numbers of genes. Primarily, they concern chromosome breaks and the exchange of material between chromosomes.

Sister Chromatid Exchange Sister chromatid exchange (SCE) occurs between the sister chromatids that together make up a chromosome. It occurs at the same locus in each chromatid and is thus a symmetrical exchange of chromosome material. In this regard, it is not strictly an aberration because the products do not differ in morphology from normal chromosome. SCE, however, is susceptible to chemical induction and appears to be correlated with the genotoxic potential of chemicals as well as with their oncogenic potential. The exchange is visualized by permitting the treated cells to pass through two DNA replication cycles in the presence of 5-bromo-2'-deoxyuridine, which is incorporated in the replicated DNA. The cells are then stained with a fluorescent dye and irradiated with UV light, which permits differentiation between chromatids that contain bromodeoxyuridine and those that do not (Figure 20.7).

The test can be carried out on cultured cells or on cells from animals treated *in vivo*. In the former case, the test chemical is usually evaluated in the presence and absence of the *S*-9 activation system from rat liver. Typically, cells from a Chinese hamster ovary cell line are incubated in a liquid medium and exposed to several concentration of the test chemical, either with or without the *S*-9 fraction, for about 2 h. Positive controls, such as ethyl methanesulfonate (a direct-acting compound) or dimethylnitrosamine (one that requires activation), as well as negative controls are also included. Test concentrations are based on cell toxicity levels determined by prior experiment and are selected in such a way that even at the highest dose excess

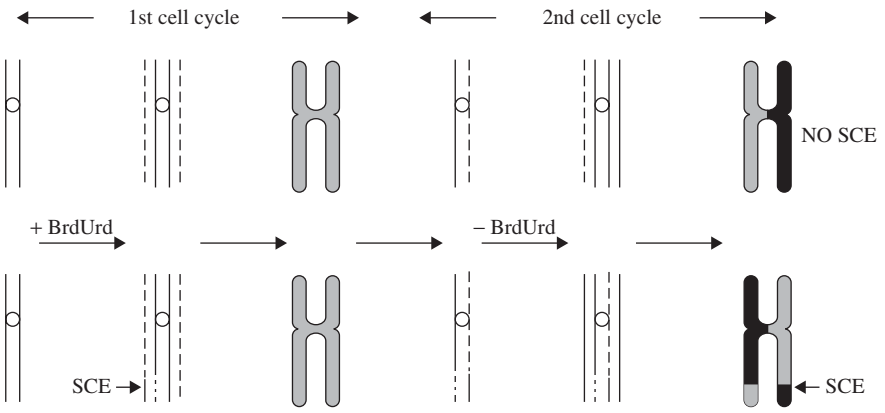

Figure 20.7 Visualization of sister chromatid exchange.

growth does not occur. At the end of the treatment period, the cells are washed, bromodeoxyuridine is added, and the cells are incubated for ≥24 h or more. The cells are then fixed, stained with a fluorescent dye, and irradiated with UV light. Second division cells are then scored under the microscope for SCEs (Figure 20.7).

The test can also be carried out on cells treated *in vivo*, and analyses have been made of SCEs in lymphocytes from cancer patients treated with chemotherapeutic drugs, smokers, and workers exposed occupationally; in several cases, increased incidence of SCEs has been noted. This is a sensitive test for compounds that alkylate DNA, with few false positives. It may be useful for detecting promoters such as phorbol esters.

Micronucleus Test The micronucleus test is an *in vivo* test usually carried out in mice. The animals are treated *in vivo*, and the erythrocyte stem cells from the bone marrow are stained and examined for micronuclei. Micronuclei represent chromosome fragments or chromosomes left behind at anaphase. It is basically a test for compounds that cause chromosome breaks (clastogenic agents) and compounds that interfere with normal mitotic cell division, including compounds that affect spindle fiber function.

Male and female mice from an outbred strain are handled by the best animal husbandry techniques, as described for acute, subchronic, and chronic tests, and are treated either with the solvent, 0.5 LD_{50}, or 0.1 LD_{50} of the test chemical. Animals are killed at several time intervals up to 2 days; the bone marrow is extracted, placed on microscope slides, dried, and stained. The presence of micronuclei is scored visually under the microscope.

Dominant Lethal Test in Rodents The dominant lethal test, which is performed using rats, mice, or hamsters, is an *in vivo* test to determine the germ-cell risk from a suspected mutagen. The test consists of treating males with the test compound for several days, followed by mating to different females each week for enough weeks to cover the period required for a complete spermatogenic cycle. Animals are maintained under optimal conditions of animal husbandry and are dosed, usually by a gavage, with several doses of less than 0.1 LD_{50}. The females are killed after 2 weeks of gestation and dissected; corpora lutea and living and dead implantations are counted. The end points used to determine the occurrence of dominant lethal mutations in the treated males are the fertility index (ratio of pregnant females to mated females), preimplantation losses (the number of implantations relative to the number of corpora lutes), the number of females with dead implantations relative to the total number of pregnant females, and the number of dead implantations relative to the total number of implantations. Mutations in sperm that are dominant and lethal do not result in viable offspring.

Related Tests Many cells exposed to test chemicals can be scored for chromosome aberrations by staining procedures followed by visual examination with the aid of the microscope. These include Chinese hamster ovary cells in culture treated in a protocol very similar to that used in the test for SCEs, bone marrow cells from animals treated *in vivo*, or lymphocytes from animals treated *in vivo*. The types of aberrations evaluated include chromatid gaps, breaks, and deletions; chromosome gaps, breaks, and deletions; chromosome fragments; translocations; and ploidy.

Heritable translocations can be detected by direct examination of cells from male or female offspring in various stages of development or by crossing the treated animals to untreated animals and evaluating fertility, with males with reduced fertility being examined for translocations, and so on. Progeny from this or other tests, such as those for dominant lethals, can be permitted to survive and then examined for translocations and other abnormalities.

20.6.6 Mammalian Cell Transformation

Most cell transformation assays utilize fibroblast cultures derived from embryonic tissue. The original studies showed that cells from C3H mouse fibroblast cultures developed morphologic changes and changes in growth patterns when treated with carcinogens. Later, similar studies were made with Syrian hamster embryo cells. The direct relationship of these changes to carcinogenesis was demonstrated by transplantation of the cells into a host animal and the subsequent development of tumors. The recent development of practical assay procedures involves two cell lines from mouse embryos, Balb/3T3 and C3h/10T1/2, in which transformation is easily recognized and scored. In a typical assay situation, cells, such as Balb/3T3 mouse fibroblasts, will multiply in culture until a monolayer is formed. At this point, they cease dividing unless transformed. Chemicals that are transforming agents will, however, cause growth to occur in thicker layers above the monolayer. These clumps of transformed cells are known as foci. In spite of many recommended controls, the assay is only semiquantitative. The doses are selected from the results of a preliminary experiment and range from a high dose that reduces colony formation (but not by >50%) to a low dose that has no measurable effect on colony formation. After exposure to the test chemical for 1–3 days, the cells are washed and incubation is continued for up to 4 weeks. At that time, the monolayers are fixed, stained, and scored for transformed foci.

Transformation assays have several distinct advantages. Because transplanted foci give rise to tumors in congenic hosts (those from the same inbred strain from which the cells were derived) whereas untransformed cells do not, cell transformation is believed to be illustrative of the overall expression of carcinogenesis in mammalian tissues. The two cell types used most (Balb/3T3 and C3H/10T1/2) respond to promoters in the manner predicted by the multistage model for carcinogenesis *in vivo* and may eventually be useful in the development of assays for promotion. Unfortunately, a large number of false-negative results are obtained because these cell lines do not show much activation capacity; it has not proved practical to combine them with the *S*-9 activation system. Furthermore, the cells are aneuploidy and may be preneoplastic in the untreated state. Syrian hamster cells, which do have considerable activation capacity, have proved difficult to use in test procedures and are difficult to score.

20.6.7 General Considerations and Testing Sequences

Considering all of the tests for acute and chronic toxicity, long and short term, *in vivo*, and *in vitro*, it is clearly impractical to apply a complete series of tests to all commercial chemicals and all their derivatives in food, water, and the environment. The challenge of toxicity testing is to identify the most effective set or

sequence of tests necessary to describe the apparent and potential toxicity of a particular chemical or mixture of chemicals. The enormous emphasis on *in vitro* or short-term tests that has occurred since the mid-1970s had its roots in the need to find substitutes for lifetime feeding studies in experimental animals or, at the very least, to suggest a sequence of tests that would enable priorities to be set for which chemicals should be subjected to chronic tests. Such tests might also be used to eliminate the need for chronic testing for chemicals that either clearly possessed the potential for toxicity or clearly do not. Although there has been much success in test development, the challenge outlined here has not been met, primarily because of the failure of scientists and regulatory agencies, worldwide, to agree on test sequences or on the circumstances in which short-term tests may substitute for chronic tests. Thus, not only are short-term tests often required; these are in addition to long-term tests. As an example, the U.S. EPA requirements for the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) include, in addition to a full battery of acute, subchronic, and chronic tests, tests to address the following three categories: gene mutations, structural chromosome aberrations, and other genotoxic tests as appropriate (such as DNA damage and repair and chromosome aberrations). It is important, however, that test sequences have been suggested and considered by regulatory agencies and, in addition, it must be considered that short-term tests do not provide all of the information needed from the longer-term tests.

20.7 ECOLOGICAL EFFECTS

Tests for ecological effects include those designed to address the potential of chemicals to affect ecosystems and population dynamics in the environment. Such tests are designed to estimate effects on field populations of vertebrates, invertebrates, and plants. The use of these tests in environmental risk assessment is discussed in detail in Chapter 27.

20.7.1 Laboratory Tests

There are two types of laboratory tests: toxicity determinations on wildlife and aquatic organisms and the use of model ecosystems to measure bioaccumulation and transport of toxicants and their degradation products.

Among the tests included in the first category are the avian oral LD_{50}, the avian dietary lethal concentration 50 (LC_{50}), wild mammal toxicity, and avian reproduction. The avian tests are usually carried out on bobwhite quail or mallard ducks, whereas the wild mammals may be species such as the pine mouse, *Paramyscus*. The tests are similar to those described under acute and chronic testing procedures but suffer from some drawbacks; the standards of animal husbandry used with rats and mice are probably unattainable with birds or wild mammals even through bobwhite quail and mallards are easily reared in captivity. The genetics of the birds and mammals used are much more variable than are those of the traditional laboratory rodent strains.

Similar tests can be carried out with aquatic organisms (e.g., the LC_{50} for freshwater fish such as rainbow trout and bluegills), the LC_{50} for estuarine and marine organisms, the LC_{50} for invertebrates such as *Daphnia,* and the effect of chemicals on the early stages of fish and various invertebrates.

Model systems, first developed by ecologists to study basic ecological processes, have been adapted to toxicological testing. In toxicology, these models were first used to determine the movement and concentration of pesticides. Typically, the model has a water phase containing vertebrates and invertebrates, and a terrestrial phase containing at least one plant species and one herbivore species. First, the ^{14}C-labeled pesticide or other environmental contaminant is applied to the leaves of the terrestrial plant sorghum (*Sorgum halpense*) then salt marsh caterpillars (*Estigmene acrea*) are placed on the plants. The larvae eat the plants and contaminate the water with feces and their dead bodies. The aquatic food chain is simulated with plankton (diatoms, rotifers, etc.), water fleas (*Daphnia*), mosquito larvae (*Culex pipiens*), and fish (*Gambusia affinis*). From an analysis of the plants, animals, and substrates for the ^{14}C-labeled compound and its degradation products, the biologic magnification or rate of degradation can be calculated.

More complex models involving several compartments, simulated rain, simulated soil drainage, simulated tidal flow, and so on, have been constructed and their properties investigated, but none have been brought to the stage of use in routine testing. Similarly, aquatic models using static, recirculating, and continuous flow have also been used, as have entirely terrestrial models: again, none have been developed for routine testing.

20.7.2 Simulated Field Tests

Simulated field tests may be quite simple, consisting of feeding treated prey to predators and studying the toxic effects on the predator, enabling some predictions concerning effects to nontarget organisms. In general, however, the term is used for greenhouse, small plot, small artificial pond, or small natural pond tests. These serve to test biologic accumulation and degradation under conditions somewhat more natural than in model ecosystems and the test chemicals are exposed to environmental as well as biologic degradation. Population effects may be noted, but these methods are more useful for soil invertebrates, plants, and aquatic organisms because other organisms are not easily contained in small plots.

20.7.3 Field Tests

In field-test situations, test chemicals are applied to large areas under natural conditions. The areas are at least several acres and may be either natural or part of some agroecosystem. Because the area is large and in the open, radiolabeled compounds cannot be used, and it is not possible to obtain a balance between material applied and material recovered.

The effects are followed over a long period of time and two types of control may be used: first, a comparison with a similar area that is untreated; and second, a comparison with the same area before treatment. In the first, case it is difficult, if not impossible, to duplicate exactly a large natural area, and in the second, changes can occur that are unrelated to the test material.

In either case, studies of populations are the most important focus of this type of testing, although the disappearance of the test material, its accumulation in various life forms, and the appearance, accumulation, and disappearance of its

degradation products are also important. The population of soil organisms, terrestrial organisms, and aquatic organisms as well as plants all must be surveyed and characterized, both qualitatively and quantitatively. Following application of the test material, the populations can be followed through two or more annual cycles to determine both acute and long-term population effects.

20.8 RISK ANALYSIS

The preceding tests for various kinds of toxicity can be used to measure adverse effects of many different chemical compounds in different species, organ, tissues, cells, or even populations, and under many different conditions. This information can be used to predict possible toxicity of related compounds from QSAR or of the same chemical under different conditions (e.g., mutagenicity as a predictor of carcinogenicity). It is considerably more difficult to use this information to predict possible risk to other species, such as humans, because little experimental data on this species is available. Some methods are available to predict risk to humans and to provide the risk factor in the risk–benefit assessment that provides the basis for regulatory action, however. Human health risk assessment is discussed in detail in Chapter 24. The benefit factor is largely economic in nature, and the final regulatory action is not, in the narrow sense, a scientific one. It also involves political and legal aspects and, *in toto*, represents society's evaluation of the amount of risk that can be tolerated in any particular case.

20.9 THE FUTURE OF TOXICITY TESTING

Because of the public awareness of the potentially harmful effects of chemicals, it is clear that toxicity testing will continue to be an important activity and that it will be required by regulatory agencies before the use of a particular chemical is permitted either in commercial processes or for use by the public. Because of the proliferation of testing procedures, the number of experimental species and other test systems available, as well as the high dose rates usually used, it is clear that eventually, some expression of some type of toxicity will be obtained for most exogenous chemicals. Thus, the identification of toxic effects with the intent of banning any chemical causing such effects is no longer a productive mode of attack. The aim of toxicity testing should be to identify those compounds that present an unacceptable potential for risk to humans or to the environment and thus ought to be banned, but, at the same time, provide an accurate assessment of the risk to humans and the environment of less toxic compounds so that their use may be regulated.

Subjecting all chemicals to all possible tests is logistically impossible, and the future of toxicity testing must lie in the development of techniques that will narrow the testing process so that highly toxic and relatively nontoxic compounds can be identified early and either banned or permitted unrestricted use without undue waste of time, funds, and human resources. These vital commodities could then be concentrated on compounds whose fate and effects are less predictable.

Such progress will come from further development and validation of the newer testing procedures and the development of techniques to select, for any given chemical, the most suitable testing methods. Perhaps of most importance is the development of integrated test sequences that permit decisions to be made at each step, thereby either abbreviating the sequence or making the next step more effective and efficient. As more data are developed and analyzed, structure–activity models should become more predictive. Some current models for predicting the potential for carcinogenesis are accurate in about 90% of cases.

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SAMPLE QUESTIONS

1. Determination of the chemical and physical properties of a test chemical are not tests for toxicity. Why is knowledge of these properties an essential preliminary for such tests?

2. The determination of the LD_{50} as a measure of acute toxicity has been much criticized. Outline the nature of these criticisms.

3. Relative to the life span of the experimental animal, what time periods for dosing would be characteristic of (a) an acute toxicity test, (b) a subchronic toxicity test, and (c) a chronic toxicity test?

4. In addition to acute, subchronic, and chronic toxicity tests, other tests may be required by regulatory agencies on a case-by-case basis or are known to be useful adjuncts to current mandatory testing protocols. Name as many of these tests as you can.

5. Briefly describe the underlying principal of the Ames test.

6. A number of tests have been devised for the measurement of the frequency and nature of chromosome aberrations. What are they?

CHAPTER 21

Forensic and Clinical Toxicology

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21.1 INTRODUCTION

Forensic toxicology and clinical toxicology are specialized applications of the basic principles of toxicology covered earlier in this book. Forensic toxicology utilizes toxicological principles within the context of legal issues and is a component of forensic science. Toxicology results can provide evidence for determination of cause of death and time of lethal exposure; source of contamination of foods, water bodies, and adulterated pharmaceuticals; role of licit and illicit drug use; and impairment in vehicular collisions, workplace accidents, and domestic disputes, among other litigation-related matters. Clinical toxicology addresses treatment and prevention of chemical poisonings of both humans and domestic and companion animals, and includes aspects of occupational and emergency medicine, poison control, and public health. Recently, a heightened role for clinical toxicology has resulted from its contribution to emergency preparedness and homeland defense. Both clinical and forensic toxicology rely heavily upon analytical chemistry, usually of analytes in complex biological matrices, and utilize many fundamentals of toxicokinetics. Exposures to specific chemicals are surmised from recognition of clinical symptoms in a process operationally the reverse of the hazard identification. Both are represented by professional societies that support mechanisms for licensure and facilities accreditation and are taught in dedicated postbaccalaureate curriculum. Each have emphasis areas characteristic of the specialization as detailed in the material that follows.

21.2 FORENSIC TOXICOLOGY

21.2.1 Overview

Empirical knowledge of acute toxicity of chemicals has existed since antiquity. Chemical poisoning was a favorite plot element of the old masters—Shakespeare's poisonings in *Hamlet* and *Romeo and Juliet* and Chaucer's recounting of poisoned wine used in *The Parson's Tale* are a few examples—and various pesticides (e.g.,

A Textbook of Modern Toxicology, *Fourth Edition*. Edited by Ernest Hodgson

arsenical rat's bane, henbane with alkaloids hyoscyamine, and scopolamine) were readily available from medieval apothecaries. However, formal recognition of poison as a murder weapon began with presentation of postmortem residue analysis as legal evidence by Mathieu Orfila of Sorbonne University, Paris, and included application, in 1840, of the Marsh arsenic test on tissues of the deceased spouse of Madame LaFarge. As in today's application of forensic toxicology, this evidence was accepted only after reasonable doubt, introduced by Orfila's own observation of detectable baseline levels of tissue arsenic, was negated by his demonstration of Mr. LaFarge's unusually high body burden. With acceptance of an arsenic detection method and passage of the British Arsenic Law (1851) restricting purchase of arsenical rat poison, aspiring poisoners adopted neurogenic phytotoxins as a new murder weapon. This led to another milestone in the late nineteenth century with development of the Stas–Otto extraction for plant alkaloids, whose premise of pH-dependent solubility continues to be used today (Figure 21.1). The launch of forensic toxicology in the United States coincides with the 1918 appointment of A.O. Gettler in the Medical Examiner's office of the city of New York. Dr. Gettler's extensive contributions ranged from diagnosis of osteonecrosis from the popular

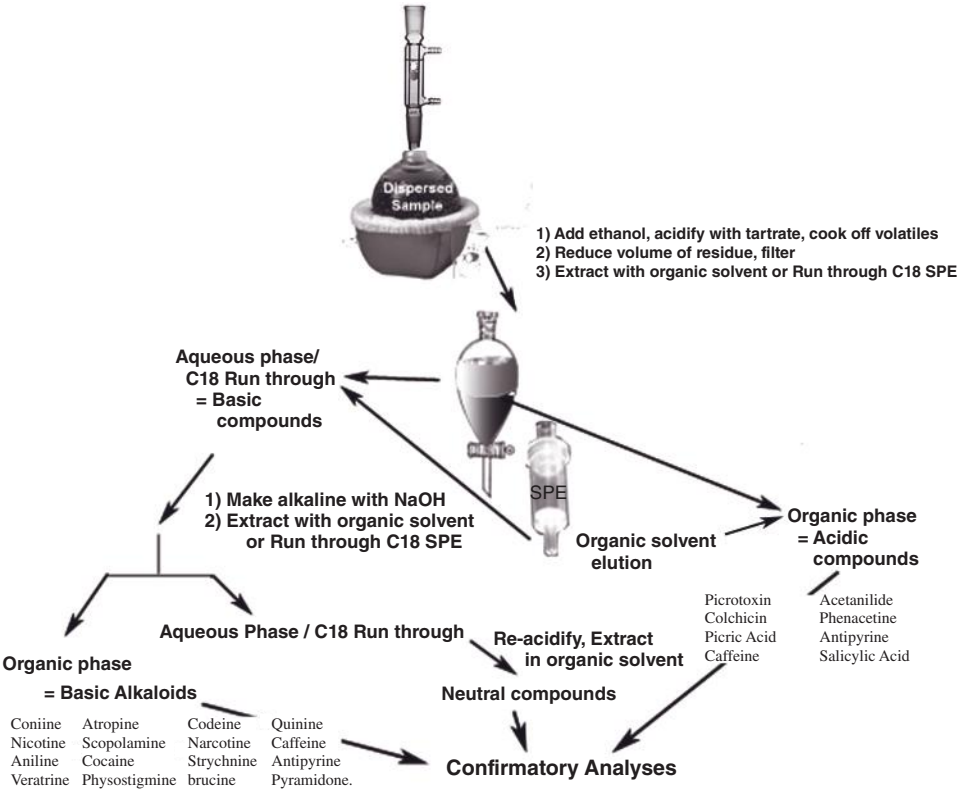

Figure 21.1 Partitioning of chemical classes in hydrophobic organic and hydrophilic aqueous phases as affected by pH as employed in the Stas–Otto procedure. Fractionation with a separatory funnel and solid phase extraction (SPE) cartridge are shown. Adapted from Autenrieth, 1921.

radium-based patent medicine "Radithor," development of methodology to quantify blood ethanol, and quality control recommendations on serotyping of degraded blood. Of broader impact was Dr. Gettler's extensive involvement in training of forensic toxicologists.

Twentieth-century advances in technology moved the field from see-taste-smell qualitative end points to instrumental measurements. The latter third of the twentieth century was marked by improved capability of and accessibility to analytical instrumentation enabled by incorporation of transistors, microchip electronics, and automation. On-site data collection enabled by equipment miniaturization eliminated some problems associated with field collection, then transport and lab analysis. Technological and electronic advances have also made it feasible to employ sophisticated instrumentation in the crime labs that previously were available only for research. Specificity of residue analysis has improved, and predictions from quantitative analysis became harder to refute. Recent expansion of forensic analysis in human performance certifications and drug use compliance has resulted in a shift in workload from predominantly postmortem chemical analysis to biomarker analysis of living tissues. Today's modern crime lab includes state-of-the-art instrumentation for analyses of a range of chemicals most relevant to forensics and is run by highly trained professionals nearly as efficient as portrayed in the popular medium.

21.2.2 Evidentiary Requirements

As with other accredited and licensed applications, performance standards require that proscribed procedures be followed. Crime labs must operate under Good Laboratory Practices (GLP). Isolation, identification, and annotation are critical for sample collection and are embodied with other information that becomes part of the sample's accession number. Chain-of-custody records documenting date of possession of signatories are mandatory for admissibility as evidence in litigation. Confidentiality requirements are strictly enforced. Most forensic laboratories operate as medico-legal components of state or local investigative agencies. Primary oversight for compliance with best practices results from facilities accreditation in the United States by the American Board of Forensic Toxicologists. If interstate activity is involved, the Department of Justice through the FBI may act in cooperation with state law enforcement agencies. Other federal agencies may have a role depending upon context, such as by FDA in cases of food and drug adulteration. Specific certification is required for conduct of mandatory urine testing of applicants for federal job positions. A recent National Academy of Science report has recommended expansion and standardization of forensic laboratory oversight (NRC, 2009).

Toxicological information relevant to litigation can be used formally as evidence in criminal and civil cases or informally in consultation with Counsel. Toxicology reports presented in court have recently been judged to be "testimonial evidence" by the U.S. Supreme Court and thus, those who generate the data in these reports may be cross-examined by defense exercising their sixth amendment rights (*Melendez-Diaz v. Massachusetts*, 2009). Forensic toxicologists that testify as expert witnesses are subject to provisions of Article VII of the Federal Rules of Evidence, especially Rule 702. This rule guides the acceptance of a witness as an "expert"

based upon "knowledge, skill, experience, training, or education" in a specialty as determined by the trial judge hearing the case, a condition decided in *Daubert v. Merrell Dow Pharmaceuticals, Inc. (1993)*. Consenting expert witnesses may be called by prosecution, defense, or be appointed by the court. Once admitted as an expert, the rule specifies that that individual's testimony be based upon facts interpreted from application of sound toxicological principles and methods. The expert toxicologist may testify to his opinion on facts presented at or before the hearing and, if the latter, may be requested to disclose those facts upon request of the court or upon cross-examination.

21.2.3 Sample Type and Chemical Classes Analyzed in Forensic Toxicology

Tissue types taken for forensic analyses depend upon the nature of needed information. Results intended for human performance assessment and determination of illicit drug use are from readily accessible, vital tissues—almost invariably blood and urine plus occasionally tears, expired air, perspiration, breast milk, feces, vaginal mucus, semen, and saliva. In addition, a wider range of tissues including internal organs can be taken during autopsy for postmortem examination. Brain, liver, and kidney are routinely collected solid tissues. Postmortem redistribution of blood requires comparison of analyte values from heart and a peripheral site, although availability of unclotted blood may be problematic at some sites. High tissue concentrations of chemical may suggest route of administration, while concentrations of parent compound or toxic metabolite sufficient to cause damage to critical organs can indicate cause of death. Stomach contents are examined in suspected oral overdoses. Hair and nails can be especially informative since their continuous growth during life and localized deposition of certain acute toxicants near time of exposure can be used to define a time interval from exposure to death. Banding in hair and nails has been used to establish time of poisoning by arsenic and heavy metals and to dispute their presence in exhumed corpses as a consequence of soil contamination. Vitreous humor is an essential sample that can be analyzed to confirm values in a clinical panel for blood, which are more susceptible to postmortem changes. For example, ethanol can be produced in decaying tissue and enter blood, thus compromising prediction of alcohol abuse from blood ethanol content. The vitreous chamber is somewhat protected from putrefaction and is thus not as susceptible to microbial ethanol contamination. Disposition of certain chemicals can elucidate circumstances of death, such as hemoglobin-bound CO below threshold (50%) in a body recovered from a burned building that would indicate death occurred for reasons other than smoke inhalation.

The presence of blood can be detected from the fluorescent product produced by hemoglobin peroxidase activity with luminal. Promising new laser-based biospectroscopic methods are in development that will enable detection of other biological fluids in the field. Blood is analyzed soon after collection and is transported to the laboratory for cell number and type and blood gases; thereafter, frozen or spotted, dried samples are prepared for archiving. Urine is chilled if not immediately analyzed to prevent loss of volatiles. Survey analyses are qualitative screens against a standardized background control with simplified procedures that

sometimes can be done in the field. If a positive sample is detected, more rigorous quantitative analysis may be done with more complex and costly instrumentation in a wet laboratory. More frequently employed analytical panels detect ethanol, various abused and therapeutic drugs (e.g., amphetamines, barbiturates, benzodiazepines, cannabinoids, opiates), common homicidal and suicidal agents (e.g., arsenic, cyanide, CO, pesticides, and thallium, the poison *du jour*), and performance enhancers (e.g., anabolic steroids, erythropoietin). Occasional environmental contaminants are monitored for specific types of litigation, such as establishing liability for illegal dumping of hazardous materials or off-site public exposure to releases implicated in causing illness or violation of policies limiting worker exposures. A well-publicized example includes hexavalent chromium contamination of drinking water portrayed in the movie *Erin Brockovich* (2000) and, more recently, exposure of National Guardsmen at a water pumping station in southern Iraq where sodium dichromate had been used prior to U.S. involvement to prevent pipe corrosion (Mancuso et al., 2008). A class of compounds of more recent interest is CWAs (chemical weapons agents, e.g., ricin, organophosphate nerve gases, phosgene, sulfur mustard).

A unique issue of forensic toxicology arises when information is needed after the decedent's body is embalmed (Levine, 2003). Components of embalming fluids, aldehyde fixatives, and disinfectants (phenol, methanol, ethanol), plus case-specific additives such as disinfectant benzaldehyde, fungicide parachlorobenzene, boric acid buffer, and eosin dyes, complicate toxicant analysis. However, in the case of the 1991 death of Kay Sybers, complete postmortem degradation of succinylcholine was evidently slowed by immediate embalming arranged by her husband who erroneously believed the preservatives would mask the poison he had earlier injected. Subsequent events can occasionally justify analysis on an exhumed corpse, such as confirmation of a *modus operandi* of a serial poisoner. Aside from previously mentioned complications associated with putrefaction and soil contamination, degradation of organic constituents by anaerobic microbial metabolism may limit their detection. Minerals will have more permanence and may be concentrated in longer-lasting remains such as bone, hair, and nails. Examples of homicidal use of selenium, arsenic, and heavy metals have been determined with exhumed material.

Residue analyses on nonbiological samples are also the subject of forensic toxicology. Chemical analysis of nitrogen is the basis of detection of gunshot residue. Product adulteration by poisonous additives is a constant problem in commerce, as evidenced by the recent supplementation of pet foods with the protein mimic, melamine. At accidental industrial releases, both state environmental officials and company representatives collect samples for future determination of liability. Analysis of accelerants by thermal desorption/gas chromatography (GC) is common in suspected cases of arson. The presence of specific sets of chemicals in a sample associated with the crime scene can give a fingerprint useful for identification of material associated with a potential suspect, for example, a particular source of chemicals used in an illicit drug synthesis lab, vegetation carried from the suspect's residence, explosives characteristic of an identified bomb maker. For these applications, sample collection must be as controlled as possible to avoid contamination, and worker safety of those obtaining samples must be of high priority.

21.3 CLINICAL TOXICOLOGY

21.3.1 Overview

Clinical toxicology is the application of toxicological principles for the purposes of diagnosing, treating, and preventing medical issues that arise from exposure to pharmaceutical agents, alcohol, and illicit drugs, biological toxins (e.g., snake and spider bites), or chemicals in the household, workplace, or environment. The clinical toxicologist is a specialist in the interaction of drugs and chemicals with the body. He or she must be familiar with the adverse effects of a large variety of therapeutic and toxic agents, and must have a thorough knowledge of the appropriate interventions for acute and chronic toxicant exposures.

21.3.2 Clinical Toxicology and Health Care

The term "clinical toxicology" is often used synonymously with "medical toxicology." After World War II, a profusion of new drugs and chemicals in the home and workplace prompted a marked increase in the rate of toxic incidents that were observed by physicians and hospitals. Recognition of the severity of the problem by the medical community was led by the American Academy of Pediatrics, who organized a nationwide committee on accident prevention to educate and assist physicians in the treatment and prevention of childhood poisoning. Largely out of these efforts, the first poison control center was established in Chicago in 1953. The classification of clinical toxicology as a subspecialty of medicine is thus relatively recent, and the role of the clinical toxicologist in health care is still evolving. Today, clinical toxicologists are doctors, pharmacists, and other members of the health-care community with special training in toxicology (see below). In hospitals and tertiary care centers (also known as specialty or referral centers), clinical toxicologists evaluate patient history and symptoms, order and interpret appropriate laboratory analyses, and consult with other members of the medical team to diagnose and plan treatment strategies for the patient. They also coordinate the practice of therapeutic drug monitoring (TDM), measuring and evaluating blood levels of medications in patients. TDM is particularly important for drugs with a low therapeutic index (TI) (see below), allowing drug dosages to be continuously adjusted so that adverse side effects can be minimized while therapeutic effectiveness is maintained.

Poison control centers are staffed by clinical toxicologists who provide 24-h, free access to information about poison exposure management and prevention to the public, as well as diagnostic and treatment recommendations to health-care providers. The American Association of Poison Control Centers (AAPCC) is an organization that certifies poison control centers and their personnel. The AAPCC also provides public and professional education about toxic agents and maintains the only poison information and surveillance database in the United States. The surveillance provided by the National Poison Data System (NPDS) allows for the monitoring of public health hazards, unusual exposure patterns, and outbreaks of public health emergencies, thus facilitating the early detection and elimination of hazardous chemical products and exposure incidents. The information obtained by the NPDS is used by public health and emergency preparedness specialists, and regulatory agencies such as the Food and Drug Administration, Environmental

Protection Agency, Consumer Product Safety Commission, and the Drug Enforcement Agency. It also serves as a valuable resource of toxicity exposure information for interested nongovernmental parties such as company product safety departments and academic medical centers.

Increasingly, clinical toxicologists are finding roles outside the hospital or poison control center in which to apply their training. Contract testing laboratories are often led by a clinical or forensic toxicologist skilled in analytical techniques. Many clinical toxicologists participate in occupational, environmental, and medical or medicolegal consulting, while others work in governmental and regulatory agencies such as the Food and Drug Administration, Centers for Disease Control, Public Health Service, Department of Homeland Security, and local health departments. Large companies—particularly in the chemical and pharmaceutical industries—employ clinical toxicologists as medical officers, health and safety officials, or in product development. Finally, due to the constant emergence of new and potentially hazardous drugs and chemicals, the prevalence of drug abuse, and the threat of terrorist attacks, there is a growing need for clinical toxicologists in academic research and teaching as our society prepares for its future.

21.3.3 Training and Certification

Most clinical toxicologists are trained as medical doctors, doctors of pharmacy, or nurses, although roles for pharmacologists and laboratory medicine specialists, or poison information specialists are increasingly being developed. Physicians who specialize in clinical toxicology must complete medical school and residency, and then complete a 2-year fellowship in affiliation with an academic medical center and/or poison control center. The expanding role of hospital pharmacists on healthcare teams requires advanced knowledge of adverse drug reactions, and most pharmacy training programs offer a clinical toxicology elective. Pharmacy students interested in specializing in clinical toxicology may choose a rotation in a poison control center as their senior practice experience. Some PharmD graduates complete residencies that focus on clinical toxicology or emergency medicine, although no formal accredited residency programs in those specialties are available. Registered nurses who wish to specialize in toxicology may complete training in an advanced practice specialty to become nurse practitioners. Generally, this requires a master's or a doctoral degree beyond the Bachelor of Science degree in nursing, although accelerated programs are available. Some programs require at least 1–2 years of clinical experience as a registered nurse for admission.

Educational requirements for specific positions in clinical toxicology vary from position to position and from state to state. For example, most poison control centers employ poison information specialists to field calls from health professionals and the public. The requirements for this position usually include certification by the American Association of Poison Control Centers as a certified specialist in poison information (CSPI). To be certified, the association requires licensure as a registered pharmacist within the state of certification, with a minimum of a bachelor's degree in pharmacy, or a registered nurse within the state, with a minimum of a bachelor's degree in nursing. On the other hand, to be a manager or director of a poison control center, usually, one must be a licensed physician, pharmacist, or nurse (although sometimes, advanced degrees in a biomedical

discipline are sufficient). Managers and directors must be board certified; physicians by the American Board of Medical Toxicology or a related discipline, and other professionals by the American Board of Applied Toxicology. Each of these boards maintains further requirements to be eligible to take their certification exams.

21.3.4 Clinical Management of Toxicant Exposure

Toxicant exposures may present in a variety of ways, ranging from an acute medical emergency to chronic conditions that have grown debilitating over time. In any emergency, basic life support is the most critical priority. Once the ABC's of airway, breathing, and circulation are addressed, supportive care (e.g., intravenous fluids, glucose, and seizure control if necessary), diagnostic evaluation and therapeutic interventions can be initiated. It is important to note at the outset that in any case where there is doubt about the diagnosis or treatment of a suspected poisoning, a call to the regional poison control center is well-advised. Cases of rare poisoning events and threats to public health should always be reported to a poison control center.

Evaluation A thorough history can provide some of the most valuable diagnostic information that can be obtained about a poisoning incident. Often, the patient is aware of the agent that he or she has been exposed to and can give details important to therapeutic planning such as the time since exposure and the dose received. In patients who are unresponsive or uncooperative, the diagnosis of poisoning must rely on the results of the physical exam and laboratory tests. General signs may be present that provide clues to the source of toxicity. For example, characteristic odors such as almonds (cyanide), garlic (organophosphates, metals such as arsenic, thallium, or tellurium), fruitiness (isopropanol, alcoholic ketoacidosis) may be detectable. An unusual appearance of the skin, such as bruising, blisters, or jaundice may also be informative. Vital signs such as temperature, pulse, respiratory rate, and blood pressure are always collected and provide diagnostic clues as well as indicate the progression or resolution of toxic effects. Neurologic signs such as confusion, agitation, sleepiness, seizures, or coma may be present depending on the agent involved. Often, patients display several signs at once that point to a specific class of poisons. This constellation of typical symptoms that are characteristic of a class of poisons is called a toxidrome. For example, the toxidrome for organophosphate toxicity can be recalled by a mnemonic known as DUMBELS, which stands for diarrhea, urination, miosis (pinpoint pupils), bronchorrhea (excessive secretions in the bronchi), bradycardia (slowed heart rate), emesis (vomiting), lacrimation (tearing), and salivation. Rapid recognition of toxidromes can help determine whether the issue is a poison and the class of poison that has been encountered.

Standard laboratory tests may reveal alterations in electrolytes, blood gases, or blood glucose concentrations, acid–base disturbances, or other changes that when considered alone would not be diagnostic of poisoning. However, these findings often contribute to toxidromes and thus aid in identifying the class of toxicant that might be present. Other clinical blood chemistry analyses such as liver and kidney function tests may be altered by specific toxicants; monitoring these throughout the therapeutic period is useful for weighing therapeutic options and developing prog-

noses. Electrocardiogram abnormalities are common with a wide variety of drugs and toxins and occasionally bear characteristic signatures that help diagnose exposure to specific toxicants.

Most diagnoses of toxicity are made based on patient history and the clinical presentation (i.e., the signs, symptoms, and results of general lab tests). Since the mainstay of treatment in chemical poisoning is supportive care, these basic findings are often sufficient to begin therapeutic intervention. On the other hand, if the physician suspects a toxicant or class of toxicant for which a specific antidote exists, he or she may desire additional confirmative information. Furthermore, for patients in acute distress, stat (urgent) diagnostic testing is necessary. Because of this need, several qualitative and semiquantitative tests have been developed to rapidly identify selected toxicants. These tests are used with the understanding that there is a balance between the need for quick decision making and the need for accuracy. The National Academy of Clinical Biochemistry has prepared a set of guidelines for two tiers of laboratory tests that should be available for patients in emergency departments. The first tier consists of "stat" (i.e., with an ideal reporting turnaround time of 1 h or less) tests for selected toxicants that are commonly encountered and for which a semiquantitative test is available (see Table 21.1). Usually, these tests are based on immunoassay technology (described in Section 21.4). The second tier of tests consists of comprehensive or broad-spectrum analyses for toxicants that may not have been identified in the first tier, or are unavailable on a stat basis, or

TABLE 21.1 Recommended "Stat" Toxicology Assays[a]

Serum (Quantitative or Semiquantitative)	Urine (Qualitative)
Acetaminophen	Cocaine
Lithium	Opiates
Salicylates	Barbiturates
Theophylline	Amphetamines
Valproic acid	Propoxyphene (Darvon)
Carbamazepine	PCP
Digoxin	Tricyclic antidepressants
Phenobarbital	
Iron	
Ethanol	
Methanol	
Ethylene glycol	
Methemoglobin, carboxyhemoglobin[b]	

[a] Adapted from Wu et al. National Academy of Clinical Biochemistry Laboratory Medicine Practice guidelines: Recommendations for the use of laboratory tests to support poisoned patients who present to the emergency department. *Clin. Chem.* **49** (3): 357–379, 2003.

[b] Not specific for any of the multiple toxicants that produce methemoglobinemia or carboxyhemoglobinemia. Detected by co-oximetry, which measures oxygenated hemoglobin as a percentage of total hemoglobin.

for which quantitative results are needed for long-term patient management. These tests entail more quantitative analytical tools such as GC/mass spectrometry (MS) and high-performance liquid chromatography (HPLC). Smaller hospitals that do not have adequate resources to perform these tests usually send samples to a local or regional reference laboratory or toxicology laboratory for analysis. For purposes of monitoring therapeutic effectiveness, it is sometimes more practical to rely on other biomarkers of toxic effect such as acetylcholinesterase activity or coagulation, rather than on the concentration of the toxicant itself.

Treatment The first goal in the treatment of a poisoned patient is to minimize further exposure. This involves removal of any material that has not been absorbed by means that are appropriate to the route of exposure, and by enhancing elimination of material that has been absorbed. As might be expected, the use of these techniques is guided by pharmaco- and toxicokinetic principles (for further discussion of these principles, see Chapters 5–9), and the choice of which method to use depends on the kinetic limitations presented by the suspected toxicant. In addition, elimination therapies are most effective when administered within the first few hours after intoxication.

Poisons that are ingested may be removed by inducing vomiting or gastric lavage with a stomach tube and saline. However, these methods are inappropriate if significant time has elapsed since ingestion (since most toxicants will have been absorbed within the first hour or so), if the patient is suspected to have ingested corrosive agents (as this promotes further damage to the mucosal lining of the esophagus), or when central nervous system function is compromised (since the patient could aspirate the stomach contents). Furthermore, vomiting may preclude the use of oral therapeutics. Rather than induce vomiting, most hospitals prefer to use chemical adsorption with agents such as activated charcoal or whole bowel irrigation. These techniques not only prevent absorption by removing the agent from the gut, but also enhance elimination by inhibiting the enterohepatic recirculation of toxicants that are normally taken up by the liver. Activated charcoal is administered as a slurry either orally or by using a nasogastric tube in single or multiple doses. A wide variety of poisons are effectively adsorbed by charcoal; notable exceptions include alcohols and heavy metals, to which it binds poorly. These agents may be removed by whole bowel irrigation. It has been proposed that whole bowel irrigation promotes a beneficial dialysis-like effect, in which low molecular weight compounds move by a diffusion gradient from the circulation back into the gut. Cathartics such as sorbitol and magnesium sulfate may be used to decrease the absorption of substances by accelerating gastrointestinal motility. Although cathartics are sometimes added to activated charcoal slurry, few clinical data exist to demonstrate their effectiveness. The elimination of weak acids and agents that are substantially excreted by the kidneys can be aided by alkaline diuresis, involving the addition of sodium bicarbonate to intravenous fluids. This method is particularly effective for reducing circulating levels of salicylates (e.g., aspirin) and chlorphenoxy herbicides. Enhancing clearance by hemodialysis (in effect, ultrafiltration) is useful for highly water-soluble, low molecular weight compounds, and those with a low apparent volume of distribution (Vd) that tend to remain in circulation rather than distribute to the tissues. Ethanol, ethylene glycol, salicylates, and lithium are often removed by dialysis. Hemoperfusion, in which

large volumes of patient blood is passed over adsorbent activated charcoal, is appropriate for more lipid-soluble drugs such as barbiturates, acetaminophen, and insecticides.

The second goal of therapeutic management of toxicant exposure is to reduce toxic effects by preventing the interaction of the toxicant with its target sites, or altering its biotransformation to toxic metabolites. Some antidotes act by binding specific toxicants and sequestering them in body compartments where they cannot reach their target sites. In the case of chelating agents, this also enhances the elimination of heavy metals. Chelating agents are negatively charged molecules that bind to the metal forming a stable complex, which is excreted in the urine. Dimercaprol, also known as BAL (British anti-lewisite), was developed for use in warfare as an antidote to the organoarsenical agent lewisite. It is still used today for the treatment of acutely symptomatic patients who have ingested arsenic, lead, and other metals. Other dithiol-containing agents used for chelation include succimer (dimercaptosuccinic acid) and deferoxamine. Calcium edentate (EDTA) is also used therapeutically to chelate metals (particularly lead), as well as calcium in cases of hypercalcemia resulting from digitalis toxicity. The synthetic pigment Prussian blue is approved by the FDA for medical use as a chelator of thallium and radioactive cesium. Immunotherapeutics (i.e., antibodies and Fab fragments of antibodies) also act by sequestering toxicants away from their site of action. Fab fragment antidotes are available for treating overdoses with certain drugs such as digoxin, and for many natural, peptide-based toxins such as snake and spider venoms.

The mechanisms of action of antidotes are as varied as those of toxicants themselves. Pharmacological antagonists, competitive inhibitors or activators of drug metabolizing enzymes, decoys, redox agents, and substances that otherwise reduce the impact of toxicants may be used as antidotes. Despite the wide variety of antidotal mechanisms, however, the actual number of effective antidotes available for clinical use is limited. Selected examples of common toxicants and their antidotes are presented in Table 21.2.

TABLE 21.2 Common Toxicant Antidotes and Their Mechanisms of Action

Toxicant	Antidote	Mechanism of Action
Acetaminophen	*N*-acetylcysteine	Enhances glutathione synthesis, may directly bind reactive metabolites
Crotalid snake venom	Fab antibody fragments	Neutralize toxin by high affinity binding and sequestration from its target
Ethylene glycol	Fomepizole (4-methylprazole)	Inhibits alcohol dehydrogenase, the primary enzyme in the biotransformation of ethylene glycol to toxic metabolites
Nitrates/Nitrites	Methylene blue	Accelerates the conversion of methemoglobin (Fe^{3+}) to hemoglobin (Fe^{2+})
Opioids	Naloxone	Opioid receptor antagonist
Organophosphates	Atropine	Muscarinic acetylcholine receptor antagonist
	Oximes	Reactivate acetylcholinesterase
Warfarin	Vitamin K	Restores synthesis of coagulation factors

Additional Considerations Health-care professionals that attend to poisoned patients must be prepared to assess toxic situations and decide on treatment options rapidly. As described above, several factors must be weighed, including the type of poison, the dose, route and time since exposure, the clinical status of the patient (including age, general health, concurrent use of prescribed/abused drugs), and the availability of medical resources and facilities. Decisions such as those regarding when and how often to order quantitative analyses, the appropriate use of elimination enhancers, and the optimal duration of antidote therapy are guided by awareness of these factors, along with a thorough working knowledge of pharmacokinetic and toxicokinetic principles. These principles are described in more detail in Part III of this book. For clinicians, predictive kinetic calculations should include consideration of factors such as the metabolic activation of compounds to toxic metabolites, the continued redistribution of the toxicant between the bloodstream and the target tissues, and the effects of the toxicant on its own clearance (such as by high-dose saturation of plasma protein binding or metabolic and excretion mechanisms). An important precaution is that poisons often adversely affect organ function, causing disproportionate changes in their blood concentrations. For example, barbiturates lower blood pressure significantly, reducing hepatic and renal perfusion and prolonging their toxicity. Finally, while the goal of therapy is to change the kinetics (absorption, distribution, metabolism, and excretion) or dynamics (interactions with target sites) of the overdosed drug or poison, it is important to note that therapeutic interventions act by their own pharmacokinetic principles. Thus, short-acting antidotes such as naloxone, an opioid receptor antagonist, must be dosed multiple times over the course of treatment, since their half-life is much shorter than that of most of the opiates with which they are intended to compete.

For some drugs, relatively small changes in systemic concentration can produce very marked changes in therapeutic and toxic response. These drugs are said to have a low TI, quantitatively represented as the ratio lethal dose 50/effective dose 50 (LD_{50}/ED_{50}). Drugs that are lethal to 50% of a test animal population at a relatively low dose (the LD50), compared to their minimum effective dose for 50% of the population (the ED50) would have a low TI and thus be likely to cause toxicity in patients within a range very close to therapeutic levels. Lithium, warfarin, phenytoin, and digoxin are examples of drug with a low TI.

Genetic factors are often unknown players in the disposition of toxicants in acute cases. Rarely is the genetic profile of an individual known at the time of treatment for poisoning. Nevertheless, interindividual variation in the response to drugs, poisons, and their antidotes may affect the clinical course and response to treatment in acute exposures. The role of genomics in toxicology is discussed in Chapter 28. With respect to clinical toxicology, it is important to keep in mind that not only may genetic variability affect toxic outcomes due to differences in metabolic and clearance enzymes, but it may also determine adverse responses in other ways. For example, polymorphisms in opioid receptor genes mediate the adverse effects (e.g., nausea) that some patients experience at therapeutic doses of morphine. Similarly, genetic variations at the receptor and even downstream of receptors at the second messenger level are thought to influence the development tolerance and dependence to opiates.

21.4 ANALYTICAL METHODS IN FORENSIC AND CLINICAL TOXICOLOGY

Qualitative screens are often solid-state antibody- or enzyme-based techniques with colorimetric end points, similar to dip-stick products used for home testing for pregnancy and blood glucose. Screens compare analytes in test sample matrices, with very little preliminary workup, to cut-off values and often have rapid responses necessary to provide information "stat" for clinical toxicology (Table 21.1). Specificity is dependent upon cross-reactivity of the antibody and is generally good for chemical class, such as amphetamines, but may be compromised for detection of a specific member of a class, such as methamphetamine. Basic principles and conduct of such immunoassays is outlined in Chapter 2. Urine is often used in screens since urinary concentrations of parent compound or metabolites are usually not well correlated with clinical symptoms. Standardized immunoassays are typically available for several analgesics and antidepressant fluoxetine, causes of the first and eighth most common human poisoning in the United States (Bronstein et al., 2008), in addition to benzodiazepines, barbiturates, and others. Urinary creatinine is measured for all samples because values below 20 mg/dL indicate potential sample dilution, an adulteration often encountered with drug testing. In clinical toxicology, urinary and blood creatinine are used to determine renal clearance, and abnormally low values indicate impaired kidney function. Another common screen used in the field for forensics relies upon photoelectric detection of ethanol oxidation in air exhaled into a breathalyzer.

Since rapid turnaround is not as critical in forensic as in clinical toxicology, while quality control is more demanding, positive samples in screens undergo follow-up confirmatory analyses. If amounts are limited, the choice of which analyses are performed is guided by prior knowledge based upon additional case history. In general, these procedures involve a preliminary preparative stage to remove analyte from its biological matrix, such as filtration to remove solids, tissue homogenization, pH adjustment, and protein precipitation, then some type of fractionation followed by instrumental measurement of an output related to concentration. Fractionation can be either low-resolution segregation into chemical classes (Figure 21.1) or high-resolution chromatographic separation (Figure 21.2). Concentration-dependent output will depend upon what type of instrument is hyphenated with the fractionation technology and is normalized to certified standards. Blood is the most frequent tissue used for such analyses and considerable guidance exists relating blood values and adverse effects, such as lethality and behavioral effects. For example, comprehensive nomograms exist for acetaminophen relating time after ingestion, plasma concentration, and severity of hepatotoxicity.

Bulk fractionation into polar versus nonpolar solvents as effected by pH is a historical procedure, but has been largely replaced by solid phase extraction. Both rely upon greater distribution of the charged conjugate base or protonated acid of Lewis acids and bases, respectively, in more polar solvents or on solid adsorbants (Figure 21.1). Elution into apolar medium is then achieved with a change in pH relative to a compound's pKa. The process is repeated until tissue components are segregated into fractions containing originally acidic, neutral, and basic compounds. Since many drugs, licit and illicit, have pKas in a range accommodated by this

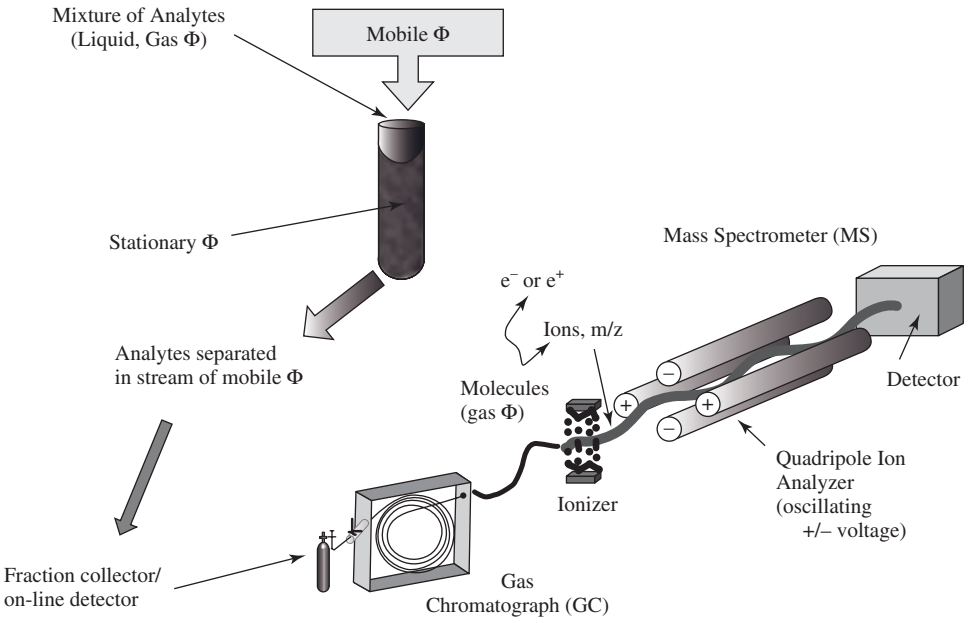

Figure 21.2 General example of analyte separation from a mixture based upon differential affinities for stationary and mobile phase (Φ) constituents. Separated analyte stream is then fed into hyphenated instrument for confirmation, such as the gas chromatograph/mass spectrometer of this example.

procedure, it can be used as a preparatory method for identification of drugs in the resultant fractions by chromatographic techniques.

Blood analysis for clinical toxicology typically starts with a general assessment of complete blood counts (CBCs) and panels of biomarkers, including intermediary metabolites, acute phase proteins, and tissue selective enzymes. Chemical assays and more targeted diagnostic tests may be performed early in treatment if specific information is available from case histories. A CBC analyzer is a dedicated flow cytometer that measures and classifies blood cells with respect to size and intracellular granularity and can distinguish erythrocytes, lymphocytes, granulocytes classes, and platelets. Hemoglobin is measured simultaneously, enabling classification of various types of anemia. Various biomarkers are measured with automated, continuous flow bioanalyzers/spectrophotometers; examples are alanine aminotransferase (ALT; increased with liver damage), albumin (decreased with inflammation), glucose (elevated with diabetes), urea (blood urea nitrogen [BUN]; elevated with poor kidney function), and electrolytes. The latter is used to detect the presence of an imbalance between serum anions and cations, the so-called anion gap calculated as $([Na^+]-([Cl^-]+[HCO_3^-]))$, and whose elevation is diagnostic for compounds that cause loss of bicarbonate, that is, metabolic acidosis. Methanol toxicity is associated with a high anion gap (>16 mEq/L) because of its metabolism to formic acid.

If the identity of poison and time or amount of administration is known, then quantitative determination of serum values will aid emergency room medical personnel to make a prognosis of outcome and chart an appropriate course of

treatment. For example, nomograms for salicylate (aspirin) and acetaminophen are commonly used. Serum analyses are also used to monitor effective levels of therapeutics given as antidotes, especially those with a low TI such as theophylline. Occasionally, other tissues are analyzed on a case-specific basis, such as neonatal meconium to determine maternal drug abuse. Clinical laboratories may also be utilized to biomonitor hazardous materials as they relate to occupational medicine, for example, as encountered by first responders with time after an accidental chemical release. Similar facilities with equivalent capabilities exist for forensic and clinical toxicological applications to veterinary medicine (Galey and Talcott, 2005).

Principles of separation for many of the laboratory instruments used in any analytical laboratory, including that for forensic and clinical toxicology, are detailed in Chapter 24 of this book. Additionally, details of quality control necessary to be considered "sound toxicological methodology" are outlined. In general, analytes adsorb with varying affinity based upon lipophilicity, volatility, size, and/or pH onto an immobile phase and are eluted into a mobile phase as conditions progressively change. Common solid phase materials are modified silicas, resins, cellulose, and other polymers of varying porosity. Compounds differentially eluted into mobile phase of either gas (GC) or liquid (liquid chromatography, LC) are separated by distance from origin on a solid support (thin layer chromatography, TLC) or time to appear before a detector for mobile phase freely flowing through a column format. The resulting chromatogram separates and localizes compounds depending upon their specific, relevant properties, which are often known from databases and can be used for identification. Both parent compound and metabolites are simultaneously detected with chromatographic procedures, and their relative abundance will depend upon type of sample. For example, psychoactive agent of marijuana, Δ^9-tetrahydrocannabinol is lipophilic (calculated K_{ow} ~10^7) and readily deposits in lipid-rich brain, while oxidized metabolite 11-*nor*-9-carboxylic acid-Δ^9-tetrahydrocannabinol is excreted in urine. For testosterone, normal amounts of androgenic C-17 S isomer are 1–4 times that of the R isomeric epitestosterone in human male urine. Ratios higher than 4:1 indicate administration of exogenous testosterone for performance enhancement. The presence of exogenous urinary testosterone is confirmed by lowered carbon isotope ratios. Testicular synthesis of testosterone from precursors with typical environmental ratios of ^{12}C:^{13}C of ~100:1 causes enrichment of product in the more reactive lighter isotope, while chemically synthesized testosterone has the same ^{12}C:^{13}C as the feedstock and is excreted into urine with the carbon isotope ratio unchanged.

The workhorse of quantitative forensic analysis is capillary GC in which deposition of stationary phase as a thin liquid or polymer film on the walls of a glass capillary greatly provides a highly efficient format. Volatile components, such as short-chain alcohols and sulfide decomposition products, can be off-gassed from intact samples into headspace that is directly analyzed by GC. Vapors and gases trapped on sampling sorbents can be injected directly from a thermal desorption attachment coupled to a GC sample port. Analysis by LC, with mobile phase propelled by high pressure (HPLC), is also frequently used. Detection methods vary from those based upon formation of colored or fluorescent derivatives, absorption spectroscopy in the ultraviolet and visible ranges, redox-coupled coulometry, and electron expulsion or capture. GC and HPLC analyses of specific chemicals in blood are also critical components of the clinical toxicology laboratory. MS hyphenated

with either GC or HPLC provides a powerful, versatile tool for simultaneous detection plus compound identification. Recent improvements in durability and size reduction of instruments performing these chromatographic techniques and analyte detection, largely funded by the Departments of Defense and Homeland Security to counter threats from CWAs, have been developed to provide essential real-time monitoring. Extensive mass spectral libraries for compounds typically encountered in forensic and clinical toxicology are readily available.

Metals can be analyzed directly in blood and urine. Solid tissue is typically ashed in a furnace or digested with strong acid and residue is redissolved for analysis. Contamination with extraneous metals is minimized by using acid-washed glassware and metal-free reagents. If remains are recovered from a metal-rich environment, such as soil, surrounding environment must be concurrently analyzed using the same workup procedures. For quantification by atomic absorption spectroscopy (AAS), dissolved metal ions are then atomized by high temperature, either generated via an oxyacetylene flame, graphite furnace, or electrothermally, and energy absorbed to excite metal valence electrons by a transecting beam of visible light is measured. Incident light of a very narrow wavelength characteristic of the specific metal analyte is produced by a hollow cathode lamp, and photon absorption is measured by a downstream spectrophotometer. A detection method that can simultaneously measure dozens of metals and metalloids, including As, Cd, Cr, Hg, Pb, Se, Tl, and U, is inductively coupled plasma–mass spectrometry (ICP-MS). This procedure also atomizes metal ions but uses an argon plasma to achieve temperatures so high that the valence electron ionizes from the metal atom. The resulting cations are then introduced into and resolved by a mass spectrometer. Sensitivity of ICP-MS approaches pg/mL (picograms per milliliter) for urine and blood, ~10-fold greater than that of AAS. Another versatile technique recently introduced for forensic analysis of metals is X-ray fluorescence (XRF) in which inner shell electrons excited to higher orbitals emit photons upon decay back to ground state that are characteristic of the specific metals in a sample. This also is a multi-element technique and can achieve resolution of ~50 μ as determined by the size of the incident X-ray beam. A unique feature of XRF analysis is the ability to determine metal speciation, that is, identification of what ligands are coordinated with a given metal.

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SAMPLE QUESTIONS

1. The first judicial application of forensic toxicology was
   a. the state-sponsored execution of Socrates
   b. a result of one of the provisions of the Magna Carta
   c. conducted by Orfila to convict Marie Lafarge of homicide using arsenic poisoning
   d. to provide support for Occupational Safety and Health Administration (OSHA) standards for benzidine dye workers

2. A comatose teenager is brought to a hospital emergency room and a screening immunoassay detects a urinary barbiturate well above cutoff value. The patient's mother reports that neither the teen nor her siblings have a history of epilepsy and that her daughter was awake and alert only an hour ago. Secobarbital has a half-life of ~30 h and a volume of distribution (Vd) of ~1.5 L/kg, while long-acting phenobarbital's half-life is ~100 h and Vd ~0.7 L/kg. Both are commonly abused street drugs.

   The patient's respiration is stabilized with a ventilator and blood pressure is maintained with IV fluids. There are no specific antidotes for barbiturates. What should be the next clinical treatment for this patient?

3. Compare how toxicokinetic information is applied to clinical toxicology versus forensic toxicology.

4. Cadaverine (1,5-diaminopentane) is a volatile decomposition product contributing to the malodor of decaying corpses. It is a basic aliphatic compound with pKa ~10. If a forensic toxicologist sought to determine whether a body contained residues of strychnine using fractions generated with the Stas–Otto procedure, would cadaverine contaminate the strychnine-containing fraction? Briefly explain your answer.

CHAPTER 22

Prevention of Toxicity

ERNEST HODGSON

22.1 INTRODUCTION

Regardless of the results of hazard assessment (Chapter 20) toxicity is always a consequence of exposure, and without exposure there cannot be a toxic effect. However, if both hazard and exposure are verified, and the risk appears to be significant, there are a range of possible actions available to reduce that risk. These actions range from outright banning of both production and use of the chemical in question through legislative means, through measures to reduce exposure, to measures to restrict effect.

Exposure can be restricted by prevention of manufacture, control of use patterns, control of application techniques, by environmental manipulation and/or by education. Effects can be restricted by prophylactic and therapeutic methods and by education. Many of these approaches are controlled in whole, or in part, by legislation while many are simply the use of common sense in the cause of good domestic and industrial hygiene. In many circumstances, particularly in the home and workplace, wisdom dictates courses of action not necessarily prescribed by law. All of these aspects, taken together, comprise the subject matter of this chapter.

Laws and regulations provide the framework for organized efforts to prevent toxicity, and sanctions are necessary to prevent those without social conscience from deliberately exposing their fellows to risks from toxic hazards. However, without a population educated to toxic hazards and their prevention, the laws cannot be properly administered. The key to toxicity prevention lies in information and education with legislation, regulation, and penalties as final safeguards. In all probability, the better educated and informed the general population is, the less likely are laws to be necessary.

22.2 LEGISLATION AND REGULATION

In the best sense, legislation provides an enabling act describing the areas to be covered under the particular law and the general manner in which they are to

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be regulated, while designating an executive agency to write and enforce specific regulations within the intent of the legislative body. For example, the Toxic Substances Control Act (TSCA) was passed by Congress to regulate the introduction of chemicals into commerce, to determine their hazards to the human population and the environment, and to regulate or ban those deemed hazardous. The task of writing and enforcing specific regulations was assigned to the United States Environmental Protection Agency (USEPA).

Legislative attempts to write specific regulations into laws usually fail. The resultant laws lack flexibility and, because they are generally not written by toxicologists, are frequently ambiguous and seldom address the problems in a scientifically rigorous manner.

It should be borne in mind that legislation is a synthesis of science, politics, and public and private pressure. It represents a society's best estimate, at that moment, of the risks it is prepared to take and those it wishes to avoid, as well as the price it is prepared to pay. Such decisions properly include more than science. The task of the toxicologist is to see that the science that is included is accurate and is interpreted logically.

This section is based primarily on regulations in the United States, not because these are necessarily the best but because, in toto, they are the most comprehensive. In many respects, they are a complex mixture of overlapping laws and jurisdictions, providing unnecessary work for the legal profession. At the same time, few, if any, toxic hazards in the home, workplace, or environment are not addressed.

22.2.1 Federal Government

The following is a summary of the most important federal statues concerned in whole or in part with the regulation of toxic substances. Further details can be obtained from the appropriate websites (e.g., www.epa.gov), including titles of all Acts administered, and actions taken under those acts.

Clean Air Act The Clean Air Act is administered by the EPA. Although the principal enforcement provisions are the responsibility of local governments, overall administrative responsibility rests with the EPA. The Clean Air Act is the law that defines EPA's responsibilities for protecting and improving the nation's air quality and the stratospheric ozone layer. Since the last major change in the law, the Clean Air Act Amendments of 1990, legislation has been passed to provide for several minor changes. The Clean Air Act, like other laws enacted by Congress, was incorporated into the United States Code (Title 42, Chapter 85).

This act requires criteria documents for air pollutants and sets both national air quality standards and standards for sources that create air pollutants, such as motor vehicles and power plants. An important action previously taken under this law was setting the standards for the now completed elimination of lead in gasoline.

Clean Water Act The Clean Water Act, which amends the Federal Water Pollution Control Act, is also administered b the EPA and provides for the protection of surface water quality, in large part through funding of municipal sewage treatment plants. However, with respect to toxicity prevention, it is more important that the act regulates emissions from municipal and industrial sources. It has as its

goal the elimination of discharges of pollutants and the protection of rivers so that they are "swimmable and fishable" and applies to "waters of the United States" subsequently defined to include all waters that reach navigable waters, wetland, and intermittent streams. It is not directly concerned with the quality of drinking since this is regulated under the Safe Drinking Water Act, also by the USEPA. While previous actions dealt primarily with point sources discharges, such as municipal sewage plants and industrial plant discharges, current actions also deal with so-called wet-weather sources including runoff from streets and farms. This act allows the federal government to recover cleanup and other costs as damages from the polluting agency, company, or individual.

Safe Drinking Water Act (1974, 1986, 1996) Specifically applied to water supplied for human consumption, this act requires the EPA to set maximum levels for contaminants in water delivered to users of public water systems but excludes private wells serving less than twenty-five people. Two criteria are established for a particular contaminant: the *maximum containment level goal (MCGL)* and the *maximum contaminant level (MCL)*. The former, the MCLG, is the level at which no known or anticipated adverse effects on the health of persons occur and that allows an adequate margin of safety. The latter, the MCL, is the maximum permissible level of a contaminant in water that is delivered to any user of a public water system. MCLs are expected to be as close to the MCLG as is feasible.

Originally focused on water treatment since the 1996 amendments, the law has also focused on source water protection and funding for water system improvements thus extending the reach of the law from source to tap.

Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) Commonly known as Superfund, CERCLA was enacted in 1980 and was amended in 1986 to deal with the many waste sites that exist across the nation. It covers remedial action, including the establishment of a National Priorities List to identify those sites that should have a high priority for remediation. This act authorizes the cleanup of hazardous waste sites, including those containing pesticides, that threaten human health or the environment. If they can be identified, USEPA is authorized to recover cleanup costs from those parties responsible for the contamination. CERCLA provides a fund to pay for the cleanup of contaminated sites when no other parties are able to conduct the cleanup. The *Superfund Amendments and Reauthorization Act (SARA) (1986)* is an amendment to CERCLA that enables USEPA to identify and cleanup inactive hazardous waste sites and to recover reimbursement of cleanup costs. One section of CERCLA authorizes the EPA to act whenever there is a release or substantial threat of release of a hazardous substance or "any pollutant or contaminant that may present an imminent or substantial danger to the public health or welfare" into the environment.

Consumer Products Safety Act (CPSA) and Consumer Products Safety Commission Improvements Act (CPSCIA) CPSA is administered by the Consumer Products Safety Commission (CPSC). The CPSA is designed to protect the public against risk of injury from consumer products and to set safety standards for such products. It provides the authority to ban products where no feasible standard can be set and to recall products that present a substantial risk. Unfortunately,

the Act is weakened by exclusion of many categories of consumer product from CPSC's authority. Such products, include food, drugs, cosmetics, medical devices, tobacco products, firearms, motor vehicles, pesticides, aircraft, and boats. These products are covered by other laws and jurisdictions, the USEPA and the Food and Drug Administration (FDA) being the most common, making a unified approach difficult, if not impossible.

Controlled Substances Act (CSA) The CSA not only strengthens law enforcement in the field of drug abuse but also provides for research into the prevention and treatment of drug abuse. CSA is administrated by the Drug Enforcement Authority (DEA) which, with the FDA, decides which substances are added to or deleted from the five schedules (classifications) of controlled substances. DEA deals primarily with law enforcement; thus, its involvement with toxicological aspects is limited.

Federal Food, Drug, and Cosmetic Act (FD&C Act) First passed in 1938 following an incident in which over 100 people died as a result of a then legal medication in which ethylene glycol was used as a solvent, the FD&C Act is administered by the FDA. It establishes limits for food additives and cosmetic components, sets criteria for drug safety for both human and animal use, and requires the manufacturer to prove both safety and efficacy. The FDA is authorized to define the required toxicity testing for each product. This act contains the Delaney clause, which states that food additives that cause cancer in humans or animals at any level shall not be considered safe and are, therefore, prohibited from such use. This clause has recently been modified to permit the agency to use more flexible risk/benefit-based guidelines. Under the Food Quality Protection Act (FQPA) of 1966 (see below) the Delaney clause is no longer applied to pesticide residues in food. This law also empowers the FDA to establish and modify the generally recognized as safe (GRAS) list and to establish good laboratory practice (GLP) rules.

Occupational Safety and Health Act This act, enacted in 1970, created the Occupational Safety and Health Administration (OSHA) and is administered by OSHA. The act concerns health and safety in the workplace. OSHA sets standards for worker exposure to specific chemicals, for air concentration values, and for monitoring procedures. Enforcement by OSHA is also provided for under the act. Construction and environmental controls also come under this act. This act provides for research, information, education, and training in occupational safety and health.

By establishing the National Institute for Occupational Safety and Health (NIOSH), the act provided for appropriate studies to be conducted so that regulatory decisions could be based on the best available information.

National Environmental Policy Act The National Environmental Policy Act (NEPA) of 1970 is an umbrella act covering all U.S. government agencies, requiring them to prepare environmental impact statements for all federal actions affecting the quality of the human environment. Environmental impact statements must include not only an assessment of the effect of the proposed action on the environment, but also alternatives to the proposed action, the relationship between local short-term use and enhancements of long-term productivity, and a statement

of irreversible commitment of resources. This act also created the Council on Environmental Quality, which acts in an advisory capacity to the president on matters affecting or promoting environmental quality.

Resource Conservation and Recovery Act (RCRA) Administered by the EPA, the RCRA is the most important act governing the disposal of hazardous wastes; it promulgates standards for identification of hazardous wastes, their transportation, treatment storage, and disposal. Included in the latter are siting and construction criteria for landfills and other disposal facilities as well as the regulation of owners and operators of such facilities. The three principal areas covered are hazardous wastes, nonhazardous solid wastes, and underground storage tanks. Farmers and commercial pesticide applicators are subject to penalties if they fail to store or dispose of pesticides and pesticide containers properly. The EPA is responsible for enforcement.

The Federal Hazardous and Solid Waste Amendments (1984) to RCRA focus on waste minimization and the phasing out of land disposal of hazardous wastes. These amendments also strengthened enforcement authority for EPA and provided for more stringent waste management standards.

Toxic Substances Control Act (TSCA) Administered by the EPA, the TSCA is mammoth, covering almost all chemicals manufactured in the United States for industrial and other purposes, excluding certain compounds covered under other laws such as Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the FD&C Act. The EPA may control or stop production of compounds deemed hazardous. Producers must give notice or intent to manufacture new chemicals or decrease significantly the production of existing chemicals. They may be required to conduct toxicity and other tests. Due to the enormous number of existing chemicals that must be evaluated, this law may never be completely applied or at least, not fully applied until new and more efficient toxicity tests are implemented. Once extensively applied, however, it will be the most important statute involving toxicology.

Statutes Affecting the Manufacture and Use of Agricultural Chemicals Because of the intense interest and concern over the use of agricultural chemicals, especially pesticides, and their possible effects on human health, they are, perhaps, the most overregulated group of commercial xenobiotics in use today. A number of laws deal almost exclusively with this use class while several others also deal with them, to a greater or lesser extent. The first law directed specifically toward pesticides in the United States was The *Insecticide Act of 1910*. This act was passed to ensure that the percentages of ingredients were as stated and that the product was efficacious. Surprisingly, it was 37 years before a law was written to replace the 1910 Act. This replacement was the *FIFRA*. First passed in 1947 and amended many times since, this act is now administered by the EPA. FIFRA regulates all pesticides and other agricultural chemicals, such as plant growth regulators, used in the United States. Establishing the requirement "that the burden of proof of a product's acceptability rested with the manufacturer," it includes the authority to establish registration requirements, with appropriate chemical and toxicological tests prescribed by the agency. This act also permits the agency to specify labels, to restrict application to certified applicators, and to deny, rescind, or modify registration. Under this act,

the EPA also establishes tolerances for residues on raw agricultural products. FIFRA was amended in 1988 requiring a reevaluation of all pesticides manufactured prior to 1984. The purposes of the 1988 amendment were to remove hazardous pesticides, and to require additional testing, primarily toxicity tests, which were not available when these early compounds were registered. Section 19 of the 1988 FIFRA amendments greatly expanded the Agency's authority to regulate pesticide storage, transport and disposal of pesticides, containers and rinsates of containers. The *FQPA* of 1996 is an amendment to FIFRA and provides a new standard for evaluating pesticides applied to food crops, in that there be "reasonable certainty of no harm" from residues found on food. USEPA is required to perform an aggregate risk assessment that combines dietary risk from a specific pesticide with those from residues in drinking water and from residential exposure. As a result of this law, USEPA is required to reevaluate all existing food tolerance residue levels based upon a number of criteria. One of these is to determine the cumulative (combined) risk of exposure to classes of pesticides having the same mechanism of toxicity, with special emphasis on infants and children. In some instances, this requires adding an additional safety factor (default value of 10) to the risk assessment for certain compounds to ensure the safety of children. This additional factor is in addition to the safety factor of 100 covering differences due to species and individual variation. Thus, if typical residue levels on a food crop is 1.0 ppm, then a tolerance of 0.01 ppm could be established, and if the additional factor of 10 were added, the tolerance could be set at 0.001 ppm. Currently, organophosphorus insecticides and several other chemical classes are undergoing this reassessment process.

While the intent of FQPA is praiseworthy, it is an excellent example of the effect of confusing legislation and regulation since specific regulations that are difficult to put on a toxicological basis are written into the law. The difficulties include deriving safety factors from toxicological data rather than using default values and the problems involved in determining (combined) risk of exposure to classes of pesticides having the same mechanism of toxicity.

The Act established the Tolerance Reassessment Advisory Committee (TRAC), composed of individuals with a variety of backgrounds and interests, to consult and make recommendations to both the USEPA and the United States Department of Agriculture (USDA). When this committee went out of existence in 1999, USEPA and USDA established a new advisory committee, the Committee to Advise on Reassessment and Transition (CARAT) to provide strategic advice on issues raised by this Act.

An Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was established to develop a comprehensive screening and testing program for pesticides and other compounds to determine potential estrogenic effects on both humans and on wildlife. EDSTAC's Final Report was presented to EPA in September 1998. EPA outlined the Endocrine Disruptor Screening Program (EDSP), which incorporated many of EDSTAC's recommendations.

FQPA is one of the most significant and far-reaching amendments ever made to FIFRA and, no doubt, will continue to generate controversy as it is put into effect.

The *Worker Protection Standard for Agricultural Pesticides* (1994) was written to protect workers from pesticide exposures. Responsibility lies with the employer and involves two types of employees: agricultural workers (e.g., harvesters) and

pesticide handlers (e.g., mixers). It requires that these people be provided safety training, access to labels, and that medical treatment be made available prior to and 30 days after the re-entry interval (REI) has expired. The types of protection offered include notification prior to applying pesticides, exclusion during applications and during an REI, and monitoring the worker's personal protective equipment (PPE). In addition, the employer is required to provide a decontamination site equipped with water and a clean change of clothes.

The Act was written to cover pesticide use on farms, forests, nurseries, and greenhouses. It does not include applications to pastures, golf courses, parks, livestock, right-of-way or home gardens, nor does it cover treatments for mosquito abatement and rodent control.

Many other legislative acts impact in whole or in part on pesticide use. They include the *Endangered Species Act* of 1973, an act written to protect endangered wildlife, and regulates pesticide use around wildlife sanctuaries. Pesticides might injure or kill endangered species if allowed to drift onto habitat, or runoff into streams, lakes, or wetlands might be found to significantly degrade endangered wildlife habitat. Also included are the Clean Water Act, the Safe Drinking Water Act, RCRA, CERCLA, and SARA, all discussed above.

Other Statutes with Relevance to the Prevention of Toxicity It should be noted that some of these statutes have been superseded by others, either in whole or in part.

- Comprehensive Employment and Training Act
- Dangerous Cargo Act
- Federal Coal Mine Safety and Health Amendment Act
- Federal Caustic Poison Act
- Federal Railroad Safety Authorization Act
- Hazardous Materials Transport Act
- Lead-Based Paint Poison Prevention Act
- Marine Protection Research and Sanctuaries Act
- Poison Prevention Packaging Act
- Ports and Waterways Safety Act

22.2.2 State Governments

Within the United States, states are free to adopt legislation with toxicological significance although their jurisdiction does not extend beyond their geographic boundaries. In other cases, the states may enforce federal statutes under certain circumstances. For example, if state regulations concerning hazardous waste disposal is neither less comprehensive nor less rigorous than the federal statute, enforcements is delegated to the states. Similarly, certain aspects of FIFRA are enforced by individual states. In some cases (California is notable in this respect), states have passed laws considerably more comprehensive and more rigorous than the corresponding federal stature.

22.2.3 Legislation and Regulation in Other Countries

It would serve little purpose to enumerate all the laws affecting toxicology, toxicity testing, and the prevention of toxicity that have been promulgated in all countries that have such laws. Legislation in this area has been adopted in most of the countries of Western Europe and in Japan. However, the recently implemented (June 2007) European Union Registration, Evaluation, Authorization and Registration of Chemicals (REACH) law is an example of the approach of another geographic region.

Although the laws in use in the United States are a complex mixture of overlapping statutes and enforcement agencies, they are probably the most comprehensive set of such laws in existence. Most other industrialized countries have legislation in the same areas, although the emphasis varies widely from one country to another. Many underdeveloped countries, due to the lack of both trained manpower and financial resources, are unable to write and enforce their own code of regulations and instead many adopt the regulatory decisions of either the United States or some other industrialized nation. For example, they will permit the use, in their own territory, of pesticides registered under FIFRA by the USEPA and will prohibit the use of pesticides not so registered.

22.3 PREVENTION IN DIFFERENT ENVIRONMENTS

Humans spend their time in many different but sometimes overlapping environments. Homes vary with climate, family income, and personal choice. The workplace varies from pristine mountains to industrial jungles, and the outdoor environment from which recreation, food, and water are derived varies through the same extremes. Each of these environments has its own specific complex of hazards, and thus requires its own set of rules and recommendations if these hazards are to be avoided.

22.3.1 Home

Approximately 50% of all accidental poisoning fatalities in the United States involve preschool children. Thus, prevention of toxicity is particularly important in homes with young children.

Prescription drugs should always be kept in the original container (in the United States and in some other countries, these are now required to have safety closures). They should be taken only by the person for whom they were prescribed, and excess drugs should be discarded safely when the illness is resolved. When children are present, prescription drugs should be kept in a locked cabinet, because few cabinets are inaccessible to a determined child. Although nonprescription drugs are usually less hazardous, they are frequently flavored in an attractive way. Thus, it is prudent to follow the same rules as for prescription drugs.

Household chemicals such as lye, polishes, and kerosene should be kept in locked storage if possible; if not, they should be kept in as secure a place as possible, out of the reach of children. Such chemicals should never be stored in anything but the original containers. Certainly they should never be stored in beverage bottles, kitchen containers, and so on. Unnecessary materials should be disposed of safely in appropriate disposal sites.

Certain household operations such as interior painting should be done only with adequate ventilation. Insecticide treatment should be done precisely in accordance with instructions on the label.

The cyclic increases in fuel costs often cause changes in lifestyle, and some of these changes carry potential toxic hazards. They include increased burning of wood and coal and the construction of heavily insulated and sealed houses with a concomitant reduction in ventilation. In the latter circumstances, improperly burning furnaces can generate high levels of CO and aromatic hydrocarbons, while even those burning properly may still generate oxides of nitrogen (NO_x) at levels high enough to cause respiratory tract irritation in sensitive individuals. These effects can be avoided by ensuring that all heating equipment (e.g., furnaces, wood stoves, heaters) is properly ventilated, maintained, and checked regularly. In addition, some ventilation of the building itself should always be provided. Less ventilation may be desirable when the temperature is either excessively high or excessively low, and more when the temperature is in the midrange, but under no circumstances should the homeowner strive for a completely sealed house.

22.3.2 Workplace

Exposure levels of hazardous chemicals in the air of work environments are mandated by OSHA as exposure limit values. The studies necessary to establish these limits are carried out by NIOSH. However, the more complete list of the better-known threshold limit values (TLVs) is established by the American Conference of Governmental Industrial Hygienists. Although TLVs are not binding in law, they are an excellent guide to the employer. In fact, they are often adopted by OSHA as permissible exposure limits (PELs). The concentrations thus expressed are the weighted average concentrations normally considered safe for an exposure of 8 h/day, 5 days/week. Absolute upper limits (excursion values) may also be included. Some exposure limits are shown in Table 22.1.

TABLE 22.1 Some Selected Threshold Limit Values (1991)

Chemical	TLV-TWA ppm	TLV-STEL ppm	TLV-C ppm
Acetaldehyde	100	150	—
Boron trifluoride	—	—	1
O-dichlorobenzene	—	—	50
p-dichlorobenzene	75	110	—
N-ethylmorpholine	5	20	—
Fluorine	1	2	—
Phosgene	0.1	—	—
Trichloroethylene	50	200	—

TLV-TWA, threshold limit value–time-weighted average concentration for a normal 8-h workday and 40-h workweek to which nearly all workers may be repeatedly exposed without adverse effect; TLV-STEL, threshold limit value–short-term exposure limit concentration. This time-weighted 15-min average exposure should not be exceeded at any time during a workday even if the TLV-TWA is within limits. Intended as supplement to TLV-TWA; TLV-C, threshold limit value-ceiling, concentration that should not be exceeded at any time.

Concentrations at or lower than those normal or working exposures are usually maintained by environmental engineering controls. Operations that generate large amounts of dusts or vapors are conducted in enclosed spaces that are vented separately or are under hoods. Other spaces are ventilated adequately, and temperature and humidity controls are installed where necessary.

Other precautions must be taken to prevent accidental or occasional increases in concentrations. Materials should be transported in "safe" containers, spilled material removed rapidly, and floor and wall materials selected to prevent contamination and allow easy cleaning.

Additional methods for the prevention of toxicity in the workplace include the use of personal safety equipment—protective clothing, gloves, and goggles are the most important. In particularly hazardous operations, closed-circuit air masks, gas masks, and so on, may also be necessary.

Preemployment instruction and preemployment physical examinations are of critical importance in many work situations involving hazardous chemicals. The former should make clear the hazards involved the need to avoid exposure under normal working conditions, and the mechanisms by which exposure is limited. Furthermore, employees should understand how and when to contain spills and how and when to evacuate the area around the spill. Locations and use of emergency equipment, showers, eye washes, and so on, should also be given, and the most important procedures should be posted in the work area.

22.3.3 Pollution of Air, Water, and Land

The toxicological significance of pollution of the environment may be work related, as in the case of agricultural workers, or related to the outside environment encountered in daily life. In the case of agricultural workers, numerous precautions are necessary for the prevention of toxicity. For example:

- Pesticides and other agricultural chemicals should be kept only in the original container, carrying the labels prescribed by EPA under FIFRA.
- Empty containers and excess chemicals should be disposed of properly in safe hazardous waste disposal sites, incinerated when possible or, in some cases, decontaminated.
- Workers should not reenter treated areas until the safe reentry period has elapsed.
- Certain workers such as applicators, those preparing tank mixes, should wear appropriate protection clothing, gloves, face masks, and so on. The development of closed systems for mixing pesticides should help protect mixers and loaders of pesticides from exposure.
- Spraying operations should be carried out in such a way as to minimize drift, contamination of water, and so on.

Pesticides have caused a number of fatalities in the past. The current practice in some countries (including the United States) of restricting the most hazardous

chemicals for use only by certified operators should greatly minimize pesticide poisoning in these locations.

Individuals can do little to protect themselves from poisoning by chemicals that pollute the air and water except to insist that discharge of toxicants into the environment be minimized. The exposure levels are low compared with those in acute toxicity cases, and the effects may be indirect, as in the increase in preexisting respiratory irritation during smog. Thus, these effects can be determined only at the epidemiologic level. Because many persons are not affected or may not be affected for years, it is often argued that environmental contamination is not very important. However, a small percentage increase may represent a large number of people when the whole population is considered. Furthermore, chronic toxicity is not often reversible. Since in most industrialized countries laws already exist to control emission problems, if such problems exist in these countries, they are usually problems of enforcement.

One of the most critical areas for the prevention of toxicity caused by environmental contamination is that of disposal of hazardous wastes. It is now apparent that past practices in many industrialized countries have created large numbers of waste sites in which the waste is often unidentified, improperly stored, and leaching into the environment. The task of rectifying these past errors is an enormous one only now being addressed.

The ideal situation for current and future practices is to reduce chemical waste to an irreducible minimum and then to place the remainder in secure storage. Waste reduction can be accomplished in many ways.

- Refine plant processes so that less waste is produced.
- Recycle waste into useful products.
- Concentrate wastes.
- Incineration. The technology is available to incinerate essentially all waste to inorganic slag. Unfortunately, the technology is sophisticated and expensive. Inadequate incineration is itself a hazard because of the risk of generating dioxins and other toxicants and releasing them into the environment. Less complex and more easily maintained incinerators will be essential if this technology is to play a prominent role in waste reduction.

Safe storage for the remaining waste may be in dump sites or in aboveground storage. In either case, such storage ideally should be properly sited, constructed, maintained, and monitored.

Because of the nature of commerce, probably none of these measures will be successful unless the laws, penalties, and incentives are manipulated in such a way as to make safe disposal more attractive economically than unsafe disposal.

22.4 EDUCATION

Because chemicals, many of them hazardous, are an inevitable part of life in industrialized countries, education is probably the most important method for the

prevention of toxicity. Unfortunately, it is also one of the most neglected. In a typical public debate concerning a possible chemical hazard, the principle protagonists tend to fall into two extreme groups: the "everything is OK" protagonists and the "ban it completely" protagonists. The media seldom seem to educate the public, usually serving only to add fuel to the flames.

The educational role of the toxicologist should be the voice of reason, presenting a balanced view of risks and benefits, and outlining alternatives whenever possible. The simple lesson that science deals not in certainty, but rather degrees of certitude, must be learned by all involved.

In terms of ongoing educational programs, there should be opportunities at all levels: elementary schools, high schools, university, adult education, and media education. Several approaches can be used to educate the general public in ideal situations:

- *Elementary schools*—Teach the rudiments of first aid and environmental concerns (e.g., proper disposal).
- *High school*—Teach concepts of toxicology (dose response) and environmental toxicology (bioaccumulation). These concepts can be introduced into general science courses.
- *University*—In addition to toxicology degrees, general courses for nontoxicology and/or nonscience majors should stress a balanced approach, with both responsible use and toxicity prevention as desirable end points. General Toxicology should be a required course in all chemically related academic programs such as chemistry, chemical engineering, and so on.
- *Media*—Encourage a balanced approach to toxicity problems. Toxicologists should be available to media representatives and, where appropriate, should be involved directly.

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SAMPLE QUESTIONS

1. Name the federal statute most important for chemical safety in the workplace. Which agency is responsible for its administration?

2. Name three federal acts important for prevention of chemical toxicity that are administered by the United States Environmental Protection Agency.

3. One means of preventive chemical toxicity is waste reduction. In general, what are the means by which this can be improved?

4. Education is important in toxicity prevention. What approaches are likely to be effective at various levels of the education process?

CHAPTER 23

Human Health Risk Assessment

RONALD E. BAYNES

23.1 INTRODUCTION

We often perform toxicological research to better under the mechanism and associated health risk following exposure to hazardous agents. Risk assessment is a systematic scientific characterization of potential *adverse health effects* following exposure to these hazardous agents. Risk assessment activities are designed to *identify, describe*, and *measure qualities and quantities* from these toxicological studies, which are often conducted with homogenous animal models at doses and exposure duration often not encountered in a more heterogeneous human population. Herein lies the challenge of risk assessment. Due to the use of default assumptions because of some level of uncertainty in our extrapolations across species, doses, routes, and interindividual variability, the risk assessment process is often perceived as lacking scientific rigor. This chapter will describe traditional practices as well as new and novel approaches that utilize more of the available scientific data to identify and reduce uncertainty in the process. The advent of powerful computers and sophisticated software programs has allowed the development of quantitative models that better describe the dose–response relationship, refine biologically relevant dose estimates in the risk assessment process, and encourage departure from traditional default approaches (Conolly et al., 1999). Although the focus of this chapter is on current and novel risk assessment methods that are scientifically based, it is critical at this point that the reader be aware of the differences between risk assessment and risk management which are summarized in Table 23.1.

Results from the risk assessment are used to inform *risk management*. The risk manager then uses this information in conjunction with factors such as the social importance of the risk, the social acceptability of the risk, the economic impacts of risk reduction, engineering, and legislative mandates, when deciding on and implementing risk management approaches.

The risk assessment may be perceived as the source of a risk management decision, when in fact, social concerns, international issues, trade, public perception, or

A Textbook of Modern Toxicology, *Fourth Edition*. Edited by Ernest Hodgson

TABLE 23.1 Comparison of Risk Assessment and Risk Management Activities

Risk Assessment	Risk Management
Nature of effects	Social importance of risk
Potency of agent	Acceptable risk
Exposure	Reduce/not reduce risk
Population at risk	Stringency of reduction
Average risk	Economics
High-end risk	Priority of concern
Sensitive groups	Legislative mandates
Uncertainties of science	Legal issues
Uncertainties of analysis	Risk perception
Identify	*Evaluate*
Describe	*Decide*
Measure	*Implement*

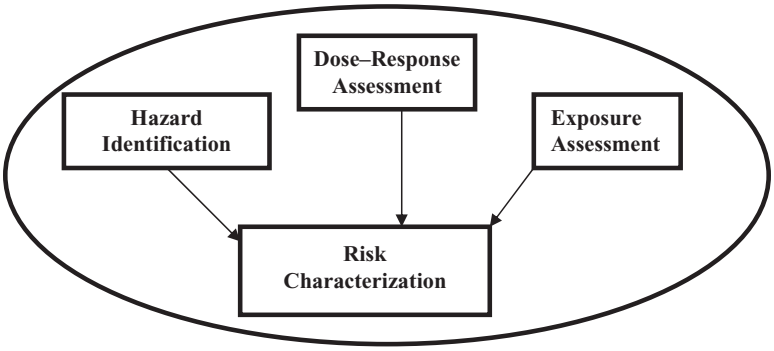

Figure 23.1 Risk assessment paradigm as per NAS and USEPA.

other nonrisk considerations may be taken into consideration. Finally, there is one activity known as *risk communication*, which involves making the risk assessment and risk management information comprehensible to lawyers, politicians, judges, business and labor, environmentalists, and community groups.

23.2 RISK ASSESSMENT METHODS

According to the National Research Council of the National Academy of Science, risk assessment consists of four broad but *interrelated* components: hazard identification; dose–response assessment; exposure assessment; and risk characterization, as depicted in Figure 23.1. The reader should, however, be aware that these risk assessment activities can provide research needs that improve the accuracy of estimating the "risk" or probability of an adverse outcome.

23.2.1 Hazard Identification

In this first component of risk assessment, the question of causality in a qualitative sense is addressed; that is, the degree to which evidence suggests that an agent elicits a given effect in an exposed population. Among many factors, the quality of the studies and the severity of the health effects should be evaluated at this stage. The following are evaluated: (1) validity of the toxicity data; (2) a weight-of-evidence summary of the relationship between the substance and toxic effects; and (3) estimates of the generalizability of data to exposed populations. Where there are limited *in vivo* toxicity data, *structural activity relationships* (SARs) and *short-term assays* may be indicative of a chemical hazard. Key molecular structures such as *n*-nitroso or aromatic amine groups and azo dye structures can be used for prioritizing chemical agents for further testing. SARs are useful in assessing relative toxicity of chemically related compounds, but there are several limitations. For example, toxicity equivalent factors (TEFs) based on induction of aryl hydrocarbon (Ah) receptor by dioxins demonstrated that SARs may not always be predictive. Inexpensive in vitro short-term tests such as bacterial mutation assays can help *identify* carcinogens, and there are other short-term test that can help identify chemicals that potentially can be associated with neurotoxicity, developmental effects, or immunotoxicity. Many of these *in vitro* studies can provide some insight into mechanism(s) of action, but there may be some *false positives* and *false negatives*. Animal studies are usually route specific and relevant to human exposure, and animal testing usually involves two species, both sexes, 50 animals/dose group, and near-lifetime exposures. Doses are usually 90, 50, and 10–25% of the maximum tolerated dose (MTD). In carcinogenicity studies, the aim is to observe significant increases in number of tumors, induction of rare tumors, and earlier induction of observed tumors. However, rodent bioassays may not be predictive of human carcinogenicity because of mechanistic differences. For example, renal tumors in male rats is associated with $\alpha_{2\mu}$-globulin-chemical binding and accumulation leading to neoplasia; however, $\alpha_{2\mu}$-globulin is not found in humans, mice, or monkeys. There are differences in susceptibility to aflatoxin-induced tumors between rats and mice which can be explained by genetic differences in expression of cytochrome P450 and glutathione s- transferase (GST) isoenzymes. Whereas humans may be as sensitive as rats to AFB_1-induced liver tumors, mice may not be predictive of AFB_1-induced tumors in humans. Epidemiological data from human epidemiological studies are the most convincing of an association between chemical exposure and disease and therefore can be very useful for hazard identification. Exposures are not often well-defined and retrospective, and confounding factors such as genetic variations in a population and human lifestyle differences (e.g., smoking) present a further challenge. The three major types of epidemiological studies available are: (1) *cross-sectional studies* involve sampling without regard to exposure or disease status, and these studies identify risk factors (exposure) and disease, but are not useful for establishing cause–effect relationships; (2) *cohort studies* involves sampling on the basis of exposure status, and they target individuals exposed and unexposed to chemical agent and monitored for development of disease, and these are *prospective studies*; (3) *Case-control studies* involve sampling on the basis of disease status. These are retrospective studies, where diseased individuals are matched with disease-free individuals.

23.2.2 Exposure Assessment

This process is an integral part of the risk assessment process; however, this will be briefly introduced in this chapter. The reader is encouraged to consult Chapter 27 in this text as well as numerous other text that describe this process in more depth. In brief, exposure assessment attempts to identify potential or completed exposure pathways resulting in contact between the agent and at-risk populations. It also includes demographic analysis of at-risk populations describing properties and characteristics of the population that potentiate or mitigate concern and description of the magnitude, duration, and frequency of exposure. The reader should be aware that exposure may be aggregate (single event added across all media) and/or cumulative (multiple compounds that share a similar mechanism of toxicity). Various techniques such as biomonitoring, model development, and computations can be used to arrive at an estimate of chemical dose taken up by humans, that is, chemical exposure. For example, the lifetime average daily dose (LADD) is a calculation for individuals exposed at levels near the middle of the exposure distribution.

$$\mathrm{LADD} = \frac{(\text{Conc. in Media}) \times (\text{Contact Rate}) \times (\text{Contact Fraction}) \times (\text{Exposure Duration})}{(\text{Body weight}) \times (\text{Lifetime})}$$

Biological monitoring of blood and air samples represents new ways of reducing uncertainty in these extrapolations. For occupational exposures, there are occupational exposure limits (OELs) that are guidelines or recommendations aimed at protecting the worker over their entire working lifetime (40 years) for 8 h/day, 5 days/week work schedule. Most OELs are presented as a time-weighted average concentration for an 8-h day for a 40-h work week. There are threshold limit values (TLVs) which refer to airborne concentrations and conditions under which workers may be exposed daily but do not develop adverse health effects. The short-term exposure limit (STEL) are recommended when exposures are of short duration to high concentrations known to cause acute toxicity.

23.2.3 Dose Response and Risk Characterization

Dose response is a quantitative risk assessment process, and primarily involves characterizing the relationship between chemical potency and incidence of adverse health effect. Approaches to characterizing dose–response relationships include effect levels such as lethal dose 50 (LD_{50}), lethal concentration 50 (LC_{50}), effective dose 50 (ED_{50}), No observed adverse effect levels (NOAELs), margins of safety, and therapeutic index. The dose–response relationship provides an estimation of the relationship between the dose of a chemical agent and incidence of effects in a population. Intuitively, a steep dose–response curve may be indicative of a homogenous population response, while less steep or almost flat slope may be indicative of greater distribution in response. In extrapolating from relatively high levels of exposure in experimental exposures (usually animals) to significantly lower levels that are characteristic of the ambient environment for humans, it is important to note the shape of the dose–response function below the experimentally observable range and therefore the range of inference. The shape of the slope may be linear

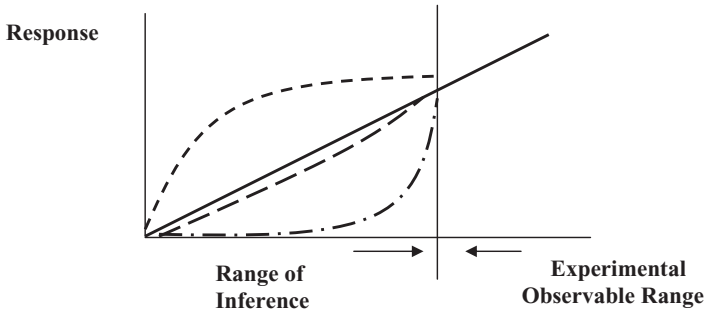

Figure 23.2 Dose–response curve, with emphasis on the shape of the dose–response function below the experimentally observable range and therefore the range of inference where people are realistically exposed.

or curvilinear, and it should be noted that the focus of risk assessment is generally on these lower regions of the dose response curve (Figure 23.2).

There is a class of curvilinear dose–response relationships in toxicological and epidemiological studies that may be described as *U-shaped* or *J-shaped curves*. Other terms such as biphasic and more recently, *hormesis* has been used to refer to paradoxical effects of low-level toxicants. In brief, these dose–response curves reflect an apparent improvement or reversal in the effect of an otherwise toxic agent. These U-shaped effects can be explained in terms of homeostatic adjustments or overcorrections in the operation of feedback mechanisms. Examples of studies with data fitting a U-shaped curve include the hormetic effect of organic lead on body growth in rats (Cragg and Rees, 1984) and peripheral nerve conduction velocity in children at low doses (Ewert et al., 1986). Similar relationships have been observed with alcohol and nicotine in humans. It has been proposed that because thresholds are inherent in U-shaped dose–response curves, the linear no-threshold extrapolation method is not an appropriate approach for regulating hormetic agents. The current risk assessment paradigm used by United States Environmental Protection Agency (USEPA) and other federal agencies does not conflict with the concept of hormesis, but it has been proposed that the risk assessor's analyses make an active consideration of the data and the application of that data in the low-dose portion of the dose–response curve for hormetic agents.

23.3 NONCANCER RISK ASSESSMENT

The noncancer risk assessment process assumes a *threshold*. For many noncarcinogenic effects, protective mechanisms are believed to exist that must be overcome before an adverse effect is manifested. At the cellular level for some toxicant, a range of exposures exists from *zero* to some finite value that can be tolerated by the organism with essentially no chance of expression of adverse effects. The aim here in risk assessment is to identify the upper bound of this tolerance range (i.e., the maximum subthreshold level). This approach involves obtaining the no observable adverse effect level (NOAEL). The NOAEL is the highest dose level that *does not produce a significant* elevated increase in an adverse response. Significance

TABLE 23.2 Comparison of Less Serious Effects and Serious Effects

Less Serious	Serious
Reversible cellular changes	Death
Necrosis, metaplasia, or atrophy	Cancer
	Clinically significant organ impairment
Delayed ossification	Visceral or skelatal abnormalities
Alteration in offspring weight	Cleft palate, fused ribs
Altered T cell activity	Necrosis in immunologic components
Auditory disorders	Visual disorders
50% reduction in offspring	Abnormal sperm

refers to biological and statistical criteria and is dependent on dose levels tested, number of animals, background incidence in the nonexposed control groups. Sometimes, there is insufficient data to arrive at a NOAEL, and a LOAEL (lowest observed adverse effect level) is derived. The NOAEL is the key datum obtained from the study of the dose–response relationship. The NOAEL is used to calculate reference doses (RfD) for chronic oral exposures and reference concentrations (RfC) for chronic inhalation exposures as per EPA. Other agencies such as the Agency for Toxic Substances and Disease Registry (ATSDR) and World Health Organization (WHO) use the NOAEL to calculate *minimum risk levels* (MRLs) and *acceptable daily intakes* (ADI). The USEPA describes the RfD as an estimate, with uncertainty spanning perhaps an order of magnitude, of a daily exposure to the human population, including sensitive subgroups that is likely to be without appreciable deleterious effects during a lifetime. In deriving reference doses, ADIs, or MRLs, the NOAEL is divided by uncertainty factors (UF) as per EPA (EPA, 1989) and ATSDR (ATSDR, 1993) and by modifying factors (MF) as per EPA.

$$\text{RfD} = \text{NOAEL}/(\text{UF} \times \text{MF}) \ldots\ldots\ldots\ldots \text{US EPA}$$

$$\text{MRL} = \text{NOAEL}/\text{UF} \ldots\ldots\ldots\ldots \text{ATSDR}$$

The calculated RfD or RfC is based on the selected critical study and selected critical end point. The risk assessor may obtain numerous studies where the toxicant may have more than one toxic end point and thus there may be many NOAELs to choose from the literature. In some instances, poor data quality may be used to exclude those end point from consideration. Also at issue is determining what is considered an adverse effect, and this has been summarized with a few examples in Table 23.2. In effect, the MRL or RfD is based on the less serious effects and no serious effects. The following are examples of effects not used in obtaining a NOAEL: decrease in body weight less than 10%; enzyme induction with no pathologic changes; changes in organ weight with no pathologic changes; increased mortality over controls that is not significant ($p > 0.05$); and hyperplasia or hypertrophy with or with out changes in organ weights.

23.3.1 Default Uncertainty and Modifying Factors

Many of the extrapolations from animal experimental data in the risk assessment process require the utilization of various uncertainty factors, because we are not

certain how to extrapolate across species, with species for the most sensitive population, and across duration. To account for variation in the general population and intent to protect sensitive subpopulations, an uncertainty factor of 10 is used by EPA and ATSDR. The value of 10 is derived from a threefold factor for differences in toxicokinetics and for threefold factor for toxicodynamics. To extrapolate from animals to humans and account for interspecies variability between humans and other mammals, an uncertainty factor of 10 is used by EPA and ATSDR, and as with intraspecies extrapolations, this 10-fold factor is assumed to be associated within toxicodynamics and toxicokinetics. An uncertainty factor of 10 is used when a NOAEL derived from a subchronic study instead of a chronic study is used as the basis for a calculation of a chronic RfD (EPA only). Note that ATSDR does not perform this extrapolation as they do derive chronic and subchronic MRLs. An uncertainty factor of 10 is used when deriving an RfD or MRL from a LOAEL and a NOAEL is not available. It should be noted that there are no reference doses for dermal exposure; however, when there are insufficient dermal absorption data, the EPA uses a default factor of 10% to estimate bioavailability for dermal absorption. A modifying factor ranging from 1–10 is included by EPA only to reflect a qualitative professional assessment of additional uncertainties in the critical study and in the entire database for the chemical not explicitly addressed by preceding uncertainty factors.

Refinements of the RfC have utilized mechanistic data to modify the interspecies uncertainty factor of 10 (Jarabek, 1995). The reader should appreciate that with the inhalation route of exposure dosimetric adjustments are necessary as this can impact extrapolations of toxicity data of inhaled agents for human health risk assessment. The EPA has included dosimetry modeling in RfC calculations, and the resulting dosimetric adjustment factor (DAF) used in determining the RfC is dependent on physiochemical properties of the inhaled toxicant as well as type of dosimetry model ranging from rudimentary to optimal model structures. In essence, the use of the DAF can reduce the default uncertainty factor for interspecies extrapolation from 10 to 3.16.

The 1996 Food Quality Protection Act (FQPA) now requires that an additional safety factor of 10 be used in the risk assessment process of pesticides to assure the safety of infants and children unless the EPA can show that an adequate margin of safety is assured without it (Scheuplein, 2000). The rationale behind this additional safety factor is that infants and children have different dietary consumption patterns than adults and infants and children are more susceptible to toxicants than adults. We do know from pharmacokinetic studies with various human pharmaceuticals that drug elimination is slower in infants up to 6 months of age than in adults, and therefore the potential exists for greater tissue concentrations and vulnerability for neonatal and postnatal effects. Based on these observations, the USEPA supports a default safety factor greater or less than 10 which may be used on the basis of reliable data. However, there are few scientific data from humans or animals to compare sensitivities on the differential responses of children and adults, but there are some examples such as lead where children are the more sensitive population. It some cases, qualitative differences in age-related susceptibility are small beyond 6 months of age, and quantitative differences in toxicity between children and adults can sometimes be less than a factor of 2 or 3.

Much of the research efforts in risk assessment are therefore aimed at reducing the need to use these default uncertainty factors, although the risk assessor is

limited by data quality of the chemical of interest. With sufficient data and the advent of sophisticated and validated physiologically based pharmacokinetic models and biologically based dose response models (Conolly and Butterworth, 1995), these default values can be replaced with science-based factors. In some instances, there may be sufficient data to be able to obtain distributions rather than point estimates.

23.3.2 Derivation of Developmental Toxicant RfD

Developmental toxicity includes any detrimental effect produced by exposures during embryonic development, and the effect may be temporary or overt physical malformation. Adverse effects include death, structural abnormalities, altered growth, and functional deficiencies. Maternal toxicity is also considered. The evidence is assessed and assigned a weight-of-evidence designation as follows: Category A, Category B, Category C, and Category D. The scheme takes into account the ratio of minimum maternotoxic dose to minimum teratogenic dose, the incidence of malformations and thus the shape of the dose response curve or dose relatedness of each malformation, and types of malformations at low doses. A range of uncertainty factors are also utilized according to designated category as follows: Category A = 1–400; Category B = 1–300; Category C = 1–250; and Category D = 1–100. Developmental RfDs are based a short duration of exposure and therefore cannot be applied to lifetime exposure.

23.3.3 Determination of RfD and RfC of Naphthalene using the NOAEL Approach

The inhalation RfC for naphthalene was 0.003 mg/m^3, and this RfC was derived from a chronic (2-year) National Toxicology Program (NTP) inhalation study in mice using exposures of *0, 10*, or *30 ppm* (NTP, 1992). Groups of mice were exposed for 5 days per week and 6 h per day. This study identified a *LOAEL of 10 ppm.* A dose-related incidence of chronic inflammation of the epithelium of the nasal passages and lungs was observed. This LOAEL concentration was normalized by adjusting for the 6-h-per-day and 5-day-per-week exposure pattern. A LOAEL of 9.3 mg/m^3 was derived by converting 10 ppm first to mg/m^3 and then duration-adjusted levels for 6 h/day and 5 days/week for 103 weeks. A UF of 3000 was used, where 10 was for the interspecies (mice to humans) extrapolations; 10 for intraspecies variation in humans; 10 for using a LOAEL instead of a NOAEL; and 3 for database deficiencies.

The oral RfD for naphthalene was 0.02 mg/kg/day, and a study by Battelle (1980) was used to calculate the RfD. Decreased body weight was the most sensitive end point in groups of Fischer 344 rats given 0, 25, 50, 100, 200, or 400 mg/kg for 5 days/week for 13 weeks. These doses were also duration-adjusted to 0, 17.9, 35.7, 71.4, 142.9, and 285.7 mg/kg/day, respectively. The NOAEL for a >10% decrease in body weight in this study was 71 mg/kg/day. The UF of 3000 was based on 10 for rats to humans extrapolation; 10 for human variation; 10 to extrapolate from subchronic to chronic and 3 for database deficiencies, including lack of chronic oral exposure studies.

23.3.4 Benchmark Dose Approach

There are several problems associated with using the NOAEL approach to estimate RfDs and RfCs. The first obvious constraint is that the NOAEL must, by definition, be one of the experimental doses tested. Once this dose is identified, the rest of the dose–response curve is ignored. In some experimental designs where there is no identifiable NOAEL but LOAEL, the dose response curve is again ignored, and the NOAEL is derived by application of uncertainty factors as described earlier. This NOAEL approach does not account for the variability in the estimate of the dose response, and furthermore, experiments that test fewer animals result in larger NOAELs and thus larger RfDs and RfCs.

An alternative approach known as the benchmark dose (BMD) approach has been developed and implemented by risk assessors as an alternative to the NOAEL approach to estimate RfDs and RfCs. This approach is not as constrained by experimental design as the NOAEL approach, and incorporates information on the sample size and shape of the dose-response curve. In fact, this approach can be used for both threshold and nonthreshold adverse effects as well as continuous and quantal data sets. This requires use of Benchmark Dose Software where the does–response is modeled and the lower confidence bound for a dose at a specified response level (benchmark response) is calculated. The benchmark response is usually specified as a 1–10% response; that is, it corresponds to a dose associated with a low level of risk, such as 1–10%.

Figure 23.3 shows how an effective dose that corresponds to a specific change of effect/response (e.g., 10%) over background and a 95% lower confidence bound on the dose is calculated. The latter is often referred to as the BMDL or LBMD, as opposed to the BMD, which does not have this confidence limited, associated with it.

Because the benchmark represents a statistical lower limit, larger experiments will tend on average to give larger benchmarks, thus rewarding good experimentation. This is not the case with NOAELs, as there is an inverse relationship between NOAEL and size of experiments. For example, poorer experiments possessing less sensitivity for detecting statistically significant increases in risk inappropriately result in higher NOAELs and RfDs which may have an unknown unacceptable level of risk. In essence, the NOAEL is very sensitive to sample size and there can also

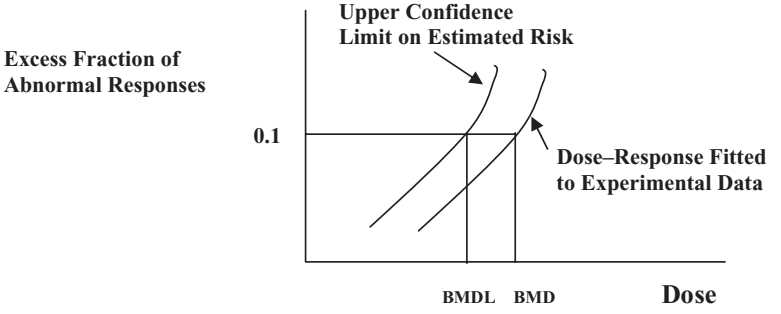

Figure 23.3 Benchmark dose determination from dose response relationship with the BMDL corresponding to the lower end of a one-sided 95% confidence interval for the BMD.

be high variability between experiments. With the benchmark dose approach, all the doses and slope of the curve influences the calculations, variability of the data is considered, and the BMD is less variable between experiments. In the BMD approach, quantitative toxicological data such as continuous data (e.g., organ weights, serum levels) and quantal or incidence data (e.g., pathology findings, genetic anomalies) are fitted to numerous dose–response models described in the literature. The resulting benchmark dose that, for example, corresponds to a tumor risk of 10% generally can be estimated with adequate precision and not particularly dependent on the dose–response model used to fit the data. Note that dose intervals are not required for BMD estimation. This will be greatly appreciated in the cancer risk assessment section of this chapter.

23.3.5 Determination of BMD and BMDL for ETU

The BMD method has been used quite extensively in assessing quantal data, and very often, this has involved analysis of data from developmental and reproductive toxicity studies. In this study example (Crump, 1984), rats were exposed to ethylenethiourea (ETU) at 0, 5, 10, 20, 40, and 80 mg/kg doses, and the number affected with fetal anomalies per number of rats were 0/167, 0/132, 1/138, 14/81, 142/178, and 24/24, respectively. The benchmark dose computation can involve utilization of any given dose–response probability model, but in this example, the quantal Weibull model was used, and the specified effect was set at 0.01 (1%) with confidence level of 0.95. The BMD was determined to be 8.9 mg/kg, and the BMDL was 6.9 mg/kg. This value is close to the NOAEL which is 5 mg/kg, but it does demonstrate that the NOAEL approximates a lower confidence limit on the BMD corresponding to an excess risk of about 1% for proportions of fetal anomalies. In fact, an empirical analysis of some 486 developmental toxicity studies have demonstrated that the NOAEL can result in an excess risk of 5% for proportions of dead or malformed fetuses per litter. The reader should at this stage recognize that the BMD approach can also be used in cancer risk assessment as we are often times working with quantal data which is ideally suited for BMD modeling.

23.3.6 Quantifying Risk for Noncarcinogenic Effects: Hazard Quotient

The measure used to describe the potential for noncarcinogenic toxicity to occur is not expressed as the probability. Probabilistic approach is used in cancer RA. For noncancer RA, the potential for noncarcinogenic effects is evaluated by comparing an exposure level (E) over a specified time period with a reference dose (RfD). This ratio is called a hazard quotient:

$$\text{Hazard Quotient} = \text{E}/\text{RfD}$$

In general, the greater the value of E/RfD exceeds unity, the greater the level of concern. Note that this is a ratio and not to be interpreted as a statistical probability.

23.3.7 Chemical Mixtures

Human populations are more likely to be exposed simultaneously or sequentially to a mixture of chemicals rather than one single chemical. Standard default approaches to mixture risk assessment consider doses and responses of the mixture components to be additive. However, it should also be recognized that components in the mixture can also result in synergistic, antagonistic, or no toxicological effect following exposure to a chemical mixture. Therefore, mixture toxicity cannot always be predicted even if we know the mechanism of all toxic components in a defined mixture. Furthermore, tissue dosimetry can be complicated by interactions at the route of entry (e.g, gastrointestinal tract [GIT], skin surface) and clearance mechanisms in the body. In essence, there are considerable uncertainties involved in trying to extrapolate effects following exposure to chemical mixtures. Several physiologically based pharmacokinetic (PBPK) models have been used to quantitate these effects and also provide some information useful for risk assessment of chemical mixtures (Krishnan et al., 1994; Haddad et al., 2001).

The 1996 FQPA has also mandated that the EPA should also consider implementing cumulative risk assessments for pesticides. Cumulative risk assessments usually involve integration of the hazard and cumulative exposure analysis, and it primarily involves cumulative nonoccupational exposure by multiple routes or pathways to two or more pesticides or chemicals sharing a common mechanism of toxicity.

Calculation procedures differ for carcinogenic and noncarcinogenic effects, but both sets of procedures *assume dose additivity* in the absence of information on mixtures:

$$\text{Cancer risk equation for mixtures: } \text{Risk}_T = \Sigma\text{Risk}_I$$

$$\text{Noncancer Hazard Index} = E_1/\text{RfD}_1 + E_2/\text{RfD}_2 + \ldots + E_i/\text{RfD}_i$$

This hazard index (HI) approach as well as others (e.g., Relative Potency Factors) is applied for mixture components that induce the same toxic effect by identical mechanism of action. In cases where there are different mechanisms, separate HI values can be calculated for each end point of concern. As the above equation indicates, the HI is easy to calculate as there is simply scaling of individual component exposure concentrations by a measure of relative potency such as the RfD or RfC, and adding scaled concentrations to get an indicator of risk from exposure to the mixture of concern. However, as noted above, this additivity approach does not take into account tissue dosimetry and pharmacokinetic interactions. Recent published risk assessments have utilized mixture PBPK models to account for multiple pharmacokinetic interactions among mixture constituents. These interaction-based PBPK models can quantify change in tissue dose metrics of chemicals during exposure to mixtures and thus improve the mechanistic basis of mixture risk assessment. Finally, the reader should be aware that this HI is different from the a term known as the Margin of Safety (MOS) which is the ratio of the critical or chronic NOAEL for a specific toxicological end point to an estimate of human exposure. MOS values greater than 100 are generally considered protective if the NOAEL is derived from animal data.

23.4 CANCER RISK ASSESSMENT

For cancer risk assessment, an assumption is held that a threshold for an adverse effect does not exist with most individual chemicals. It is assumed that a small number of molecular events can evoke changes in a single cell that can lead to uncontrolled cellular proliferation and eventually to a clinical state of disease. This mechanism is referred to as "nonthreshold" because there is believed to be essentially no level of exposure to such a chemical that does not pose a finite probability, however small, of generating a carcinogenic response. That is, no dose is thought to be risk free. Therefore ,in evaluating cancer risks, an effect threshold cannot be estimated. For carcinogenic effects, the U.S. EPA uses a two-part evaluation: (1) the substance is first assigned a weight-of-evidence classification and then (2) a slope factor is calculated.

1. *Assigning a weight-of-evidence:* The aim here is to determine the likelihood that the agent is a human carcinogen. The *evidence* is characterized separately for human studies and animal studies as *sufficient, limited, inadequate, no data,* or *evidence of no effect*. Based on this characterization and on the extent to which the chemical has been shown to be a carcinogen in animals or humans or both, the chemical is given a provisional *weight-of-evidence* classification.

The U.S. EPA classification system (EPA, 1986) shown in Table 23.3 below has been revised in the EPA (1996) proposed guidance and more recent draft guidance (EPA, 1999).

This system was also adapted from the approach taken by the International Agency for Research on Cancer (IARC). This alphanumeric classification system has been replaced with a narrative and the following descriptor categories: "*known/likely,*" "*cannot be determined,*" or "*not likely*." These EPA (1996) guidelines indicate that not only are tumor findings an important consideration, but also structure–activity relationships, modes of action of carcinogenic agents at cellular or subcellular level as well as toxicokinetic and metabolic processes. These revised guidelines also indicate that the weighing of evidence should address the conditions under which the agent may be expressed. For example, an agent may "*likely*" be carcinogenic via inhalation exposure, but "*not likely*" via oral exposure. The narrative will summarize much of this information as well as the mode of action information.

TABLE 23.3 Weight of Evidence Designation Based on EPA(1986) Guidelines

Group	Description
A	Human carcinogen
B1 or B2	Probable human carcinogen
C	Possible human carcinogen
D	Not classifiable as to human carcinogenicity
E	Evidence of noncarcinogenicity for humans

Note: B1 indicates that limited human data are available; B2 indicates sufficient evidence in animals and inadequate or no evidence in humans.

2. *Quantifying Risk for Carcinogenic Effects:* In the second part of the evaluation, the EPA (1986) guidelines required that quantitative risk be based on the evaluation that the chemical is a known or probable human carcinogen, a toxicity value that defined quantitatively the relationship between dose and response (*slope factor*) is calculated. Slope factors have been calculated for chemicals in classes A, B1, and B2. Sometimes, a value is derived for those in Class C on a case-by-case basis. The slope factor is a plausible upper-bound estimate of the probability of a response per unit intake of chemical over a lifetime. Slope factors have been accompanied by the weight-of-evidence classification to indicate the strength of evidence that the chemical is a human carcinogen.

Development of a slope factor entails applying a model to the available data set and using the model to extrapolate from high doses to lower exposure levels expected for human contact. There are a number of low-dose extrapolation models which can be divided into distribution models (e.g., log-probit, Weibull) and mechanistic models (e.g., one-hit, multihit, and *linearized multistage*). EPA 1986 guidelines for carcinogen risk assessment are currently being revised, and it is very likely that the new guidelines will encourage the use of bioloically based models for cancer risk assessment. The previous guidelines (EPA, 1986) recommended that the linearized multistage model, which is a mechanistic model, be employed as the default model in most cases. Most of the other models are less conservative. The proposed biologically based models attempt to incorporate as much mechanistic information as possible to arrive at an estimate of slope factors. In essence, after the data are fit to the selected model, the upper 95th percent confidence limit of the slope of the resulting dose response curve is calculated. *This represents the probability of a response per unit intake over a lifetime* or that there is a 5% chance that the probability of a response could be greater than the estimated value on the basis of experimental data and model used. In some cases, slope factor based on human dose–response data are based on "best" estimate instead of upper 95th percent confidence limit. The toxicity values for carcinogenic effects can be expressed in several ways:
The slope factor is expressed as q_1*

$$\text{Slope factor} = \text{risk per unit dose} = \text{risk per mg/kg/day}$$

The slope factor can therefore be used to calculate the upper bound estimate on risk (R)

$$\text{Risk} = q_1 * \left[\text{risk} \times (\text{mg/kg/day})^1\right] \times \text{exposure}\,(\text{mg/kg/day})$$

Here, risk is a unit-less probability (e.g., 2×10^{-5}) of an individual developing cancer and exposure is really chronic daily intake averaged over 70 years: mg/kg/day, and this can be determined if we can determine the slope factor and human exposure at the waste site or occupational site. The EPA usually sets a goal of limiting lifetime cancer risks in the range of 10^{-6}–10^{4} for chemical exposures, while the FDA typically aims for risks below 10^{-6} for general population exposure. It is therefore quite likely for very high exposures for the accepted EPA range of risk to be exceeded. The EPA range is considered protective of the general and

sensitive human population. It should be noted that these orders of magnitude are substantially greater than those used in estimating RfD and RfCs in noncancer risk assessment.

Because relatively low intakes (compared to those experienced by test animals) are most likely from environmental exposure at Superfund sites, it generally can be assumed that the dose–response relationship will be linear on the low-dose portion of the multistage model dose–response curve. The above equation can apply to these linear low-dose situations. This linear equation is valid only at low risk levels (i.e., below estimated risk of 0.01). For risk above 0.01 then, the one-hit equation should be used:

$$\text{Risk} = 1 - \exp(-\text{exposure} \times \text{slope factor})$$

As indicated above, biologically based extrapolation models are the preferred approach for quantifying risk to carcinogens, although it is possible that all the necessary data would not be available for many chemicals. The EPA (1986) guidelines have been modified to include the response data on effects of the agent on carcinogenic processes in addition to data on tumor incidence. Precursor effects and tumor incidence data may be combined to extend the dose–response curve below the tumor data, that is, below the range of observation. Thus, a biologically based or case-specific dose response model is developed when there is sufficient data or a standard default procedure when there is insufficient data to adequately curve-fit the data. In brief, the dose–response assessment is considered in two parts or steps: range of observation and range of extrapolation, and the overriding preferred approach is to use the biologically based or case-specific model for both of these ranges. In the first step of this process, the lower 95% confidence limit on a dose associated with an estimated 10% increase in tumor or nontumor response (LED_{10}) is identified. When human real-world exposures are outside the range of the observed or experimental data, this serves as the point of departure or marking the beginning for the extrapolating to these low environmental exposure levels. Please note that these procedures are very similar to the benchmark procedure for quantitating risk to noncarcinogenic chemicals. In the second step, the biologically based or case-specific models is preferred to be used in extrapolations to lower dose levels provided there is sufficient data. If the latter is not the case, then default approaches consistent with agent chemical mode of action are implemented with the assumption of linearity or nonlinearity of the dose–response relationship. The linear default approach is a departure from the 1986 guidelines which used the linearized multistage (LMS) procedure but is based on mode of action of action or alternatively there is insufficient data to support a nonlinear mode of action. In brief, it thus involves drawing a straight line from the point of departure (LED_{10}) to the origin (i.e., zero). When there is no evidence of linearity or there is a nonlinear mode of action, the default approach is the margin of exposure (MOE) analysis. The MOE approach computes the ratio between the LED_{10} and the environmental exposure, and the analysis begins from the point of departure that is adjusted for toxicokinetic differences between species to give a human equivalent dose.

Finally, it should be noted that prior to the FQPA in 1996, the Delaney clause prohibited the establishment of tolerances or maximum allowable levels for

food additives if it has been shown to induce cancer in man or animals. This is an important change in regulations because pesticide residues were considered as food additives. Because of the FQPA, pesticide residues are no longer regarded as food additives, and there is no prohibition against setting tolerances for carcinogens.

23.5 PBPK MODELING

PBPK modeling has been used in risk assessment to making more scientifically based extrapolations, and at the same time help to explore and reduce inherent uncertainties. Historically, pharmacokinetics has relied on empirical models, and in many instances, this process offers little insight into mechanisms of absorption, distribution, and clearance of hazardous agents and does not facilitate translation from animal experiments to human exposures. For example, dose scaling using by body weight or size may often overestimate or underestimate toxicant levels at the target tissue. PBPK models can help predict tissue concentrations in different species under various conditions based on *independent* anatomical, physiological, and biochemical parameters. In these analyses, physiological parameters such as organ volumes, tissue–blood partition coefficients, and blood flow to specific tissue compartments described by the model, are calculated or obtained from the literature and integrated into the model. Monte Carlo analysis, a form of uncertainty analysis, can now be preformed, and this allows for the propagation of uncertainty through a model which results in estimation of the variance of model output. This can be achieved by randomly sampling model parameters from defined distributions; some parameters such as cardiac output, metabolic, and log P parameters, may have a log normal distribution, while other parameters may be normal or uniform. In essence, the Monte Carlo analysis, when coupled with PBPK, characterizes the distribution of potential risk in a population by using a *range* of potential values for each input parameter (not single values) as well as an estimate of how these values are distributed (Clewell and Andersen, 1996). Using these approaches, uncertainty is identifiable and quantifiable, and this can reduce inappropriate levels of concern in reporting the risk of chemical exposure. These mathematical modeling approaches also help identify areas of potential scientific research that could improve the human health assessment.

In recent years, there have been significant efforts at harmonization noncancer and cancer risk assessment (Barton et al., 1998; Clewell et al., 2002), and in this respect, PKPD modeling can be a very useful tool in the risk assessment process. For example, recall that noncancer risk assessment addresses variability in a population by dividing the NOAEL by 10, whereas the cancer risk assessment does not address this quantitatively. PBPK modeling coupled with Monte Carlo analysis is one approach as described in the previous paragraph that will help address this level of uncertainty in the risk assessment. In conclusion, it should be noted that the use of PBPK modeling has been utilized with very few toxicants, but it is hoped that risk assessment policy would encourage the use of this tool as well as other appropriate models to integrate mechanistic information and the pharmacokietics (dosimetry), and pharmacodynamics (dose response) of toxicants. These improved quantitative risk assessments would ultimately provide scientifically sound information that will influence the risk management decision process.

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SAMPLE QUESTIONS

1. **a.** Cancer risk assessment does not assume a threshold dose.
b. PBPK models can reduce the uncertainty factor in an RfC calculation.
c. The bench mark dose approach is an improvement on the NOAEL approach.

2. **a.** Obtaining the slope factor is critical to cancer risk assessment.
b. The uncertainty factor only takes into account animal to human extrapolation.
c. Hazard identification takes into account bioassays and epidemiological data.

3. Define the following terms: NOEL; NOAEL; LOAEL; RfD.

4. The following dose–response from a chronic mouse study is being considered for use in a noncancer risk assessment. Use the data set to answer the questions below.

Data set.	Dose (mg/kg)	0	1	2	4	8	16
	Response	0	0	0	X	X	X*

X* is a significant increase in hepatic necrosis.

a. What is the NOAEL?
b. What is the LOAEL?
c. What is the RfD?