CHAPTER 13
Toxicologic Testing Methods

In general all toxicity testing methods can be divided into two categories. The first category consists of tests that are designed to evaluate the overall effects of compounds on experimental animals. The individual tests in this category differ from each other basically in regard to the duration of the test and the extent to which the animals are evaluated critically for general toxicity. The tests are identified as acute, prolonged, and chronic toxicity tests.

The second category of tests consists of those tests that are designed to evaluate in detail specific types of toxicity. The prolonged and chronic tests do not detect all forms of toxicity but they may reveal some of the specific toxicities and indicate the need for more detailed studies. Also, the intended use of a compound may require that an estimate of the order of safety from certain specific toxicities be investigated. The second category of toxicity tests has been developed to fill these needs. Examples of specific toxicity tests are: (1) those that determine the effects on the fetus in a pregnant animal, that is, teratogenic tests, (2) tests to determine effects on reproductive capacity of the animals, that is, reproduction tests, (3) tests to determine effects on the genetic code system, that is, mutagenic tests, (4) tests to determine the ability of agents to produce tumors, that is, tests for tumorigenicity and carcinogenicity, (5) tests to determine local effects of agents when they are applied directly to the skin and eyes, (6) tests to determine the effect of agents on various behavior pattern of animals, that

is, behavioral or neurotoxicity tests, and (7) tests to determine the effects on the immune system, that is, immunotoxicity tests. These tests will be described in the order listed.

Certain technical and procedural factors are common to the conduct of all types of toxicity studies on experimental animals. These are the preparation of a suitable form of the chemical for administration purposes, the selection of the route or routes of administration to be used, and the species of animals to be used in the study. All of these factors are collectively considered since each influences the others.

It is essential that the chemical and physical characteristics of any test material used in a toxicology study whether actue, prolonged, or chronic be thoroughly evaluated for its composition. The purity of the material and its major contaminant should be thoroughly evaluated both from the standpoint of the initial chemical or mixture and from the standpoint of its durability throughout the testing protocol. In repeat studies, the stability of the chemical should be understood and the products of degradation evaluated because of their own unique toxicity or their ability to alter the toxicity of the test material. Chemical analyses should be conducted before the initiation of the prolonged study to ensure that the test material is stable for the period of its intended use. This evaluation can be conducted concurrently with the study but detection of instability during the study could invalidate the results. If the material is stored only under certain conditions, e.g., frozen storage, special arrangements for storage should be made. Instability of the test material may result in lower than expected doses and the exposure of the animal to degradation products may occur. Methodologies need to be developed for evaluating the compound and/or its metabolites in the carrier whether it is mixed with feed or in an aerosol.

In order to formulate a preparation of a compound for administration to animals, it is desirable to take advantage of the solubility characteristics of the compound. Water or physiological saline (0.9% NaCl in water) is always the solute of choice since these solvents permit administration by all routes. When this is not possible because of solubility limitations, it is necessary to resort to the use of vegetable oils such as corn oil or even organic solvents, of which propylene glycol is commonly used. Whenever possible the use of suspensions or emulsions should be avoided except for oral administration. Regardless of the route of administration, the volume required to administer a given dose is limited by the size of the animal that is used. It is desirable to keep the volume of each dose uniform within and between groups of animals. When rats or mice are used the volume administered by the oral route should not exceed 0.005 ml per gram of animal. Even when aqueous or physiological saline solutions are used for parenteral injection the volumes that are tolerated are limited, although

such solutions are ordinarily thought of as being innocuous. The intravenous LD_{50} of distilled water in the mouse is approximately 0.044 ml per gram and that of isotonic saline is 0.068 ml per gram of mouse.

When a compound is to be administered by inhalation, special techniques for generating test atmospheres are necessary. Dose estimation becomes very complicated. The methods usually involve aerosolization or nebulization of fluids containing the compound. If the agent to be tested is a fluid that has an appreciable vapor pressure, it may be administered by passing air through the solution under controlled temperature conditions. Under this condition, dose is estimated from the volume of air inhaled per unit time, the temperature of the solution, and the vapor pressure of the agent involved. Gases are metered from reservoirs. When particles of a solution are to be administered, unless the particle size is less than 2 μm the particles will not reach the terminal alveolar sacs in the lungs. A variety of apparatuses and chambers exists to perform studies for detecting effects of irritants or other toxic endpoints when they are administered by inhalation.

The simplest method of administering an agent to animals is via the oral route, either by intubation or by incorporating the agent in the feed. Admixture of the test agent in the feed is precluded if it is unstable or if it reacts with the components of the feed to any degree that would alter the properties of the compound. Also, in some cases the presence of a test chemical in the feed may affect the acceptability of the feed by the test animals. The approximate quantity of feed consumed daily by most species of animals used in toxicity tests is known. Liquids or solids that are soluble in water may be added to the drinking water of the test animals. In each case the daily dose is calculated by monitoring the daily intake of feed or water.

It previously has been indicated that, with the exception of acute toxicity tests, most tests are conducted for the purpose of determining the nature of any toxicity that can be produced by repeated dosing of animals over an extended period of time and for estimating the degree of safety of a material for man. Some guidelines should be considered whenever experimental studies are contemplated.

1. Wherever practical or possible, use one or more species that biologically handle the material qualitatively as similarly as possible to man. For this, metabolism, absorption, excretion, storage, and other physiological effects should be considered.

2. Where practical, use several dose concentrations on the principle that all types of toxicologic and pharmacologic action in man and animals are dose-related. The only exception to this should be the use of a single, maximum dosage if the material is relatively nontoxic; this concentration

should be a sufficiently large multiple of that which is attainable by the maximum applicable hazard exposure route, and should not be physiologically impractical.

3. Effects produced at higher doses (within the practical limits discussed above) are useful for delineating mechanism of action, but for any material and adverse effect, some dose concentration exists for man or animal below which this adverse effect will not appear. This biologically insignificant concentration can and should be set by use of a proper safety factor (uncertainty factor) and competent scientific judgment.

4. Statistical tests for significance are valid only for the experimental units (e.g., either litters or individuals) that have been mathematically randomized among the dosed and concurrent control groups. It is to be understood that statistical significance may be of little or no biological importance and, conversely, that important biological trends should be further examined even in the absence of statistical significance.

5. Effects obtained by one route of administration to test animals are not a priori applicable to effects by another route of administration to man. The routes chosen for administration to test animals should, therefore, be the same as those by which man will be exposed. Thus, for example, food additives for man should be tested by admixture of the material in the diet of animals.

It is therefore apparent that there are no fixed rules regarding the selection of specific species of animals for toxicologic tests. In the absence of some specific reason for the use of relatively expensive species, such as nonhuman primates, dogs, or cats, most toxicity studies are performed on rats, mice, guinea pigs, and rabbits; these animals are relatively inexpensive, readily obtainable, and easily handled, and there is a considerable amount of toxicologic information regarding the effects of most chemical entities on these species.

Details of the tests summarized in this chapter as well as the importance of care and maintenance of experimental animals are beyond the scope of this chapter but can be found in the textbooks listed in Chapter 15.

ACUTE TOXICITY TESTS

The single test that is conducted on essentially all chemicals that are of any biologic interest is the acute toxicity test. The test consists of administering the compound to the animals on one occasion. The purpose of the test is to determine the symptomatology consequent to administration of the compound and to determine the order of lethality of the compound.

The initial procedure is to perform a series of range-finding doses of the compound in a single species. This necessitates selection of a route of administration, preparation of the compound in a form suitable for administration by the slected route, and selection of an appropriate species. It has already been stated that the intended use of the compound suggests the most suitable route for the initial tests. However, even if the intended use of the compound does not involve the oral or parenteral routes, at least the oral route is used in addition to other routes for comparative purposes with other related compounds and for estimating the use of this route for subsequent, more extensive and prolonged toxicity studies. It is apparent that the data which will be obtained are limited to those routes of administration that are used in the experimental procedure. The compound may be less toxic following oral administration than it is following intramuscular administration because it is not absorbed or is poorly absorbed from the gastrointestinal tract or is detoxified by passage through the liver following absorption from the gut. Lethality following intraperitoneal administration may be less than that following intravenous administration as a result of liver detoxication of the compound or by enterohepatic cycling of the compound unless the toxic effect of the compound is localized in the liver.

Essentially all initial, acute toxicity tests are performed on either rats or mice because of their low cost, their availability, and the fact that abundant reference toxicologic data for most compounds are available for these species. However, when subsequent studies are to be performed on other species such as rabbits or guinea pigs, a procedure similar to that used with rats or mice is followed to obtain an estimation of the order of lethal toxicity in these additional species of animals. Regardless of the species selected, all test animals should be in a state of good health and should be observed for a period of time (1 week for rats or mice, 3 to 4 weeks for dogs) in the laboratory or central animal quarters prior to the tests.

The classical sequence for determining the acute toxicity of a new compound consists of an initial dose-range-finding experiment, a subsequent experiment to narrow the range of effective doses for measurement of lethality, and finally a definitive experiment for establishing the dose–response curve for lethality.

The initial rough dose-range-finding experiment involves selecting doses based on suspected toxicity which is obtainable only from a knowledge of the structure of the compound and prior information on the toxicity of structurally related compounds. When rats or mice are used, only two animals are used for each dose. By using logarithms of the dose, a range of doses is experimentally determined that will produce death versus no effect or minimal effects. The animals are observed for a minimum of 24 hr. If the animals appear to be healthy at the end of 24 hr, they are set

aside and observed at daily intervals for at least 1 week for appearance of delayed toxicity.

A second series of tests then is performed on the same species using four animals in each test group in an attempt to narrow the range of doses so that data are obtained for doses that produce death in less than all of the members of a high-dose group and at least signs of intoxication in a low-dose group. The animals are followed as in the initial experiment.

When the data from the preliminary dose-finding tests are obtained, the final experiment is performed. A total number of animals of similar body weight and of the same sex or equal numbers of both sexes are selected and assigned randomly to test and control groups so that each group contains 10 animals (rats or mice). Each group is administered different doses of the formulation which previously has been estimated to produce between 10 and 90% mortality. The sequence of effects following the administration of the compound has already been observed so that time of onset of signs as well as time of death or time of recovery from signs is recorded accurately. The LD_{50} is then statistically determined according to methods described in Chapter 2.

When animals are exposed to the varying concentrations of the test compound via inhalation, the lethal concentration required to produce death in 50% of the animals is determined. During the test, the duration of exposure is always kept constant. Usually the experiment involves a 4-hr exposure period and the animals then are observed for 2 weeks after exposure. The LCT_{50} (lethal concentration time) is then determined. The LCT_{50} in this case is the concentration of the test agent which is required to kill 50% of the animals when exposure is for 4 hr.

A list of typical types of effects that may be observed or elicited during experimental determination of the acute toxicity of a compound on rats and mice is found in Table 13.1.

From these data the experienced toxicologist can arrive at certain conclusions regarding the site and mechanisms of action of compounds undergoing the test.

When suitably extensive observations are made of the signs of animals used for acute toxicity tests, it is possible to estimate the minimal symptomatic or toxic dose, the maximal tolerated dose for which the animals recover completely from all effects of the chemical, and the dose that produces no effect in the test species. On the basis of such information, estimations of the duration of action of single doses may be made for use in subsequent repeated dose types of toxicity tests.

In the acute toxicity study, every effort is made to obtain information that can be used for subsequent prolonged toxicity studies. If subsequent, more prolonged experiments are planned using rats or dogs, the acute test

TABLE 13.1 Physical and Observational Examination of Animals in Toxicity Studies

- Activity
  - Dec locomotor activity
  - Inc locomotor activity
  - Jumping
- Bizarre reaction
  - Circus movements
  - Aimless wandering
  - Backward movements
  - Waltzing
  - Nuzzling
  - Licking compartment walls
  - Shovel-nose movements
  - Glassy-eyed stare
  - Circling movements
- Phonation
  - Inc phonation
  - Dec phonation
  - Abnormal phonation
- Sensitivity to pain
  - Inc sensitivity
  - Dec Sensitivity
  - Analgesia
- Sensitivity to sound
  - Inc sensitivity
  - Dec sensitivity
  - Reactivity
- Sensitivity to touch
  - Inc sensitivity
  - Dec sensitivity
  - Pinnal reflec depr
  - Pinnal reflec abs
- Social interaction
  - Inc exploratory behavior
  - Dec exploratory behavior
  - Inc rearing frequency
  - Inc speed of rearing
  - Dec rearing frequency
  - Dec rearing height
- Abnormal tail
  - Rigid tail
  - Straub tail
  - Limp tail
- Aggressive behavior
  - Inc—specied toward same species
  - Dec—species
  - Inc—people toward experimenters
  - Dec—people
- Ataxia
- Convulsions
  - Tonic convulsions
  - Clonic convulsions
  - Mixed convulsions
  - Subconvulsions
  - Subconvulsive movements
- Muscle tone
  - Inc muscle tone—trunk
  - Dec muscle tone—trunk
  - Inc muscle tone—limbs
  - Dec muscle tone—limbs
- Paralysis
- Somatic response
  - Inc preening
  - Dec preening
  - Rubbing nose
  - Inc scratching
  - Dec scratching
  - Writhing
- Postural reflexes
  - Placing reflex depr
  - Placing reflex abs
  - Grasping reflex depr
  - Grasping reflex depr
  - Righting reflex depr
  - Righting reflex abs
- Prostration
  - Prostration
  - Loss of consciousness
- Tremors
  - Tremors—rest and movement
  - Tremors—movement only
- Exophthalmos
- Eye irritation
  - Eye opacity
  - Blinking—excessive
  - Iritis
- Corneal reflex
  - Corneal reflex abs
  - Corneal reflex depr
- Lacrimation
- Nystagmus
- Pupillary light reflex
  - Pupillary reflex abs
  - Pupillary reflex depr
- Photophobia

(continues)

TABLE 13.1 (*Continued*)

Pupil size	Dec respiration depth
Mydriasis	Irregular respiration
Miosis	Cardiac
Defecation	Heart rate
Inc defecation	Pulse
Dec defecation	Nasal discharge
Diarrhea	Body temp
Bloody stool	Inc rectal temp
Salivation	Dec rectal temp
Salivation	Cyanosis
Dry mouth	Motor deficit
Urination	Mot def inclined strip
Inc urination	Mot def roa-rod
Dec urination	Mot def horizontal wire
Hematuria	Piloerection
Apnea	Death
Dyspnea	No effect
Respiration	Special Functional tests
Inc respiration rate	Conditioned-avoidance behavior
Dec respiration rate	Conditioned-reflex response
Inc respiration depth	

Note. Abbreviations used: dec, decreased; inc, increased; depr, depressed; abs, absent.

should be conducted first on rats; from these data an estimate of a single nonlethal dose of the compound can be determined for dogs. In this manner similarities and dissimilarities in signs between species can be evaluated. Usually chemical methods for determining the concentration of a new chemical in biologic tissue are not available, but if an analytical method is available and an experiment is conducted on dogs some information on distribution of the chemical in the various body fluid compartments and organs can be obtained; this information can be correlated with signs. It is possible also to determine the route of excretion and the half-life of the compound in the animal, provided the analytic methodology is sufficiently specific and sensitive.

The LD_{50} values should be reported in terms of the duration over which the animals were observed. That is, if the test animals were observed for 24 hr after the administration of the compound the results represent a 24-hr LD_{50}. Whenever the interval of time is not indicated, it is generally assumed that the animals were followed for 24 hr. Occasionally it is desirable to follow the animals for periods considerably longer than 14 days. For example, it is well known that tricresyl phosphate will produce a neurological syndrome in animals after a single dose but only after an interval of 10

to 14 days. Carcinogenic compounds and those compounds used in the treatment of carcinoma frequently show delayed toxicities and delayed deaths. Although single dose 24-hr or 7-day LD_{50} values usually can be determined for such compounds, the quantity of the compound required to produce short-term deaths is large and has little practical value for comparison purposes with other similarly acting compounds. Whenever deaths are delayed, the compound is strongly suspected of having the potential for accumulation when repeated doses are given at a daily rate. Whenever such a compound is to be studied in prolonged types of studies, it is useful to determine the effects and lethality of several daily doses. Such an LD_{50} can be obtained by administering four or five daily doses and then following the animals for 4 weeks. At least two different routes of administration are used on groups of four animals for each test done. In this manner the accuracy of estimating the range of doses required in prolonged toxicity studies is increased greatly.

In vitro replacement methods for acute toxicity testing are difficult to define. A number of *in vitro* strategies have been suggested to replace whole animal acute toxicity testing; however, none have been accepted for regulatory purposes. A variety of procedures have been suggested to reduce the number of animals necessary and to refine protocols to minimize pain and suffering of animals. Included among these protocols are a variety of modified LD_{50} tests, e.g., approximate lethal dose method, the up-and-down method, the British Society of Toxicology Protocol, and the fixed dose procedure.

Although many regard the issue of alternative methods for animal testing as no more than animal rights activism that is solely based on humanitarian reasons, animal testing nonetheless continues to be widely debated. There are three recognized alternatives, the so-called 3 R's: Reduction, Refinement, and Replacement. Reduction of the number of animals used in testing and refinement of existing testing methods to minimize the pain and suffering of animals represent the short-term objectives. Replacement of animal testing with nonanimal based methods, e.g., *in vitro* ("in glass," test tube) methods, is the ultimate goal; however, genuine validated and regulatory accepted nonanimal alternative methods to replace whole animal toxicity testing are still more of a goal than a reality.

PROLONGED TOXICITY TESTS

The objective of the prolonged toxicity tests generally is to evaluate and characterize all effects of compounds when administered to the experimental animals repeatedly, usually on a daily basis over a period of 3 to 4

months. When the chemical under investigation is a drug, the pharmacologic effects particularly are evaluated. When food additives are under investigation, a prolonged test usually is followed by the chronic or special types of tests; therefore, the prolonged test supplies additional information which can be utilized in designing the long-term chronic test. As the duration of the toxicologic test increases from the single administration type of test to the multiple repeated dose type of test, two practical factors are encountered that limit the design of the experiment and the types of animals used. The first is that the available routes of administration are limited because the route which is used must be suitable so that repeated administration of the compound does not induce harmful effects in the animals. The second factor is that the properly designed prolonged experiment involves the use of the species of animals from which blood and urine samples can be obtained at intervals for clinical chemistry and hematology without inducing significant harm to the experimental animals.

The route of administration of a test compound usually is limited to the oral route whenever a compound is given on a daily basis for several weeks. Although some studies have been performed in which the test agent has been given by oral intubation, or by gastric intubation, that is, by inserting a tube through the mouth into the stomach and injecting the agent through the tube, oral intubation generally is avoided unless there is reason to believe that a mixture of the chemical with the feed or water alters the absorption or chemical properties of the agent. The usual procedure is to administer the agent by incorporating it into the feed or water. It is rare that a prolonged experiment is conducted in which the test agent is injected by a hypodermic needle intraperitoneally, intramuscularly, subcutaneously, or intravenously on more than a few occasions. Prolonged repeated tests involve the application of analytical techniques for determining effects on blood chemistry and blood cells, urine chemistry, and specific organ function. Table 13.2 summarizes the type of analytical and functional tests that commonly are involved.

Since these types of tests are incorporated in prolonged animal studies, it is apparent that blood and urine samples must be obtainable from the animals without harming the animals. All of the tests described in the table can be readily performed in the dog even at 1-week intervals. All of the tests wtih the exception of some of the function tests listed in the table can be performed at 1- to 2-week intervals in rats. Consequently the species most commonly used in prolonged toxicity studies are the rat and the dog, providing data on two species, one of which is not a rodent. An exception is when a new compound is under investigation and there is no reason to believe that a different species would be more similar in metabolic function

TABLE 13.2 Analytical and Functional Tests Employed in Prolonged and Chronic Toxicity Tests

Test
Hematology
Erythrocyte count
Total leukocyte count
Differential leukocyte count
Hematocrit
Hemoglobin
Blood chemistry
Sodium
Potassium
Chloride
Calcium
Carbon dioxide
Serum glutamate–pyruvate transaminase[a]
Serum glutamate–oxalacetic transaminase[b]
Serum alkaline phosphatase[a]
Serum protein electrophoresis
Fasting blood sugar
Blood urea nitrogen
Total serum protein
Total serum bilirubin
Serum albumin
Urine analyses
pH
Specific gravity
Total protein
Microscopic examination of sediment
Glucose
Ketones
Bilirubin
Special function tests
Bromsulphalein retention[a]
Thymol turbidity[a]

[a] Liver function tests.
[b] Test for injured tissue cells.

to the human. Only rarely are prolonged toxicity tests performed on subhuman primates, the goat, the hamster, or the guinea pig.

Prolonged toxicity tests involve the evaluation of all animals for gross pathologic and histologic effects at least at the end of the experiments. Also, during the experiment any animals that become ill or moribund are killed and a complete necropsy is performed. Table 13.3 summarizes the

TABLE 13.3 Pathologic and Histologic Examinations Commonly Performed in Prolonged and Chronic Toxicity Tests

Weights
Body
Thyroid
Heart
Liver
Spleen
Kidneys
Adrenals
Testes with epididymis
Histologic examinations
Adrenals
Heart
Liver
Large intestine
Small intestine
Spleen
Ovary
Mesenteric lymph nodes
All tissue lesions
Pituitary
Thyroid
Kidneys
Stomach
Pancreas
Urinary bladder
Testes

tissues that generally are examined by gross observation and histologic techniques.

It generally is necessary to precede any prolonged toxicity tests with suitable short-term dose-finding experiments. In performing preliminary tests, information also will be obtained regarding potential or actual organ damage, which would indicate a need to incorporate suitable monitoring techniques in the prolonged test. The preliminary test involves administering the test chemical by stomach tube or in the diet on 4 to 7 successive days. The animals are then observed for a minimum of 7 weeks. Suitable doses are selected and are given to groups of three to four rats so that most of the animals in the high-dose group die during the experiments. All animals that die during the experiment are necropsied as soon as possible. All remaining live animals at the end of the experiment are killed and are

also necropsied. All abnormal lesions are histologically examined. From the data obtained a range of doses is estimated for use in the prolonged study.

The usual dietary feeding toxicity test of the prolonged type involves the use of three groups of test animals plus an additional control group. Each group contains 10 to 20 male and an equal number of female rats or, if dogs are used, each group contains 3 to 4 male and an equal number of female dogs. The test diet is prepared separately for each group by blending the test agent with a commerical laboratory ration on a weight to weight basis. One of the test groups of animals will receive a low-level concentration, one group will receive an intermediate concentration, and one group will receive a high concentration of the test agent. When the experiment is conducted on dogs, it is frequently convenient to incorporate the daily dose of the test agent in a bolus of moist, canned dog feed. When rats are used, fresh diets are prepared weekly. If the presence of feed alters the chemical properties or action of the test agent, it is necessary to fast the animals for 6 to 12 hr and then administer the agent by oral intubation. All animals are housed individually, observed daily, and weighed each week. Those animals that become moribund during the experiment are killed and undergo complete pathological examination. Whenever the test chemical is incorporated in the diet or drinking water, it is necessary to measure the amount of feed or water consumed daily so that the dose can be estimated. Clinical hematology, blood chemistry, and urine analysis are performed at least at 4-week intervals and just prior to the termination of the experiment. Records are maintained throughout the experiment of all general and pharmacological manifestations in the animals. At the termination of the experiment all animals are killed and subjected to complete pathological examination. Also prior to termination the animals are subjected to complete examination of the eyes for evidence of corneal or lens abnormalities.

One of the objectives of the prolonged toxicity tests is to attempt to demonstrate some form of toxic effect at least in the high-dose group. Such effects may occur early in the experiment or may not occur for the duration of the experiment. If severe toxicity occurs early in the experiment, the selection of the dose schedules was in error. Therefore in rat experiments an additional 10 male and an equal number of female rats often are included along with the regular high-dose group at the beginning of the experiment. During the course of the experiment if the regular high-dose group develops severe toxicity and shows evidence of becoming moribund, then the supplemental animals that have been added to the group are removed from the high-dose schedule and are continued in the experiment at a lower dose schedule. In this manner the reversibility of the toxic effect can be evaluated and the experiment can be continued using a more realistic maximal toler-

ated dose. In contrast to this particular situation, if during the course of the experiment the regular high-dose group of animals fails to develop toxicity, then the supplemental animals may be shifted from the high-dose schedule to a still higher dose schedule in order to ensure that the experiment will show some form of significant toxicity. In the ideal experiment in which the high-dose group of animals develops some toxicity toward the end of the experiment, the supplemental animals in this group can be removed from the test diet and reversibility of lesions produced by the test chemical can be evaluated. When new drugs are subjected to the prolonged toxicity tests, it is not uncommon for the animals on the high-dose schedule to show, either during the test or at necropsy at the end of the test, some altered function of certain organs, abnormal blood chemistry or hematology, or even an abnormal urine analysis. In these situations it is of considerable value to determine the degree of reversibility of all of these effects.

Whenever an analytical method is available for determination of a chemical under investigation and particularly if the method is applicable to biological sample material, it should be used in these studies. When such analytical data are obtained, they are invaluable for any subsequent studies that may be performed for longer periods of time in animals as well as for possible initial studies in humans.

With the exception of carcinogenesis, and some forms of cytotoxicity, there is considerable evidence indicating that tests performed on animals by daily administration of a compound at high-dose concentrations for periods of 3 to 4 months will reveal most forms of toxicity to adult animals. There are of course certain types of toxicity, such as mutagenesis, teratogenesis, and effects on reproduction of animals, that involve particular modification of the standard prolonged toxicity test; however, none of these involves extending the exposure time of the animals to the test agent beyond a 3- to 4-month time period.

An important additional application of the acute and prolonged tests is to provide toxicity information when two or more chemicals are simultaneously encountered. In fact, in the real world it is indeed rare that persons are exposed only to a single xenobiotic on any occasion. The subjects of summation of actions, potentiation of actions, and antagonism of actions when two or more chemicals are simultaneously involved have been discussed in Chapters 3 and 4. In order to detect these types of actions it is standard practice to perform at least the acute type of toxicity test and, if appropriate, prolonged tests for each compound alone and in combination, usually in 1:1 proportions. The type of chemicals that are commonly subjected to these combination tests are drugs, agricultural chemicals, and food additives. In addition, special test protocols may be followed when the possibility exists that one agent may influence the effect of another

when they are not administered simultaneously. For example an agent that produced microsomal enzyme induction would be expected to influence the rate of microsomal biotransformation of a second compound administered at a later date, thereby influencing its dose–response relation. Thus test protocols for chemical interactions can take on a variety of forms.

CHRONIC TOXICITY TESTS

The primary reasons for conducting animal toxicity tests that are of a year or more duration are to demonstrate, first, the absence of toxicity when the doses involved represent some practical concentration and, second, the carcinogenic potential of a compound. Every effort should be made to use as the highest dose schedule the maximum no-effect dose, and if the agent is a potential food additive one dose schedule should be at least 100 times the dose contemplated for humans. The exact nature and duration of chronic tests again are predicated on the nature of intended use of the chemical. There is at present no method that can be universally applied to establish the most adequate duration of chronic toxicity tests. The basis of chemical-induced toxicity proposed in this book is that toxicity is related directly to the concentration of the chemical at the effector site. Therefore, a sufficiently small concentration of any chemical should be compatible with the biologic system for indefinite periods of time. The thesis that if a given concentration of a chemical produces toxicity in 1 month, then one-half of that given concentration of the chemical would produce the same toxicity in 2 months is not tenable. Consequently chemicals that are to be administered to humans over periods of months or years should be tested in experimental animals over comparable periods of time (in terms of relative lifetime of animals versus man), by comparable routes of administration, and by comparable doses, as well as by excessive doses.

By the time a new chemical is considered for chronic toxicity studies, information has been obtained regarding the nature of its toxicity and its tolerable as well as lethal repeated doses. Also in the case of new drugs, by this time, it should be feasible to give a few doses of the chemical to selected human subjects under controlled experimental conditions in order to obtain enough confidence to indicate that the absorption, metabolic disposition, and duration of action of the compound are similar between the human and the species selected for the chronic toxicity studies. Except for its academic value, there is little rationale for performing 1- to 2-year chronic studies on a species of animal that grossly differs from the human in its ability to absorb, distribute, metabolize, or excrete the compound. If one species of animals is found to fulfill the requirements for approximating

the human species, chronic toxicity studies on the species would be more meaningful. Because of the time, effort, and expense involved in conducting chronic toxicity studies in animals, any preliminary effort that is expended in determining the most suitable species for the tests is well spent.

For the same reasons cited in the section on prolonged toxicity studies, when there is no rationale to support the use of a specific species of animal, the rat and the dog are the species most commonly used. The duration of the tests is usually not shorter than 1 year, and if carcinogenicity is to be evaluated the tests should be at least 2 years in duration in rats. For practical reasons the tests generally involve incorporation of the agent in the diet (with the exception of tests for dermal toxicity or inhalation toxicity). Similar numbers of animals are used in the chronic tests regardless of whether the duration of the study is for 1 year or longer. When studies are conducted on rats a minimum of three test groups plus one control group are used. Equal numbers of male and female animals randomly are assigned to each test and control group, and the control group often contains twice as many animals as each test group. A common example of a chronic rat study is to have 50 males and 50 females in each test group with 100 males and 100 females in the control group. The test groups are divided into low-, intermediate-, and high-concentration dosage schedules. The diets are prepared as in the prolonged type studies and the animals are started on the diets when the rats are at weaning age. When dogs are used the only difference is that the test groups and the control group usually consist of 10 male and 10 female animals each. The dogs are started on the diet when they are growing adults. Purebred beagles bred for research/testing purposes are used almost exclusively. All animals are individually housed.

Prior to and during the course of the chronic toxicity tests, it is important that clinical evaluation of the animals be made daily early in the experiment and at least weekly for the remainder of the experiment by persons knowledgeable in animal behavior and untoward signs. All animals are weighed at weekly intervals. Feed and water consumption are noted, as well as the nature of the excreta. Appropriate biochemical tests are essential, but do not necessarily need to be performed at routine intervals. Clinical blood chemistry tests, urinalysis, and blood cell counts should be performed at 6- to 12-week intervals or when animals become ill or show evidence of effect from the test chemical. Routine special types of biochemical analyses of sample material, such as blood or urine, from apparently healthy animals probably are indicated only when there is reason to suspect that the chemical under investigation is capable of producing specific toxic effects for which biochemical methods are clinically of diagnostic value. It is frequently desirable to obtain evidence regarding the reversible nature of chronically induced toxicities. During the course of chronic toxicity studies, if it becomes

apparent that the animals in a high-dosage group are progressing toward lethal effects from the agent involved, that group of animals may be subdivided into paired groups in which administration of the chemical is discontinued in one of each pair. At the time of death of one of the pair, the other is killed; complete pathological evaluation is made on the animals and an evaluation is made regarding the reversible nature of the toxicity.

All animals in chronic toxicity studies eventually are subjected to complete pathological evaluation. Those that die during the experiment as well as those that are killed at the end of the experiment are examined by necropsy, and tissue sections for histologic examination are prepared from samples of all types of tissues from each animal.

Since the pathological examination may reveal abnormal findings that were otherwise unsuspected, it is necessary to have a control group consisting of animals that were exposed to all conditions to which the experimental animals were exposed except for the chemical under investigation. This is particularly important when vehicles are used as solvents or suspending agents for the purpose of administering the chemical to the experimental animals. Also, because the pathological findings may indicate an otherwise unsuspected chemical-induced toxicity, it is desirable to include extra animals in the experimental group from which the test chemical is withdrawn at the time of the termination of the experiment. When the pathological results become available, such reserve animals may subsequently be killed to permit evaluation of the degree of reversibility of the pathological effect.

A variety of special endpoints can be incorporated into chronic toxicity studies either as complements to standard antemortem and postmortem observations collected for core group animals or as satellite group investigations. In some cases, it may be more appropriate to study one or more of these endpoints in separate mechanistic repeated dose studies. These endpoints include such things as cell proliferation and cell cytokinetics, enzyme-altered foci, enzyme induction, and DNA–protein aduct.

TERATOGENIC TESTS

A teratogen has been defined in Chapter 8 as a chemical that increases the occurrance of structural or functional abnormalities in offspring if administered to either parent before conception, to the female during pregnancy, or directly to the developing organism. Many chemicals have been shown to cause embryotoxicity of some form. Some agents are predominantly lethal whereas others are predominantly able to produce malformations of the fetus. The difference in type of embryotoxic effect induced by various chemical agents is mainly in dose requirements and the

time during gestation when the fetus is exposed to the test compound. Frequently all of the forms of embryotoxicity can be manifested in a given teratologic test in different groups of animals that receive different dosage concentrations of the test compound.

Many factors other than chemical agents have been shown to initiate abnormal development of the fetus when introduced during pregnancy in laboratory animals. Some of these factors are dietary deficiencies, viral infections, hyperthermia, hormonal imbalance, and various stress conditions. Consequently any teratogenic test method must include provisions for adequate evaluation of normal incidences of occurrence of teratogenic effects. Although many chemical agents are known to be capable of producing teratogenic changes in laboratory animals, only a few compounds have been shown to produce such effects in man. Table 2 in Chapter 8 is a selected list of some known human teratogens and their clinical effects.

Malformations of the fetus due to chemicals are rare when exposure of the mother to a compound occurs only prior to the implantation of the fertilized ovum. Also in the early stages of undifferentiated cell multiplication the cells of the fetus are not susceptible to teratogenic effects of chemicals. Thus, the stage of development of the fetus determines susceptibility to teratogenic agents; specific damage occurs readily during organogenesis. The earliest that terata have been produced in the rat is in the 7-day-old fetus. This effect was shown using actinomycin D. Specific organs in the fetus are most susceptible to the effects of chemicals that are given to the mother only on a specific day during the pregnancy and administration of the agent 1 or 2 days before or after the critical day decreases susceptibility of the organ to the agent. When organogenesis reaches completion, chemical-induced malformation of organs does not occur; rather, the effects that are obtained are either growth retardation or the same types of acute toxicity that are observed in adult animals. Only a limited number of studies have been performed to evaluate effects on the fetus that are manifested as alterations in metabolic function or behavior of the animals. Toxic effects on the embryo are experimentally obtained by administering the agent to the mother. The embryo relies on the maternal organism for growth and maintenance. At different times, the placenta may act as a more or less efficient barrier to transfer of an agent from the mother to the embryo. The excretory function or even the endocrine function of the placenta may be directly influenced by the chemical, thereby resulting in an indirect detrimental effect on the welfare of the embryo. If an agent produces toxicity to the mother, such an effect would be expected to influence the intrauterine environment of the fetus. For example, the degree of vitamin A deficiency in the rat necessary to produce severe malformations in the fetus is poorly tolerated by the mother. In some cases maternal protection

of the fetus could be brought about by rapid excretion or detoxification of foreign agents. There is some evidence that enzyme induction is an important factor since pretreatment of mice with the antibiotic drug mitomycin reduces the expected teratogenicity of this substance when given during the period of organogenesis.

Mice, rats, and rabbits are the test animals most frequently used in teratogenicity tests. They are used not only because of prior experience with these species but also because of their availability in most laboratories and because the number used can be great enough to satisfy the statistical requirements. The smallest number of rats usually involved is 10 females for each group. This would yield approximately 100 fetuses; if this number produces equivocal results the experiment is repeated with at least 20 females in each group. The range of doses that is selected for the groups should be such that the highest dose is not seriously toxic to the mother and the lowest dose is without determinable effect on the mother. The principles described in the introduction to this chapter should be adhered to. The sequence of all teratology tests is: (1) produce gestation, (2) confirm gestation and administer test chemicals, (3) establish teratogenic effect.

In the production of gestation the time of mating of the animals should be limited as much as possible to enable the accurate estimation of onset of pregnancy. Artificial insemination techniques have been used with considerable success. Rats or mice with regular estrus cycles should be selected by prior daily check of their vaginal smears and mating should be done in the proestrus stage. Gestation is verified by observation of sperm in the vaginal fluid and development of a permanent diestrus. In mice a vaginal plug will appear and last for about 24 hr. In the rat physiologic bleeding may be observed on the 14th day. In the rabbit, the mating act followed by the presence of sperm in the vaginal fluid is acceptable evidence of onset of pregnancy. Palpable fetal masses in the abdomen are evident in about 15 days. The gestation period in mice is 21 days; in rats it is 21 to 22 days, and in rabbits 30 to 35 days.

All tests should be conducted in two species involving at least three dose concentrations in different groups of animals. In addition to negative controls a positive reference control is recommended. The choice of a reference is based on the existence of established uniformity of teratogenic action of the reference compound in the species under test. Each of the two reference compounds commonly used has some drawbacks. Trypan Blue is one, and its action varies with the production lot sample of the agent, and with various strains of mice. High dosage of vitamin A is the preferred positive control because its action is consistent in mice, rats, and rabbits and it induces abnormalities of several organs including the central nervous system, eyes, face, skeleton, and viscera. However, its teratogenic

effects have not been shown in humans. When rats or mice are used, the animals are dosed by administering the test chemical, placebo, or positive control agent by daily dosing from the 7th to the 15th day of pregnancy. The animals are weighed daily under careful conservative handling conditions and are examined for evaluation of their general state of health. Pregnant females are always housed separately.

The pregnancy is interrupted just prior to the calculated date of delivery by removal of the pups by caesarean section. With mice the section is performed on the 20th day, with rats on the 21st day, and with rabbits on the 31st day. The mother undergoes complete necropsy. All live and dead fetuses are counted and inspection of the uterus is made for evidence of fetal resorption sites. All fetuses are thoroughly and systematically examined for evidence of malformations. Abnormalities of tissue organization require evaluation of histologic sections of the tissues. A number of special techniques for sectioning the fetuses for further examination for malformations are available. If no fetuses are found in the test animals, abortion should be considered as having taken place and the experiment would need to be repeated using smaller doses.

The traditional types of malformations may be obvious. Some of these are cleft palate, renal agenesis, and club foot. A variety of malformations observed in humans and experimental animals have been described. All degrees of effects may occur; the effect of a test chemical may be only that of increasing the incidence of normally occurring malformations. The final evaluation of the test rests on proper statistical evaluation of the data and scientific judgement.

The selection of animals for the tests described so far is based on similarity of reproductive physiology with that of humans. This involves the use of a placental type of test animal. Few species other than the rat, mouse, and rabbit have been used to any extent because there is no real evidence that one test species has advantages over the others. Cats, dogs, and primates present handling problems in regard to following the pregnancy.

One test involving a nonplacental species is that which is conducted on the developing chicken embryo. The test is open to criticism because it shows a high number of false positives when compared to the mammalian tests, and little confidence can be placed in negative results. Many factors influence the results obtained, among which are pH, specific gravity, coagulation effect, and ionic strength of the test solution. The chick embryo test also is used as an index of general toxicity. The test involves initial selection of fertile, white leghorn, chicken eggs. Verification of fertility and hatchability is obtained by the use of control groups of the eggs. A minimum of 20 eggs is randomly selected for each dose. At 1 day of age the test chemical is injected into the yolk of the eggs by insertion of a hypodermic needle

through a drilled hole in the center of the large end of the egg shell over the air cell. The volume of material injected does not exceed 0.1 ml and the injection is made under sterile conditions. The hole in the shell is covered with a small piece of plastic tape. The injected eggs then are put in an incubator tray with the large ends up. The incubator is maintained at 38°C and a relative humidity of 60%. The eggs are candled on the 5th day and every day thereafter. The eggs with dead embryos are removed and examined for a pathological condition. On the 17th day the eggs are transferred to the hatcher and kept at 37°C until they hatch. During the first 7 days after hatching the yolk is absorbed. The live chicks are examined for gross abnormalities and are followed for 2 to 6 weeks for mortality and weight change as well as for the appearance of delayed damage.

REPRODUCTION TESTS

A well designed and executed reproduction study on experimental animals constitutes a sufficiently comprehensive single protocol so that essentially all of the toxicities of any chemical, with the exception of carcinogenicity, are obtainable. Information obtained from adequate studies designed for evaluating the effects of compounds on fertility and general reproductive performance yields information about the effects of chemicals on neonatal morbidity and mortality and on teratogenesis. Also, since studies concerned with effects on reproduction frequently are conducted on more than one generation of test animals they can yield information on mutagenesis.

The factors involved in evaluating chemical-induced effects on fertility and reproduction have been reviewed by a number of government agencies. A multigeneration test which provides for accumulation of effects of the agent to the point at which toxicity is manifested has been recommended. Tests for effects on reproduction have as their final objective an estimation of effects on fertility, on gestation, and on the offspring. Thus there are three segments to any complete reproduction toxicity test. Effects of compounds on fertility are reflected by toxicity in the parent male or female or both, and may be the direct result of altered gonadal function, estrus cycles, mating behavior, conception rates, and effects the early stages of gestation, such as implantation of the fertilized ovum. The second segment of the test is concerned with the development of the fetus, its degree of normality, including teratogenic and mutagenic effects, and intrauterine mortality. The last segment of the test is concerned wtih effects on the mother, such as effects on lactation and acceptance of the offspring, and with the offspring with respect to its growth, development, and sexual maturation. The result is an overall evaluation of the toxicity of the test

compound on multiple systems within the animals. The minimum safety evaluation protocol for determining no-effect concentrations of any food additive or chemical residue should involve a study of animals who have been exposed to the test substance from the time of conception to the time they produce their own offspring, plus a study of the progeny during development and growth. A schematic presentation of such a protocol for a three-generation study for effects on reproduction is shown in Fig. 13.1.

In the figure F_o animals (rats) are maintained after weaning until the females reach a weight of 180 g and the males 275 g. This is identified as the growth period. The time required for growth is approximately 5 weeks but will vary with different strains of rats. During this time the animals are maintained in groups, each of which consists of a minimum of 10 males and 20 female animals with the males and females caged separately. Control and treated groups are included in the study. The test compound is incorporated in the diet or drinking water. During the last 2 weeks the estrus cycles are followed in the females and at the end of the 5 weeks the females are

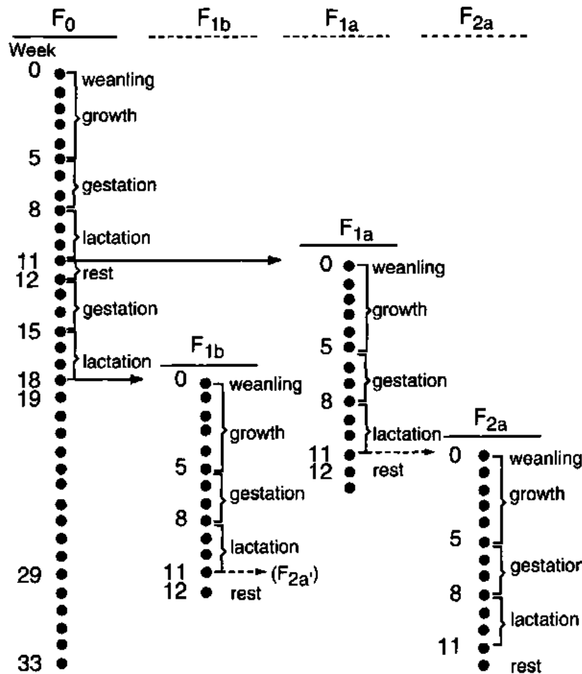

FIGURE 13.1 Generations and time intervals involved in a three-generation study of effects on the reproduction process in rats.

exposed to the males. The occurrence of copulation is established by daily examination of vaginal fluid for sperm and this finding is considered Day 0 of pregnancy. Pregnant females then are housed separately in individual cages. Dosing of the test animals and the vaginal fluid examinations are continued to check for the absence of new estrus periods in order to further establish that pregnancy had occurred on the Day 0. Near the end of the period of organogenesis, on Day 13, half of the females from both control and test groups are killed and examined for number and distribution of embryos, empty implantation sites, and embryos undergoing resorption. At the same time a complete necropsy is performed and histological sections are made of all lesions that are observed. The remaining animals are continued as test and control animals and allowed to litter normally. The duration of gestation is calculated (approximately 21 days) and the animals are observed at delivery time. The live-born and still-born pups of the F_{1a} generation are counted, weighed, and examined for abnormalities. The F_0 generation of animals is continued on the test and control diets while nursing the pups. The F_0 animals have now produced and nursed their first litter in 12 to 14 weeks and data have been obtained on their fertility, pregnancy, parturition, and lactation. After 1 week of rest they may be remated for the initiation of an F_{1b} generation.

The F_{1a} pups have been exposed continuously to the test compound during embryogenesis, fetal development, and lactation. When the F_{1a} pups are weaned the test diet is started on this generation and the animals are followed for growth for 5 weeks. At this time they are tested for reproductive performance as in the previous F_0 generation. The same procedure as used in the F_0 generation is followed so that an identical observation can be made on the F_{2a} generation. Studies of the effect of combinations of pesticides in reproduction studies for six generations of mice by a procedure similar to that described for the rat, except that the second litter from each generation was used for each subsequent generation, have indicated that there was no justification for continuing the tests for more than three generations.

All animals carried through reproduction studies should eventually be killed and subjected to complete gross and microscopic pathological examination. All still-born pups should be examined for skeletal abnormalities. The three-generation reproductive study will supply data on accumulation of test compounds regarding effect on reproduction. Reproduction studies which include determination of the reproductive capacity of the F_{1b} animals can be performed in a total of 30 to 36 weeks. Such studies provide good approximations of no-effect dosages that are so essential to evaluation of potential hazard. It would seem that if test groups from the F_0 generation

were carried through on continued test diets for an additional 18–24 months even the potential for carcinogenicity could be evaluated.

As observed with other types of toxicity it appears that there are both strongly and weakly active compounds in regard to effects on reproduction. Furthermore, the spontaneous occurrence in control animals of still-born pups and teratogenic effects on pups necessitates that evaluation of the data be subjected to rigorous statistical procedures. Strongly active compounds can readily be demonstrated to produce significant effects. Accurate data on weakly active compounds necessitate careful conduct of the test procedures on adequate numbers of animals. The evaluation of the absence of an effect on reproduction involves a probability evaluation and the exercise of scientific judgment in interpretation of the results obtained.

A properly conducted reproduction experiment employing an adequate number of animals and at least three different test doses, the largest of which represents a nearly maximum tolerated dose which produces no significant effect, represents the degree of perfection that is currently obtainable in experimental toxicology for determination of the no-effect dose.

MUTAGENICITY TESTS

Mutagenesis is the induction of those alterations in the information content (DNA) of an organism or cell that are not due to the normal process of recombination. In higher organisms, genetic damage can occur in both somatic and germinal cell lines. Somatic mutations in a developing organism may lead to abnormal differentiation of its cells. Alterations in the duplicating somatic cells of an adult may lead to cancer. This type of genetic damage is important to the individual animal but will not appear in its progeny. Genetic alterations in germ cells, on the other hand, are passed on to further generations. Effects from germinal mutations range from death to abnormally developed offspring.

There are two general types of alterations of genetic material that can occur either spontaneously or by chemical induction: (1) point mutations and (2) chromosome aberrations. A point mutation is defined as an alteration in a single nucleotide pair in the DNA molecule and usually leads to a change in only one biochemical function. Point mutations are detected by changes in phenotypes and their locations can be determined by genetic mapping. Chromosome aberrations include structural mutations (breaks and rearrangements of chromosomes) and changes in chromosome number. These alterations can be detected by cytological examination of the chromosomes. Different mutagens produce varying degress of the two basic types of mutations. Hydroxylamine is an example of a mutagen that produces

primarily point mutations but under certain conditions also can cause chromosome aberrations. On the other hand, radical-producing mutagens produce mainly chromosome aberrations. The problem of man's contact with increasing numbers of substances in the environment, including drugs, food additives, and pollutants, has led to increased interest in the development of methods for detecting possible mutagenic effects of these substances. A detailed survey of mutation test systems, from bacteriophage to mammals, can be found in books such as *Chemical Mutagens—Principles and Methods for Their Detection* and the third edition of *Principles and Methods of Toxicology*. The important question for the toxicologist is which methods should be included in the framework of routine toxicology testing.

Submammalian systems, including such organisms as bacteria, yeast, plants, and insects, offer many advantages to the geneticist. They are simple systems, allowing detailed analysis of their genetic composition. Large populations are necessary in mutagen testing and with a bacterial system (Ames Samonella Assay) about 5×10^8 bacteria can be exposed to a mutagen in one petri dish. The *in vitro* bacterial mutagenesis tests also play a role as surrogate tests for carcinogenicity and are discussed in greater detail in this chapter under Carcinogenicity Tests. Because of their simplicity and economy, submammalian systems can be valuable in quick screenings of large numbers of chemicals. The one serious disadvantage is that despite having the same genetic material, microorganisms lack the physiology and metabolism of mammals. However, this disadvantage has been overcome partly in most *in vitro* tests for mutagenesis simply by the addition of the mammalian metabolizing systems (as liver homogenates) to the growth medium. An advantage to the use of the submammalian systems is that they are capable of characterizing the type of genetic damage caused by a given chemical. All such tests provide important information about possible genetic risks for man. Any complete study of a compound for its mutagenic potential should involve *in vitro* tests on submammalian species as well as *in vivo* mammalian assays. Three *in vivo* methods will be described because of their practical application; the dominant lethal assay, the host-mediated assay, and an *in vivo* cytogenetics assay.

The Dominant Lethal Assay

A dominant lethal mutation is one which kills an individual heterozygous for it. It indicates that genetic damage has occurred in the form of structural or numerical chromosome aberrations. A procedure for screening chemicals in the dominant lethal test is to treat male mice or rats with a subtoxic dose (generally one-fifth of the LD_{50}) of the chemical being tested. The males are sequentially mated with groups of untreated females. The sequen-

tial mating makes it possible to determine the sensitivity of the male germ cells in different stages of spermatogenesis. The females are killed about 14 days after mating, dissected, and scored for corpora lutea, early fetal deaths, late fetal deaths, and total implantations. A mutagenic index (MI) often is calculated as follows.

$$MI = \frac{\text{early fetal deaths}}{\text{total implantations}} \times 100$$

A criticism of the dominant lethal test is that it screens for chromosome aberrations but cannot detect point mutations. If a compound does not produce a significant amount of chromosome breakage, it will escape screening. The majority of the compounds found to be mutagenic by this method are known alkylating agents. The main advantage of the method is its ability to test the sensitivity of mammalian germ cells *in vivo* at different stages of development.

A number of agents including pharmaceuticals, food additives, pesticides, and extracts of air and water pollutants have been tested using this method. Many investigators have studied the effects of caffeine in various mutagenic test systems, due to the belief that the purine-like compound might be incorporated as a base analog into DNA. Positive results were found in bacteria and plants, contradictory results were found in Drosophila, positive results were found in human cells *in vitro,* and contradictory results were found in mammalian tests *in vivo.* The administration of nontoxic single or repeated doses of caffeine to male mice produced no distinct mutagenic effects in the dominant lethal assay. In addition caffeine failed to induce synergistic mutagenic effects with x rays or with alkylating agents.

The Host-Mediated Assay

The host-mediated assay typically uses microorganisms as indicators of genetic damage within a mammalian test system. There are some substances that are not mutagenic in microorganisms but are converted to active mutagens in mammals. Other substances may be mutagenic in microorganisms but are detoxified by mammalian systems to forms that are nonmutagenic. The host-mediated assay takes into account the metabolism of the potenial mutagen by the host mammal. An indicator organism such as Salmonella or Neurospora is injected intraperitoneally into the host mammal which may be a mouse, hamster, or rat. The animal is treated with the potential mutagen which is administered by an alternate route of administration. After an appropriate period of time a sample is withdrawn from the peritoneal cavity of the host and the mutation frequency in the microorganisms is measured. Simultaneously, the mutagenic action of the compound is

tested *in vitro* on another culture of the microorganisms and the mutation frequency *in vitro* is compared to the frequency obtained from the host-mediated assay.

Dimethylnitrosamine (DMNA), a known animal carcinogen, had no mutagenic activity *in vitro* but when tested in the host-mediated assay it was mutagenic. DMNA is activated by oxidative hydroxylation to its mutagenic form. Pretreatment of animals with the hepatotoxic chemical carbon tetrachloride, prior to treatment with DMNA, reduced the mutation frequency to control levels, indicating that the activation of DMNA probably takes place in the liver. Besides providing information on the metabolism of chemical mutagens, the host-mediated assay is a means for detecting point mutation in a mammalian test system. Further development of the assay includes the use of mammalian cells instead of bacterial cells as an indicator for mutagenic activity. Another method available that detects point mutations in mammalian cells is the specific locus test in mice, but this assay requires scoring thousands of mice to locate mutations at seven recessive gene sites and would not be suitable as a routine screening method.

In Vivo Cytogenetics

Many methods are available, both *in vitro* and *in vivo*, for microscopically examining mammalian cells for chromosome aberrations. Information derived from *in vitro* cytogenetic studies, in which the agent in question is introduced into an existing culture of mammalian cells, is considered by many to be of ancillary value only. *In vitro* tests, however, do allow for more accurate regulation of doses and length of time of exposure to the chemical. A test for detecting chromosome abnormalities is the *in vivo* test which permits direct examination of cells from treated experimental animals or even humans who have been exposed to various agents. Tissues that are commonly studied are bone marrow for effects on mitosis, lymphocytes for damage occurring before DNA synthesis, skin fibroblasts for long-term effects, gametocytes for effects on meiosis, and amniotic fluid cell cultures for damage to developing offspring. The advantage of cytological studies is that they can be useful in the course of clinical trials of drugs in man.

Guidelines for cytogenetic methods in mutagen screening, as well as other tests for mutagenesis, have been developed by numerous governmental agents, including the U.S. FDA and the U.S. EPA. In addition, several international organizations have developed guidelines. Most recommend the use of mice or rats for *in vivo* testing. The animals are treated acutely or repeatedly at several dose concentrations and then killed, and their bone marrow cells are examined in metaphase. Newer protocols for scoring and

reporting of abnormalities have simplified interpretation and comparison of results.

The greatest inadequacy of most mammalian test systems is their relative insensitivity. In order to detect low levels of effects, it becomes necessary to use either very large populations of animals or high concentrations of the mutagen, both conditions not being feasible in mammalian *in vivo* testing. Another drawback is that point mutations are not easily detected. It has become clear that a battery of tests should be performed in deciding whether an agent is safe for human use. Of the three mammalian systems discussed, no single method is best. A negative result in one test should be confirmed. Most investigators agree that a positive result in any of the three mammalian tests is enough evidence to suggest the agent as potentially dangerous to man.

CARCINOGENICITY TESTS

The methods for evaluation of carcinogenic risk have been reviewed in detail by the National Academy of Sciences of the National Research Council, by the joint committee of the Food and Agriculture Organization of the United Nations, by the U.S. Food and Drug Administration, by the U.S. Environmental Protection Agency, and by a number of international organizations, including IARC and OECD. A tumor is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of normal tissues. The two basic types of tumors are described as benign or malignant. The cells of a benign tumor are structurally, characteristically identical with those of the normal tissue from which it originates; it is confined to the area in which it originates and its growth is only expansive and space-occupying, that is, it does not invade neighboring tissues. In contrast, the cells of a malignant tumor are not typical of those structures from which they arise and they have a tendency to invade neighboring tissues. The malignant tumor is prone to develop secondary growths at a site in the animal distant from the origin of the primary tumor. These secondary growths are referred to as metastatic tumors, and a primary tumor that produces such secondary tumors is described as a metastasizing tumor. All tumors that metastasize are considered malignant. The two types of malignant tumors are carcinomas, which arise from epithelial (surface) cells, and sarcomas, which in general arise from supporting or connective tissues.

It is common to describe the chemicals that produce any type of tumor as tumorigenic agents, and tumorigenic agents that produce malignant tumors are called carcinogenic agents, regardless of whether the tumor is a

sarcoma or a carcinoma. Furthermore, in recent years, because of the unresolved problems in establishing whether benign tumors in animals are or are not potentially malignant, the term "carcinogenic agent" has gradually been used to identify an agent that is capable of producing any type of tumor. The response in animals to a carcinogenic substance may consist of (1) an increased incidence of tumors of a type seen in normal control animals, (2) an occurrence of a type of tumor not seen at all in control animals, or (3) a combination of an increased incidence of normal tumors and the occurrence of a different type of tumor from those seen in normal animals.

Tests for carcinogenicity are interpreted in terms of effect of the compound on the incidence of occurrence of tumors in experimental as compared to control groups of animals. The inclusion of negative control groups of animals is essential since there are physical agents, viruses, and chemical agents that are present in the environment that are capable of inducing tumors at least in experimental animals. Chemical-induced carcinogenesis in man is substantiated only through epidemiological studies from occupational, environmental, or medical exposure. In most instances where a compound is known to be a carcinogen in man the carcinogenicity has been confirmed in laboratory animal studies.

Some carcinogenic compounds, such as certain polycyclic aromatic hydrocarbons, the aminoazo dyes, and dimethyl nitrosamine, are active in several species of animals at relatively low concentrations of exposure. In animal experiments for carcinogenicity as with other types of toxicity it is usually not difficult to demonstrate the action of the compound when it is strongly carcinogenic. That is, it is not necessary to employ maximally tolerated doses. However, many agents have been investigated which would best be classified as weak carcinogenic agents so that there is significant evidence of tumorigenicity only when maximally tolerated doses of the compounds are administered to maximal numbers of animals. Even then the evidence for carcinogenic effect of a compound is based only on an increased incidence of normally occurring tumors in the test animals as compared to the controls. It is the weak carcinogenic agent that presents the greatest challenge in the design and conduct of toxicity tests. Occasionally compounds are reported to be carcinogenic on the basis of inadequate data. Thus it is necessary that all such tests be reported on proper numbers of randomly selected test animals where the experiments were adequately controlled and the lesions obtained were established by proper pathological examination.

Regarding the nature of those chemicals that are carcinogens, about half are polycyclic aromatic hydrocarbons or their derivatives. The other half consists of many diverse agents such as nitrogen mustards, certain epoxides,

and ethylene imines. It is also well established that although certain compounds are not tumorigenic or are weak tumorigens, they can be biotransformed in suitable species to active tumorigens. For example, 2-acetylaminofluorene is a carcinogen in every species that forms the *n*-hydroxy derivative and this derivative is a more potent carcinogen than is the parent compound. There is good evidence that some of the transient intermediates involved in the sequence of biotransformation reactions lead to the formation of agents with potential carcinogenic properties.

Carcinogenic studies usually are performed in the mouse and the rat although dogs, rabbits, guinea pigs, and nonhuman primates have been used. Although a number of short-term tests, including transgenic mice and neonatal mice, currently are being evaluated, there is no short-term test that has been successful in evaluating tumorigenic potential for chemical agents. Most of the studies involve lifetime tests or at least exposure of the animal to the chemical for a significant portion of the lifetime of the animal. Tests in the rat last 2–3 years, and those in the dog last 7 years. Therefore tests utilizing dogs generally are impractical. However, consideration should be given to the dog in evaluating the potential tumorigenicity of compounds related to the aromatic amine bladder carcinogens because of the particular susceptibility of the dog to these types of chemicals. Pathogen-free animals (rats or mice) are not preferred over standard laboratory animals because they are more susceptible to infection and have an unestablished duration of life under various test conditions. Random bred animals are commonly used. Since most of the tests for carcinogenicity are performed on food additives or pesticides, the oral route of administration is used and the test agent is incorporated daily in the diet or in the water. All commercial diets vary in composition from time to time and contain compounds of varying general toxicity as well as compounds with carcinogenic potential. It is feasible to prepare synthetic diets from chemically defined substances but only a few studies have been conducted with such diets. Although synthetic diets are more expensive than standard rations this is a minor factor in terms of the total cost of carcinogenic tests. Positive control groups of animals are desirable and negative control groups are necessary. It is recommended that the control group be at least as large as any test group. The size of the control group can be calculated as $\sqrt{N}$ times the number of animals per dosage group, where N is the number of dose groups; that is, 100 controls would be used for four dosage groups of 50 animals per dosage group. Prior studies of at least 90-day duration should be performed to accurately estimate at least three dose concentrations to be used. One dose generally is the maximally tolerated dose and the other two dose concentrations are frequently one-third and one-ninth of the maximally tolerated dose. All animals are weighed at weekly intervals and

at the time of weighing they are examined physically. During the test, as the animals undergo growth and maturing, the dose should be adjusted to maintain a constant dosage per unit weight of the animal. The World Health Organization report on Carcinogenic Food Additives (1961) suggests a schedule for maintaining uniform dosage concentrations of test compounds.

All animals that become seriously ill during the course of the experiment are killed and complete clinical chemistry and necropsy are performed. A diagnosis of the cause of illness is recorded. If tumors become evident during the course of the experiment, the time of appearance, characteristics, and type of tumor should be recorded. At the end of the experiment the usual procedure is to kill all animals; the final conclusion that can be reached is based on adequate pathological examination of all tissues and identification of all lesions in the animals.

Because of the expensive and time-consuming nature of *in vivo* tests for mutagenicity and carcinogenicity, there is a need for simple and rapid methods which would evaluate or at least screen compounds in regard to these toxicities. One such *in vitro* method consists of a simple bacterial test for the detection of mutagens. This test (the Ames Salmonella Assay) consists of adding the suspected mutagenic compound to a nutrient agar which then is seeded with a specially developed mutant strain of *Salmonella typhimurium;* unlike its parent strain, the mutant requires added histidine in its nutrient medium in order for growth of the bacteria to occur. In the presence of a mutagenic chemical, the mutant strain of salmonella tends to revert to its original strain. This reversion to the prototype is readily detectable since the reverted organisms will grow in the absence of significant amounts of histidine. The test is conducted on agar in a petri dish; wherever an organism reverts to its prototype, it develops a colony of the organisms, and the colonies can be simply counted. By the use of various concentrations of the test compound, the mutagenic potency of the compound can be quantitated. Since there is a spontaneous rate of reversion of the test organisms, suitable control tests are always conducted so that the rate of spontaneous reversion will be taken into account in the interpretation of the results of a test.

If the assumption is made that many carcinogens are mutagens because carcinogens produce cancer by producing somatic mutation, then the *in vitro* test for detecting mutagens becomes a test for potential carcinogenicity. Furthermore, since many carcinogens require metabolic activation, the *in vitro* test using the salmonella histidine mutant has been modified by including in the test the addition of a biotransformation system. This is done by adding to the test medium a homogenate of rat or human liver. In this manner, a wide variety of carcinogens that require metabolic activation can be detected in the test as mutagens. At the present time many com-

pounds known to be carcinogens as a result of *in vivo* tests in animals have been tested by the *in vitro* method, and about 60–70% of them have been shown to be mutagens. Also, many noncarcinogens have been tested by the *in vitro* test procedure, and less than 10–20% have been found to be mutagenic. Thus it appears that this indirect *in vitro* test for carcinogenicity has merit. By the use of different specially developed tester strains of salmonella, it has been shown that one particular strain can be used to detect mutagens causing base pair substitutions. Other strains can be used to detect frame shift mutants.

Other similar short-term tests have been developed involving both mammalian and submammalian cells. None of these tests either alone or in combination appear to be any more sensitive or specific than the Ames Salmonella Assay. A number of carcinogens appear to work through nongenotoxic mechanisms and may involve such factors as interaction with receptors or alteration in hormonal status of the animal. In other cases, production of highly specific proteins has led to certain types of tumors resulting from irritation and/or cell proliferation in experimental animals. The general bioassay for carcinogenicity can detect, but does not differentiate between, a genotoxic or a nongenotoxic mechanism. Cell transformation, as well as alteration in the immune or hormone system, may lead to promotional events that enhance the initiation process. The biology of cancer is complex but, certainly, is a multistage process which involves, at least, initiation, promotion, and progression.

Two issues considered routine in a cancer bioassay which continue to be debated are (1) the maximally tolerated dose, and (2) *ad libitum* feeding. The maximally tolerated dose, initially incorporated into the cancer bioassay to compensate for the large number of animals needed to develop the necessary statistical power for detection of cancer at low human exposure equivalents, may not be appropriate. The data suggest that a number of defense mechanisms such as DNA repair, metabolic detoxification, and hormonal imbalance are impaired (or overloaded) and as a result tumors develop. Ongoing research also indicates that obese animals develop significantly more tumors than calorically restricted animals.

Recent information from the U.S. Food and Drug Administration and the National Toxicology Program suggests that multispecies evaluation for tumorgenesis may not be necessary. The FDA has suggested, at least in the case of drugs and pharmaceuticals, that 80% of cancers detected in a bioassay program have occurred in the rat (male and female) while the National Toxicology Program has suggested that up to 95% of the tumors detected in their bioassay program appear to be in male rats and female mice. In both cases, the potential for a reduction in total number of animals may be on the horizon.

SKIN AND EYE TESTS

It has been seen that chemical injury can result from a variety of routes of exposure, including absorption through the skin. Substances that come in contact with the skin and eyes also can produce local effects at the site of contact. Literally thousands of chemical entities in the form of soaps, detergents, emulsifiers, cosmetics, solvents, and a large variety of dyes are available for general human use. Every substance that comes in contact with the body has some potential for affecting the body at least at the site of contact. Because of this it is desirable to obtain information on the relative ability of these substances to produce injury in the course of customary intended use and in the course of reasonably anticipated misuse. The experimental basis for safety assessment of topical agents has been reviewed by a number of individuals and government agencies. As with the other routes of administration of chemical agents, the capacity of a compound to produce injury to the skin or eyes involves measurement of the type and degree of effect. The major local types of effects than can occur are primary irritation, corrosion, cutaneous sensitization, phototoxicity, and photoallergy. Animal tests have been developed for the purpose of acquiring data in regard to each of these types of toxicity.

Single chemical agents can produce more than one type of local reaction. For example, the difference between an irritant and a corrosive effect is usually a matter of degree of effect, depending on concentration of the agent and frequency of insult of the skin with the agent. Basicially, an irritant effect is a reversible effect and a corrosive effect is one which causes visible destruction and irreversible alteration in the tissue at the site of contact. In contrast to these effects cutaneous sensitization and photoallergy involve the immunogenic mechanism. In the case of cutaneous sensitization the response occurs in the absence of exposure to light, whereas in the case of photoallergy the reaction occurs only after exposure of the sensitized area to light. Phototoxicity does not involve the immunogenic mechanism but does involve irradiation of the area with light. By the use of animals tests it is generally not difficult to demonstrate the occurrence of skin or eye toxicity when the compound under test is strongly capable of producing any of the listed types of effects. However, when a compound is only weakly capable of producing harmful effects or even when no effect is obtained, all of the previously described factors inherent in statistical evaluation of the data preclude establishing absolute safety of the compound as a result of either animal or human tests.

Tests to evaluate these parameters were initially developed in 1944 and applied quantitative methods to skin and eye tests by using an arbitrary weighted scale for describing the degree of the various effects observed.

The protocols for these tests have been altered over the years to reduce the number of animals and the potential pain but essentially follow the same principles.

Primary Irritation—Skin

The animals that have been widely used for the detection of irritant properties of chemicals are the albino rabbit, the albino guinea pig, and the white mouse. Whenever a chemical is applied by repeated dermal application, the objective of the test is to detect both topical and systemic effects. When compounds are absorbed significantly from the skin, the LD_{50} may be determined. When rabbits are used the duration of the tests may vary from a minimum of 3 days, involving application of the agent on one occasion, to as long as 2 years. Whenever the study is of the prolonged type, the animals are followed in a manner similar to the general prolonged studies previously described; that is, the animals are subjected to hematological, clinical chemistry, and urine tests. When carcinogenic effects are to be evaluated the skin tests are extended for a period of 2 years, during which the agent is painted on the skin at least twice weekly for the duration of the test.

The acute dermal test is usually a 3-day test. It is conducted on a limited number of albino rabbits which have been divided into two equal groups. The area over the back of each animal extending from the base of the neck to the hind-quarters is shaved or depilated. In one group an area of approximately 2 $in.^2$ of the bare skin is abraded by making minor incisions through the surface layer of cells; that is, the incisions are not sufficiently deep to disturb the derma or produce bleeding. If the test material is a liquid, 0.5 ml or less of the material is placed beneath a 1×1 in. gauze pad which is secured in place over the shaved area of the skin of both the abraded and nonabraded animals. If the agent is a solid, it is dissolved in a suitable solvent such as vegetable oil or water and 0.5 g of the substance is introduced under the gauze pad. Whenever a solvent is used a control group of animals exposed only to the solvent is included in the test. The animals then are generally immobilized in restraining stocks and the entire trunk of each animal is wrapped in a nonabsorbent binder for the subsequent 24 hr. After the 24-hr time interval the binder and the pad are removed, and the area of exposure is evaluated and is reevaluated 48 hr later.

Evaluation of skin effects involves using a scoring system for the degree of redness and the degree of edema at the site of application of the gauze pad. The scoring system used is commonly that which has been published in the Federal Register in Section 191.11 of the Federal Hazardous Sub-

stances Act of the United States. The scoring involves relative assignment of separate numbers for the degree of erythema and the degree of edema formation as follows: no erythema, 0; slight, barely perceptible erythema, 1; well-defined erythema, 2; moderate to severe erythema, 3; and severe, beet red, erythema with injuries in depth, 4; no edema, 0; slight, barely perceptible edema with raised edges, 2; moderate edema with the surface raised approximately 1 mm, 3; severe edema with the area raised more than before and extending beyond the area of exposure, 4. The scores obtained for both erythema and edema at each scoring period and for both the abraded and intact skin are listed and the mean for each group and for each type of effect is calculated. All eight mean values then are added together and divided by 4 since there are four mean values for each effect (erythema and edema), thereby giving a final numerical figure which is the primary irritation score. When systemic toxicity is to be determined in addition to local toxicity, the animals are treated on one occasion as indicated above except that the area of exposure is washed after the gauze pad is removed and the animals are followed for the subsequent 14 days for evidence of local and systemic effects.

In prolonged, repeated administration studies for periods of 3 months duration, young albino rabbits of either sex are randomly divided into three groups of three to six animals in each group plus an equal number of control animals. The area of exposure on the skin of at least two animals in each group is abraded. The test material is applied directly to the skin 5 days/week for a total of 60 applications. The entire trunk of each animal is wrapped as in the previous test and the wrapping is secured with adhesive tape. Collars are placed on the animals to prevent ingestion of the test material. Scoring of the observed topical effects is performed at intervals during the test and the animals are observed for systemic effects. At the end of the exposure sequence the animals are killed and complete necropsies are performed. Histologic sections of the skin exposure area as well as of all abnormal tissues are evaluated.

The investigation of possible carcinogenic effects from repeated topical administration of chemical agents involves studies varying in duration from 3 months to 2 years duration. The animals commonly used are white mice, and the test includes both positive and negative control groups of animals plus a minimum of two test groups which are assigned to high- and low-dosage concentrations of the agent. The number of animals in each group is from 25 to 50 males plus an equal number of females, all of which are individually housed. The skin of each animal is shaved over the back of the base of the neck and between the scapulae. The agent is painted or dropped from a pipette on the exposed skin. At each application the volume of material applied is 0.1 ml, and the animals are treated two times weekly

for the duration of the experiment. The animals that survive the experiment as well as those that become ill during the experiment are necropsied. Histopathological examination is made of all tissues as described in the conventional chronic toxicity tests. Special attention is directed to evaluating the animals during the course of the study for the appearance and nature of skin tumors in the animals. These tests are commonly modified to include procedures to detect whether the chemical being tested acts by initiating or promoting the occurrence of tumors.

Cutaneous Sensitization Tests

Topical application techniques for the determination of skin sensitization have been in common use for the past 40 years. The method of Landsteiner (Chapter 9) which involves intradermal injection of the test material in guinea pigs is commonly used, although it does not represent true topical sensitization. The method of Roudabush, which readily differentiates between irritant effect and sensitization, will be described.

White male guinea pigs are used. The hair is clipped from the lower back of each animal, and the agent is applied by placing on the exposed skin 7 drops of a 1.0% or a 0.1 M solution of the agent in a 1:2:7 mixture of guinea pig fat : dioxane : acetone. If the test agent reacts with either the dioxane or the acetone, these solvents are omitted. The fat helps to prevent the mixture from drying out or evaporating. Five or more animals are used in each test group and negative controls are included. One day after the application of the agent the hair stubble is removed with a depilatory and the area is scored for degree of redness and swelling, as described in the acute rabbit test, and is compared to untreated areas and control animals. The readings are repeated 2 days after the initial application following which 10 drops of the test mixture (the sensitizing dose) are applied to the same area on the animals. After an additional 2 days the procedure is repeated without taking readings of effects on the skin. The animals are left untreated for 3 weeks following which the hair is depilated on the right shoulder of each animal and 7 drops of freshly prepared mixture (the challenge dose) are dropped on the skin. On each of the next 2 days readings are taken as before. The numerical scores are averaged for the animals in each group. The scores that were made on the 2 days following the initial application of the test mixture are used as an index of the irritant properties of the agent. The scores that are taken following the challenge dose are compared with the irritant scores. If the challenge scores are two times the irritant scores, the compound is considered a mild sensitizer. If the challenge scores are two to four times the irritant scores, the compound is considered

a moderate sensitizer. Compounds that have high sensitizing activity will show sensitizing scores that are four to seven times the irritant scores.

Phototoxicity and Photoallergy Tests

In both of these types of toxicity the effect follows exposure of the skin to light energy. The light energy involved is that containing the ultraviolet wavelengths. The agent plus the light energy results in excitation of the agent; that is, the basic energy state of the compound is raised to a higher energy level. The excited form is transient and in the process of returning to the original state the released energy results in alteration of the biologic cell. In the phototoxic reaction the energy transfer directly leads to cell damage. In the photoallergy reaction the free energy promotes hapten formation which can result in the appearance of antigen so that true allergic sensitization of the cells can develop. Subsequent exposure of the skin to light results in repeated hapten formation and the resultant antigen–antibody response is the cause of the cell damage.

Much of the available information regarding contact photosensitivity has been obtained from human studies. In such studies the test material is applied to a gauze pad which then is attached by adhesive tape to the skin of the subject. The pads are referred to as patches and the test is called a patch test. The patches are applied in pairs, usually on the lower portion of the back, where they are left in contact with the skin for 1 to 2 days. At the end of this time one of the patches is removed, and the skin beneath the patch is scored for irritant effect and then irradiated with specified amounts of ultraviolet light. One to 2 days later the other patch is removed and comparisons of the dermal effects of the two tests are made according to the scale described above for degrees of erythema and edema. This type of test is used in the clinic to differentiate between irritant and photosensitization responses and to diagnose suspected photosensitization. It also has been used to investigate sensitizing potential of suspected new offensive compounds in humans.

Tests for determining the sensitizing properties of new compounds generally involve initial procedures conducted on guinea pigs. The procedure for a typical guinea pig test is to apply a 2.0% concentration of the test compound in absolute alcohol to the skin on the shaved back of the neck of white guinea pigs. This is repeated for five daily applications; following each exposure the skin is irradiated with ultraviolet light. Each day before the next application, the exposure area is examined for erythema and edema and this information is used as an index of irritant and phototoxic effects. At the end of the application period and after an additional 10 to 14 days the challenge application of 0.1% of the test material in olive oil is applied

to the normal appearing site of previous exposure. The area is again irradiated and skin readings are taken the following day. The degree to which the skin response following the challenge dose plus irradiation is in excess of that observed following only the initial application plus irradiation indicates sensitization of the animals. In such tests the duration and type of irradiation must be limited to prevent excessive erythema, that is, sunburn. Control animals that have been given both the solvent and the same exposure to light are incorporated in the experiment.

Eye Tests

One of the general types of toxicity that can occur following systemic administration of chemical agents is effects on the structure and function of the eyes. Thus the general prolonged and chronic tests that have already been described incorporate in the protocol suitable procedures for examination of the eyes during the course of the experiments. An example of a compound that in small doses will produce dose-related bilateral cataracts in rats and dogs after chronic exposure is the herbicide Diquat. Most compounds that are generally available for human use are tested for irritant effects following topical application of the agents to the eyes (rather than systemically induced eye toxicity) of experimental animals. The Draize procedure for eye irritation is commonly employed for evaluating the irritant capacity of liquids and solids. The procedure is to instill 0.1 ml or less of the liquid or 100 mg or less of the solid in the lower conjunctival sac of one eye in each of at least six rabbits. The eyelids then are held together for 1 sec and released. The eye is not washed at this time and the other eye in each animal serves as the control. The eyes then are examined at 1, 2, and 3 days after exposure to the agent. The eyes may be washed with physiological saline at the end of the first day. The degree of local reaction is assigned a numerical score according to an illustrated guide for grading eye irritation. Copies of the guide are available from the Superintendent of Documents, U.S. Government Printing Office, Washingtion, DC. These guides are color photographs of rabbit eyes that exhibit various degrees of corneal opacity, iritis, and conjunctival effects to which numerical values have been assigned. Each animal is graded separately and any finding regarding the cornea, except for a slight dulling of normal luster, or any obvious inflammation of the iris or the conjunctiva is regarded as a positive reaction. If only one animal exhibits the positive reaction, the test is regarded as negative. If only two or three of the animals show a positive reaction, the test is considered as inconclusive and is repeated using new animals. If four or more of the animals show a positive reaction,

the test is considered as positive. Occasionally the test is repeated three times; in this case if, in the last test, any animal shows a positive effect the compound is regarded as an irritant. All degrees of irritant effect may be obtained with various agents and the scoring procedure assists in documenting the degree of irritant action of the compound on the eye. The nature of the scoring of the Draize test is completely subjective, and this is at least in part the cause of considerable interlaboratory variation in results obtained on any single compound. Objective methodology involves measurement of corneal thickness, the corneal and conjunctival water content, and permeability of the blood vessels in the eye. The latter measurement is done by use of a dye which is injected into the blood stream and which rapidly binds to the blood proteins. Whenever injury to the capillary wall is great enough to allow seepage of the blood proteins containing the bound dye, the dye will be present in the tissues and fluids of the eye in an amount proportional to the degree of capillary damage. Although the objective tests are not necessary for screening programs, they would certainly add quantitative information wherever more definitive tests are desired.

BEHAVIOR TESTS

Some of the more recent additions to the tests for toxicity are those that are designed to demonstrate effects on behavior of animals. Although many drugs have been designed to modify behavior following even single doses, the use of behavior tests in toxicology has developed because of the need to determine these effects following long-term repeated exposure to small concentrations of a large variety of agents that are not drugs and that do not produce detectable effects following a single dose or a few doses of the agent. Some types of behavioral tests can be incorporated into the standard prolonged or chronic toxicity test, since it has been emphasized that animals in these tests should be on a schedule for observation in regard to the detection of physical or behavioral abnormalities. In time, those effects of considerable magnitude should become obvious to the alert animal experimenter. However, the standard prolonged or chronic test is not intended to detect the more subtle type of behavioral toxicity. Rather, both general and specific tests that give reliable and reproducible data have been developed for this latter purpose.

The general tests all involve the quantitative evaluation of "activity" and specifically locomotor types of activity of experimental animals. The amount of movement that an animal displays is determined by many factors,

and in various environments takes the form of a complex sequence of acts of a structured and definable nature. The various locomotor types of behavior tests have been organized according to the environments that are employed in the actual tests. These include (1) the running wheel, (2) the open field, (3) the home cage, and (4) the residential maze.

The running wheel is the oldest test and consists of a small closed compartment that contains the animal and its feed and water. Attached to the compartment is a wheel which is designed so that the animal can enter the inside of the wheel and run along the inside of the rim, whereby the weight of the animal causes the wheel to revolve. The wheel is attached to a counter so that the number and/or the speed of rotation can be recorded. The running wheel technique has been used to record diurnal activity in animals, and it can detect central nervous system stimulant drug-induced effects. It also can detect some types of brain damage when the damage is associated with hyperactivity.

The open field technique consists of an open cage with a flat bottom that is marked off with grid lines. After an animal is placed on the grid, as he crosses from one square to another an observer can record the act, or the grid and floor can be constructed so that the activity will be mechanically or electronically recorded. Basically, the test quantifies the exploratory activity of the animals in an unfamiliar environment; consequently, the test usually involves observation of the animal for a short period of time, perhaps only a few minutes. It is a simple test that has been shown to demonstrate hyperactivity from brain damage in young rodents following exposure to inorganic lead salts.

The home cage environment consists, as the name indicates, of a standard animal cage in which the animal is reared and in which the activity of the animal as he goes about feeding, grooming, and socially interacting (if more than one animal is housed in the cage) is recorded by an observer or by mechanical–electronic recording devices. The residential maze is similar to the home cage environment except that the cage is modified by the addition of partitions that form corridors and rooms. Movement of the animal through the corridors and rooms is recorded by electronic devices. Activity of the animals is less variable in the residential maze than in the home cage and there is no loss in the sensitivity of the test. The test has been used to detect nocturnal hyperactivity in young rats that have been exposed to high concentrations of carbon monoxide. If several animals are tested simultaneously in the same residential maze, the test may include evaluation of agents on social behavior.

The specific tests that are used to measure the effect of chemical agents on behavior vary from the use of the classical operant techniques to tests involving direct measurement of evoked (usually visually evoked) brain

electrical potentials. The operant techniques generally follow the principle that behavior patterns can be manufactured through the use of various rewards or punishments. For example, an animal can be taught to press a lever or respond in some fashion to visual stimuli if the lever-pressing or response to the visual stimulus is associated with a reward, such as a pellet of feed or avoidance of a punishment such as an electrical shock. The shock or the feed are contingencies that create and reinforce the behavior pattern. Such behavior patterns are very stable, but the entire conditioning procedure requires time and effort on the part of the experimenter; thus the test is not readily useful in screening procedures that may involve the use of large numbers of animals. Such tests should be used if it is desirable to measure effects on visual performance, on fine motor control, or even on discrimination. Tests for discrimination are those in which the operant conditioning involves discrimination by the animal between sequential patterns or shapes of lights.

The uses of such tests in toxicology have been limited. The heavy metals, and more particularly mercury and lead salts, and the brain stimulants of the amphetamine type are examples of agents tested by these procedures. In every case, the technique involves first teaching an animal the complex behavioral test, and then quantitating any alteration in the conditioned behavior after the test compound has been administered. Suitable positive and negative controls should be included in the experimental protocol. Recent developments have involved the use of behavioral tests to detect teratologic damage in the form of altered learning ability and altered sensory–motor interactions in the progeny of animals treated with lead salts.

IMMUNE TESTS

The immune system is a multicell organ system comprising granulocytes, macrophages, lymphocytes, mast cells, and soluble mediators. The cells in this system are located in the peripheral blood, lymphatic fluid and organized lymphatic tissues, including bone marrow, spleen, thymus, lymph nodes, tonsils and gut-associated lymphoid tissues. Immune tests therefore involve specific evaluation of xenobiotic induced adverse effects on one or more of these types of cells or organs. In addition much can be learned by appropriate clinical tests and pathologic evaluation in the standard prolonged or chronic test. Immunopathology can be incorporated in any prolonged or chronic test by including spleen, thymus and lymph node weights and histopathology. In fact, because of the structural division of the spleen and lymph nodes into thymus-dependent and thymus-independent compartments, histological examination or immunocytochemical staining may

indicate preferential effects for T or B cells. Likewise, microscopic examination of the thymus may reveal effects on thymocyte viability.

The subtlety of xenobiotic induced alterations on the immune system may not be detected in routine protocols; therefore, several in vitro and in vivo tests have been developed for evaluating cell mediated and humoral immunity. Chemical-induced immune dysfunction includes immunosuppression and sensitization (allergy). Mice and rats are the primary species used in these tests. A list of tests commonly employed to assess immune function is presented in Table 13.4. Two of these tests will be discussed briefly: the mixed lymphocyte response (MLR) assay and the natural killer (NK) cell assay.

Immunopathology generally includes selected organ weights (spleen, thymus, and lymph nodes), histopathology of bone marrow, thymus, spleen, and lymph nodes, and hemogram and spleen cellularity. Because of the structural division of the spleen and lymph nodes into thymus-dependent and thymus-independent compartments, microscopic examination or immunocytochemical staining may indicate preferential effects for T or B cells. Likewise, microscopic examination of the thymus may reveal effects on thymocyte viability.

The mixed lymphocyte response (MLR) assay is a sensitive indicator of chemical induced immuno-suppression. It is a measure of the proliferative response of T lymphocytes in the spleen to surface antigens on allogenic cells. Briefly the assay involves preparing single cell suspensions of splenocytes obtained from the spleens of chemically treated and control mice.

TABLE 13.4 Assays to Assess Immune Function

Mouse	Rat	Nonhuman primate	Human
Surface marker	Surface marker	Surface marker	Surface marker
NK cell activity	NK cell activity	NK cell activity	NK cell activity
Hematology	Hematology	Hematology	Hematology
Lymphoid organ weight	Lymphoid organ weight	—	—
Primary antibody response	Primary antibody response	Serum Ig	Serum Ig
CTL or DHR	CTL	—	DHR
MLR and mitogen assays	MLR and mitogen assays	Mitogen assays	Mitogen assays
Host resistance	Host resistance	—	—

Note. NK, natural killer; CTC, cytotoxic T lymphocyte; Ig, immunoglobulin; MLR, mixed lymphocyte response; DHR, delayed hypersensitivity response.

The suspensions are made in a specific tissue culture medium and are called the "responder" populations of cells. An additional suspension of splenocytes from the DABA/2 strain of mice is made to which is added mitomycin-C (MMC) which inactivates the cells. These inactivated cells are then washed free of the MMC and resuspended in the culture medium. The final suspension is referred to as the "stimulator" population of cells. A specific number of stimulator cells are then added to a specific number of responder cells in each of the wells in a 96-well tissue culture-treated plate which is then incubated under 5% carbon dioxide for 5 days. Eighteen hours before the end of the incubation period each well is treated with tritium (as 3H TdR). At the end of the incubation period the cells from all wells are harvested and the incorporation of tritium into the cells is determined in a beta-scintillation counter. The mean counts from the population of responder cells with stimulator cells minus the mean counts from the population of responder cells without stimulator cells are determined. The MRL response is then represented by comparing control groups with chemically exposed groups.

Natural killer (NK) cells are lymphoid cells that are capable of lysing some types of tumor cells. These cells play a major role in inhibiting the growth of primary tumors and the development of metastases. In addition NK cells may be involved in the control of immune regulation of microbial infections. The NK cell assay is a procedure which is designed to estimate the lytic activity of single cell suspensions prepared from lymphoid tissue usually obtained from mice.

The procedure for conducting the NK cell assay is similar to that of the MLR assay. The target cells are from the YAK-1 cell line. These cells are radiolabeled with sodium chromate-51 and are suspended in isotope-free nutrient solution. This suspension of target cells is added to the wells in a tissue culture plate which contain a suspension of unlabeled effector cells. The effector cells are prepared as a single cell suspension from the appropriate tissue from control mice or mice that have been pretreated with a xenobiotic agent. After a 4-hr incubation period the amount of chromate-51 liberated into the nutrient solution is determined by a suitable isotope counting technique. Appropriate controls are included in each assay so that specific cell lysis due to killer-cell activity can be determined with the formula.

$$(\%)\text{ specific cytolysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

Many variations on the types of tests described in this chapter have been reported. Laboratories that perform these tests on a commercial scale acquire considerable expertise in conducting the tests and they frequently

apply simple but important techniques that may be known only in that facility. Experienced personnel that work with the animals become the real experts in conducting the tests, such that sometimes they are able to sense an illness in any of their animal patients before the clinical tests become positive. Accurate and detailed accounts of all observations made during the course of the experiment supply valuable leads regarding effects that may not have been anticipated at the initiation of a study. Thus there is an art involved in the conducting of toxicological tests that is acquired through actually doing the tests; this aspect of the subject cannot be described in a book. Any investigator who is entering an area of toxicological testing should consult the original articles describing the procedures. The investigator also would benefit immeasurably by visiting laboratories that are conducting the type of tests of interest.

In addition to guidelines concerning the design of a toxicology study, many U.S. and international regulatory agencies have issued regulations governing the manner in which nonclinical hazard assessment studies are to be conducted, documented, and reported. These Good Laboratory Practice regulations (GLPs) are intended to assure the quality of the data generated and reported for nonclinical studies. The regulations require that the studies be conducted according to written protocols and standard operating procedures. Analytical chemistry studies are required to characterize the test and control materials. Study procedures and data must be clearly and completely documented and reported. In order to assure compliance, the GLPs provide for disqualification of laboratories that do not comply with the regulations. Data from the laboratory that has been disqualified will not be used by the regulatory agencies during the safety assessment of a chemical. Therefore, laboratories conducting studies for regulatory submission must take great care to comply with the appropriate GLP regulations.

CHAPTER 14
Clinical Toxicology

Clinical toxicology is primarily associated with two areas in toxicology which apply to clinical medicine. The first involves the documentation, diagnosis, and treatment of harmful effects of chemicals on humans. The second involves the acquisition and interpretation of epidemiologic data and the estimation of risk associated with exposures of human populations to chemicals of all types.

DOCUMENTATION OF CHEMICAL-INDUCED ILLNESS AND DEATH

Documentation of causation in deaths as well as less severe toxicities when chemicals are believed to be involved is the element of toxicology commonly referred to as Forensic Toxicology. It utilizes the data obtained from pathologic facilities and analytical chemical laboratories. The pathology facilities identify the gross and histologic evidence obtainable from individual cases. The analytical chemical laboratories analyze blood and tissue samples for the presence of chemicals suspected as being involved and supply information on the clinical significance or lack of significance of the results obtained. When the pathologic and laboratory findings are consistent with the known effects of the suspected chemical(s), this information is used to establish causation. In the case of illness, such data help to

define the nature of therapy that would be used. In the case of death such data are the main reliable documentation for statistics on chemical-induced deaths. However, it must be recognized that a chemical may be the cause of a cancer which is eventually lethal, in which case the relationship between chemical causation and death cannot be established by the forensic laboratory. In this latter situation the causal relationship between exposure to the chemical and death or other delayed toxicity can be estimated only by epidemiologic procedures. Such procedures include case reports, in which clusters of cases appear to be associated with specific occupations, habits, living conditions, etc., proportionate mortality studies, in which the proportion of deaths in a study group is compared with the general, nationwide proportion of the same type of deaths, and cohort studies, in which the exposed study group is compared with a similar unexposed group in the same facility. By such procedures the risk of occurrence of a disease or death associated with exposure to each chemical is estimated. When the causation of chemical-induced harm is established, the estimation of risk that is taken by exposure to various amounts of the chemical can be determined. This subject of risk is considered later in this chapter.

The sources of documentation of the role that chemicals play in clinical medicine are medical records and death certificates. Accuracy of these sources becomes very important when those data are used for statistical, legal, or regulatory purposes. Incorrect interpretation of these data, whether by the authors or the public media, can result in unsupported claims regarding chemical causation of illness and death in humans. In order to improve the accuracy of such documentation, illnesses and deaths should only be recorded as being chemical-related when there is reasonable proof of the relationship. Such proof includes supporting chemical analytical data when it is appropriate and exclusion of other possible causes of the illness or death.

DIAGNOSIS OF ILLNESS

The diagnosis of illness associated with exposure to chemicals follows the same procedures used for the diagnosis of any illness except that it requires that the clinician familiarize himself with the effects of the suspected chemical(s) on humans. This is a difficult task because, except for drugs and some very commonly encountered xenobiotic agents such as alcohol and carbon monoxide, it is common to find very little direct quantitative information about the harmful effects of chemicals on man. Most of the detailed information is based on experimental programs conducted in animals, in which case the clinician frequently relies on the extrapolation

of the animal data to man. This is a valid procedure since it is generally accepted that effects observed in suitable experiments in animals will occur in man. Conversely, effects that occur in man can usually be demonstrated in the experimental animal.

The clinician who familiarizes himself with toxicology may establish a diagnosis regarding causation of a suspected chemical-induced illness by the following steps. First, regarding exposure, establish that exposure to the suspected chemical did, in fact, take place. This is done by defining the parameters of the exposure, including sources, routes, concentrations involved, frequency of exposure, and chemical analytical information available. Second, regarding the nature of the illness, evaluate the nature and extent of the signs and symptoms as described by the patient and determined by physical and laboratory diagnosis. If these are not demonstrable at the time of examination, evaluate the extent to which they have been supported by qualified medical personnel on some prior occasion. Third, regarding temporal relations, from a detailed medical history, outline the time relationships involved in the onset and duration of the illness in relation to exposure to the chemical. Review all medical records from current and prior physician visits and hospitalizations. Fourth, regarding literature information, review the clinical and toxicologic literature on the suspected chemical(s). Evaluate the consistency or lack of consistency of the literature relevant to the patient's clinical effects. Apply weight to the extent that the literature is adequate and appropriate for use in the current case. Fifth, regarding other possible causes, evaluate concomitant and preexisting illnesses from other causes including their sequel and their possible relation to the current illness or complaints. Determine other chemicals possibly involved including other environments and industrial exposures. From the foregoing five steps, make a probability conclusion regarding causation of the illness in the current case.

TREATMENT

The treatment of chemical-induced illness depends upon whether the illness is due to an acute overdose or accumulation of the chemical which is present in the patient at the time the diagnosis is made. Under these conditions treatment is directed toward decreasing the body load of the causative agent while maintaining adequate vital functions (respiration and cardiac function). The various methods of decreasing the body load of the chemical are considered in Chapter 11 on antidotal agents. In some cases where the antidotal therapy may be hazardous to the well being of the patient, particularly when the causative agent may have a very short half-

life in the patient, it may be prudent to simply support the vital signs and avoid the use of antidotal agents. When the illness is a consequence of prior exposure to a causative agent that may no longer be present in the patient, it is treated according to appropriately accepted practice as if no chemicals were involved. For example, gastric ulceration from aspirin is treated as ulceration from other nonchemical causes, and kidney failure from carbon tetrachloride is treated as kidney failure from other nonchemical causes. In all cases treatment of chemical-induced illness is directed toward the production of minimal additional insult to the patient and maximal recovery rate.

RISK ASSESSMENT

Risk assessment in toxicology refers to the estimation of the probability of occurrence of a harmful effect(s) resulting from exposure to a chemical agent. If complete dose–response data on humans were available for a given chemical, then the estimation of the risk involved with exposure to that chemical would be simple and direct. However, in most cases the amount of toxicologic data obtained directly from humans is very minimal and the clinician depends on other sources of data which can be suitably extrapolated to man.

When risk estimation is based on extrapolation of data from animal experiments to humans, it is performed by making two assumptions. One is that all toxicities with the exception of mutagenesis and carcinogenesis occur only when the body load of the agent exceeds a "threshold" level. This is an assumption that is consistent with most animal experimental data. The second assumption is that in the case of mutagenesis or carcinogenesis the dose–response relation is linear to a virtual zero dose; that is no threshold is involved. When tumorigenicity or mutagenicity is the toxicity of concern, risk estimation is commonly made for state or federal regulation purposes. This is done by using data from mandated, long-term studies in specified strains of rats and/or mice. Such studies are commonly referred to as lifetime studies in rodents, in which the chemical is fed to the animals in their diet or water or is incorporated in the atmosphere 5 days each week for 2 or more years. The data that are obtained are then applied to models for extrapolation outside the range of the actual data, assuming no threshold is involved. In this manner risk associated with exposures to doses well below possible experimental dose levels are mathematically estimated. Figure 14.1 shows these concepts in graphic form.

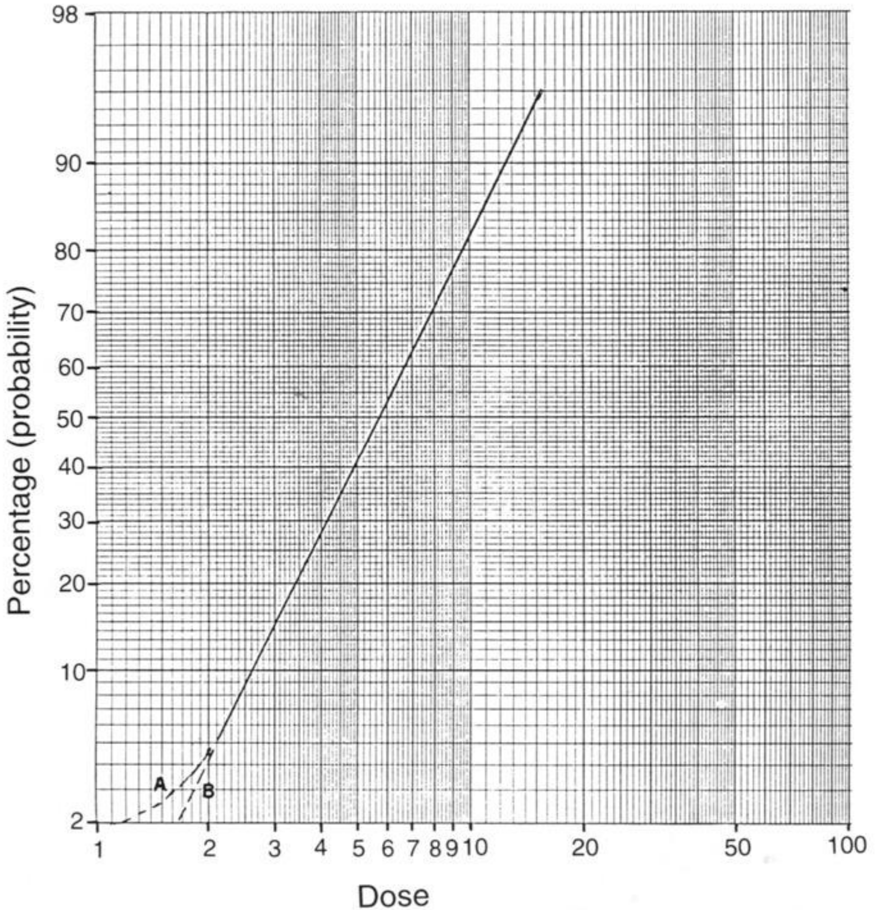

FIGURE 14.1 Dose–probability plot of the occurrence of a hazard (i.e., death or illness) for a hypothetical chemical. Solid line represents the best fitting line for the acquired data. A, low-dose extrapolation under zero threshold conditions. B, low-dose extrapolation under threshold conditions.

It is probably not very important as far as the overall estimation of risk is concerned whether or not the concept of thresholds exists for certain toxicities. This is because extrapolation of data is used only to estimate risks that are infinitesimally small, that cannot be scientifically verified, and have no practical use except for regulatory and legal purposes. Risk estimation for regulatory purposes enables the regulatory agency to estimate a lifetime dose of each chemical that the agency will

then mandate as being safe. Such a safe dose of a carcinogenic substance for man is usually a level which is estimated to cause one cancer in 100,000 to 1,000,000 exposed humans above the normal background risk of cancer.

HAZARD VERSUS RISK

There are at least five different ways through which direct data on the harmful effects of chemicals on humans become available. First, chemicals that are intentionally given to man, such as therapeutic drugs, eventually produce direct evidence of dose–response relations not only for therapeutic effects but also for harmful effects. In fact, the nature and severity of harmful side actions of each drug are usually the determining factors regarding whether the drug can continue to be used in humans. Good examples are the teratology produced by thalidomide and the damage to the eighth cranial nerve produced by specific antibiotic drugs. Second, certain chemicals are abusively used under either intentional or accidental conditions, in which case dose–response relations for lethal as well as sublethal acute and chronic toxicities become recognized. Good examples of these types of toxicities are the liver damage from chronic abusive use of ethyl alcohol and the optic nerve damage from accidental use of methyl alcohol. Third, the unintentional, incidental exposure of humans to chemicals in the workplace, home, or recreational environment have resulted in various mild to severe chemical-induced toxicities. Some examples are the acute depressant effects of trichlorethylene on the central nervous system, the vinyl chloride monomer-induced carcinoma of the liver, and asbestos-induced lung tumors. The fourth way by which data on chemical effects become available is through the catastrophic accidents that periodically occur, in which large populations are unintentionally exposed to chemicals that are recognized for their toxicity. A list of such catastrophies is given in Table 1.1. Finally, in the early and mid-nineteenth century, limited, controlled experiments were intentionally conducted on humans for the purpose of demonstrating dose–response relations for some minor toxicities such as skin irritation.

As indicated above, the principal source of information regarding the harmful effects of most chemicals on man is through animal experiments, in which case conclusions of effects on man are by inference. It is generally accepted in toxicology that properly conducted experiments conducted on suitable animals produce data that are generally applicable to man. In the absence of direct data on humans, it is not acceptable to assume that effects observed in animals will not occur in humans. The process of extrapolation of animal data to man for the purpose of determining a dose–response

relation basically involves correction of the data for differences in size, i.e., body weight or surface area, in the two species and incorporation of a safety factor. Various mathematical models have been devised to aid in this procedure. Only in recent years has a significant effort been directed toward the development of *in vitro* toxicity test protocols as substitutes for the animal tests.

INTERRELATIONS OF HAZARD, RISK, SAFETY, AND BENEFIT

In toxicology a hazard is a harmful biologic effect. Various chemicals by virtue of their structure possess the ability to produce specific hazards when introduced into biologic systems under specified conditions. The study of these structure–activity relations is an important part of toxicology. Risk, however, is the probability that a hazard will occur when humans are exposed to the agent. All chemicals possess hazards, hence all chemicals present risks when used by humans. Safety is a probability evaluation of the assurance that humans can be exposed to a specific chemical without the occurrence of significant hazards. A benefit associated with a chemical is presumed to be the reason for the existence of the chemical. That is, a chemical exists (such as a naturally occurring agent) or comes into being (usually as a result of industrial efforts), its hazards are defined, the risks are estimated, and the safety is evaluated. If there are no benefits, real or virtual, then it is unlikely that there will be exposure of humans or a need for an estimation of the order of safety of the compound. The final determination of the order of safety of each chemical will be a function of the type of hazards involved, the extent of risk encountered, and the nature of the benefits to humans that accompany the existence of the chemical. Each of the above factors can have a range of significance in arriving at a conclusion about the safety of a compound. For convenience that range of significance can be perceived as being of great (high) importance or of little (low) importance in regard to making a conclusion about safety.

Hazards of high importance are such effects as carcinogenesis and mutagenesis, because such effects, once induced, are irreversible and are presumed not to have a threshold below which the risk disappears. Safety in this case can only be defined in terms of acceptance of an arbitrary level of risk. Hazards of low importance are all other forms of toxicity, because unless they are overwhelming they are reversible and because they have a threshold below which risk disappears, in which case safety is simply a function of dosage. However, in the former example the arbitrary level of acceptable risk is tantamount to acknowledgement of a threshold even

though it may be represented by an extremely low risk (such as 1 in 10^{-5} or 1 in 10^{-6}) that cannot be experimentally demonstrated. Next, if the benefits are sufficiently high even though the hazards and risks are also high the chemical may still be used in some circumstances. For example, many of the chemotherapeutic agents commonly used in the treatment of cancer are also known to be carcinogenic. The philosophy here is that the benefits of using these compounds exceed the risk that they will produce additional cancer.

In clinical toxicology the adage may well be that no chemical is harmful if it is handled properly and no chemical is safe if it is not handled properly. Proper handling determines the exposure dose, and therefore the degree of safety involved in any scenario.

CHAPTER 15
Information Sources in Toxicology

The ultimate sources of information in toxicology are the same as they are in any science. They are the scientific data created throughout the world under laboratory or field conditions which are interpreted and reported by the original investigators in printed form in scientific journals. These reports constitute the primary journal literature. It is important to recognize that all other printed sources of toxicologic information such as textbooks, special reports, monographs, and handbooks, as well as the electronic databases, do not create scientific information; rather, they are mechanisms for locating, referencing, organizing, systematizing, condensing, abstracting, or reviewing the primary journal literature. Each is therefore, in a sense, a product that involves a third party. As such, each is subject to the third party errors of omission and commission. Consequently, the serious student as well as the established investigator who is seeking specific scientific information in toxicology should ultimately direct his investigation to locating and reading the original sources in the primary journal literature. This is frequently initiated by a search procedure which is greatly facilitated by third party printed resources and by electronic databases.

Although toxicology has its own specific literature sources, since it is such a diverse science it borrows freely from a host of other sciences which

are the origins of data and concepts relevant to toxicology. This subject has been discussed in the introductory chapter of this book. Consequently, the information sources for toxicology many times involve disciplines with which an investigator may have only a superficial acquaintance. For example, issues on poisoning from drugs are directly identified with emergency medicine and pharmacology as well as toxicology; also, whereas groundwater contamination from hazardous wastes may be identified with geology and engineering, it is also identified with toxicology. Hence, the overall base of published toxicologic information becomes exceedingly broad and perhaps even limitless. This condition makes it unlikely that any single comprehensive text of toxicology could be created that would embody more than a fraction of the composite literature on the subject. The past 30 years have seen an explosion in the volume of toxicologic information and it appears to continue to expand.

Information sources in toxicology have become so abundant and all-inclusive that some information can be obtained on the toxicity of almost every chemical available to man. The nature of the information desired largely determines the most likely sources of the answer. For example, information on the clinical effects of chemicals commonly used in industry would probably be available in symposia or texts on industrial and occupational medicine. If the effects of a common drug are being sought, the search should begin with the comprehensive pharmacology texts that are available. If the search involves information on an uncommon chemical, it may be more expedient to initiate the search through computer database systems. For general information, excellent textbooks on the subject of toxicology are readily available.

Whenever a search of the literature is conducted and a variety of information has been obtained on a specific chemical agent, the investigator will become aware of apparent conflicts in opinions or conclusions. This condition is confusing to a new investigator. Usually the original sources of data as reported do not conflict; rather, it is due to interpretation and extrapolation of data without proper precautions. A fundamental fact in experimental science is that no properly conducted scientific experiment yields erroneous data; however, it is not uncommon to encounter conclusions that go beyond or are only superficially supported by the data.

The book *Information Resources in Toxicology,* edited by Philip Wexler (2nd edition, Elsevier Publishing Co., 1988, 510 pages) essentially contains worldwide coverage of the subject. Part 1 of that book contains a brief discussion of the history of toxicology and toxicology information systems. The book then identifies other books, special documents, and many principal journal articles and newsletters. This is followed by identification of the electronic information sources, information handling, legislative, regula-

tory, and compliance issues in the United States. Part 2 gives international sources of information in toxicology, from 17 countries throughout the world.

In addition to the above book, continuously updated reference sources of toxicology information such as the Information Industry Directory (published by Gale Research, Inc.), which is updated annually, are very useful. This directory is an international guide to organizations, systems, and services involved in the production and distribution of information in electronic form. It is interdisciplinary in scope and thereby helps resolve a basic problem in locating toxicologic information.

Since this is an introductory text the remainder of this chapter will simply recommend a very limited list of reference texts which may be considered as additional reading in the general field of toxicology, followed by a list and brief description of the most common computer database systems.

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DATABASE SYSTEMS

TOXLINE (Toxicology Information Online). Produced by the Toxicology Information Program of the National Library of Medicine, National Institutes of Health, Bethesda, MD. It consists of a collection of bibliographic files containing three million citations on virtually all aspects of toxicology. The bibliographic citations, of which a great many contain abstracts, are from four major secondary sources and 12 special collections. The four secondary sources are MEDLINE (Medical Information Online), BIOSIS, International Pharmaceutical Abstracts, and Toxicological Aspects of Environmental Health. The special literature collections include the Environmental Mutagen Information Center File, Environmental Teratology Information File, Epidemiology Information System File, Aneuploidy File, Hazardous Materials Technical Center File, International Labour Office File, two subfiles of Toxicology Research Projects, Toxicology Document and Data Depository, Toxic Substances Control Act Test Submissions, Poisonous Plants Bibliography, and National Institute for Occupational Safety and Health Technical Information Center database. TOXLINE is updated monthly and is available online through MEDLARS (Medical Literature Analysis and Retrieval System). It is also available on CD-ROM from private companies.

TOXLIT (Toxicology Literature) is a National Library of Medicine database containing bibliographic citations, primarily in English, which at the present time are exclusively from *Chemical Abstracts.* It contains about two million citations. It consists of two parts: TOXLIT65 contains citations from 1965 to 1980 and TOXLIT FILE contains citations from 1980 to the present. It is updated monthly.

TOXNET (Toxicology Data Network) is produced by the Toxicology Information Program of the National Library of Medicine. It contains factual data banks. Files available include a Hazardous Substances Data

Bank (HSDB), a Registry of Toxic Effects of Chemical Substances (RTECS), a Chemical Carcinogenesis Research Information System (CCRIS), an Environmental Teratology Information Center Backfile (ETICBACK), an Environmental Mutagenesis Information Center Backfile (EMICBACK), a Directory of Biotechnology Information Resources (DBIR), a Toxic Chemical Release Inventory (TRI), a Developmental And Reproductive Toxicology file (DART), a Genetic Toxicology data base (GENETOX), and an Integrated Risk Information System (IRIS). TOXNET also permits the user to create and maintain chemical records online, and allows interactive review and editing of data records. It has electronic mail capability and is available to National Library of Medicine MEDLARS system users.

POISINDEX is a corporate product developed by Micromedex, Inc. (Denver, CO) together with the Rocky Mountain Poison and Drug Center and the University of Colorado Health Sciences Center. It is available on CD-ROM for personal computer use and on tape for mainframe systems. It is an information retrieval system that provides identification and ingredient information on approximately 750,000 products or substances including drugs, commercial products, plants, venoms, and mushrooms. It also contains approximately 850 clinical treatment protocols. It is available by subscription.

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