PURDUE PESTICIDE PROGRAMS

Purdue University Cooperative Extension Service

PESTICIDE TOXICOLOGY
Evaluating Safety and Risk

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Public Debate About Pesticides and Human Health

The public perceives "pesticides" as a unique class of chemicals more "dangerous" than chemicals in prescription and over-the-counter medications and more "toxic" than chemicals that occur naturally in food and the environment. Actually, pesticides are *intentionally designed* to be toxic to plant, animal, or microbial pests—e.g., weeds, rodents, bacteria, insects, fungi—just as antibiotic drugs are *intentionally designed* to be toxic to specific disease bacteria. Many natural chemicals in our food supply can also be toxic to living organisms.

The private sector, academia, and regulatory agencies worldwide combine expertise to assess pesticide safety and risk potential. Specialists in toxicology, human and veterinary medicine, epidemiology, chemistry and biochemistry, statistics, physiology, anatomy, pathology, and molecular biology evaluate numerous studies and other information. Conclusions drawn by these scientific communities represent their best professional judgment based on many years of education, research, and experience. It is impossible to test or prove safety under every imaginable scenario. However, the overall testing program is comprehensive; and it examines the responses to pesticide levels much higher than man or animal would normally encounter.

The public is expected to place confidence in scientific and regulatory professionals, yet as a society we are ill-informed on the mandatory, comprehensive evaluation process that precedes the registration of every pesticide product. Innumerable media discussions center on the impact of pesticide residues in food, air, or water on past, present, or future human diseases and disorders. And without the necessary tool—that is, knowledge—to sort out the media hype, we have no basis on which to judge the validity of what we hear and read. Our lack of knowledge, coupled with the misperception of pesticides as an exceptionally dangerous class of chemicals, provides fertile ground for debate among groups with conflicting opinions on the benefits and risks of pesticide use.

The debate is polarized, contentious, and speculative. The underlying issues center on how "safe" or "dangerous" pesticides are. That's it. That's what the

public wants to know, once and for all: Just tell us whether or not the benefits of pesticide use justify any risks their use might pose! But it's not that easy.

There exists a common misperception that pesticides can be classified as "safe" or "not safe." But no chemical, either natural (produced by plants or other organisms) or synthetic (produced by man), can be determined completely safe. Researchers, state and federal regulators, manufacturers, and public health officials continually grapple with the possibility that pesticides might contribute to the development of human disease or trigger adverse environmental effects. The effort to develop conclusive evidence of safety is ongoing, but absolute safety can never be guaranteed. The U.S. Environmental Protection Agency (EPA) regulates the pesticide product registration process and mandates studies to determine the conditions under which pesticides can be used with minimal risk and maximum benefit. The findings dictate the conditions under which the product can be marketed.

The public forum often is slanted by an incomplete understanding of the principles of toxicology and the pesticide registration process. Addressing public concern requires comprehension of the science and the regulatory system; and appreciation for the judgments and predictions derived from scientific data is essential. This publication is intended to foster a better understanding of the role of toxicology in the safety evaluation and registration processes.

The Science of Toxicology

Toxicology is the scientific study of the harmful effects of chemicals on living organisms: humans, animals, and plants. Toxicological testing evaluates whether short-term exposure to a pesticide will produce acute effects (e.g., eye and skin irritation, death) and whether long-term, continual exposure will cause chronic effects (e.g., impaired liver function, reproductive abnormalities, cancer).

Toxicological evaluations are conducted with experimental animals exposed to various levels of the pesticide for various lengths of time, from hours to years. Results often lead investigators to additional research on the interaction of the pesticide with biological systems. Understanding the biological mechanisms that underlie effects observed in animals allows toxi-

cologists and risk assessors to predict the chances of harm to human populations exposed to the pesticide.

Consideration of exposure levels and effects produced at specific doses is essential in determining toxicity. Exposure, in and of itself, does not necessarily produce harmful effects; for instance, people who are exposed to low levels of pesticides in their food or drinking water, or through contact at the workplace, usually suffer no harm. But harm is to be expected when people are exposed, accidentally or otherwise, to much higher levels that have been shown to produce adverse health effects in laboratory animals.

The dose/response concept is familiar: High doses are likely to produce detectable injury, while low doses may produce little or no injury. For example, ingesting one or two sleeping tablets might have a beneficial effect, while consuming a bottle of them could be lethal. One glass of beer may not affect an adult, but the same amount could easily intoxicate a child. A little salt improves the taste of food, but a lot could cause serious health consequences—even death.

Toxicologists follow the basic premise that all chemicals, both natural and synthetic, can prove toxic at some dose. Those such as table salt cause adverse effects only at high doses, while others (e.g., cyanide) exhibit toxicity at a very low dose. When toxicologists determine the gradation of effects resulting from increasing doses of a pesticide, they have established a dose-response for that pesticide.

The duration and magnitude of *exposure* determine the severity of the poisoning. In other words, the increment of time during which exposure to the dose occurs (duration), plus the size and number of doses (magnitude) combine to determine the severity of the poisoning. Although the terms *dose* and *exposure* sometimes are used interchangeably, they are not synonymous. Example: A *dose* of oral medicine is prescribed by the doctor, but the patient is not *exposed* to the medication unless and until he swallows the *dose.* A pesticide will trigger an adverse response when a person is exposed *long enough* to a dose *large enough* to cause harm.

Pesticide and Animal Interaction

It is important when investigating the toxicology of a pesticide to understand the effects of the chemical on the animals—and vice versa. The genetic, physiologi-

cal, anatomical, and biochemical variability among and within animal species helps scientists understand why a pesticide may be highly toxic to rats but nontoxic to dogs or people, for example, or why another is toxic to rats and mice but not to fish or birds.

Pesticides cannot be categorized as "safe" or "dangerous" to humans merely because they are classified as substances that kill pests. Each active ingredient has its own unique chemical structure and toxicological characteristics. Pesticides with very similar chemical structures in many instances produce dramatically different effects. One chemical may generate a highly toxic effect while another may exhibit no toxicity whatsoever to the same animal at the same dose. Laboratory studies are useful in predicting and explaining the toxicity of a pesticide.

Effect of the Chemical on the Animal

Effects on animals are determined by the chemical structure of the pesticide, its action mechanism, and the fate of the chemical within the animal. Not all animals react to all pesticides in the same manner; and response can be species- or individual-specific. The system of one animal species may metabolize a pesticide to a nontoxic metabolite, whereas that of another species may not (species-specific response); and individual animals of a species also can respond differently (individual-specific response).

Species-Specific

Pesticidal effects often vary with the species of animals studied; for instance, one species may exhibit skin irritation and another, liver disease. The degree of sensitivity may vary, as well; two species may react dermally, for example, with one exhibiting severe sensitivity compared to the other's mild skin irritation.

Individual-Specific

Individual animals within a species can exhibit dissimilar responses to the same pesticide. Toxic effects can vary with the size, sex, age, and general health of the test animals.

Effect of the Animal on the Chemical

An animal's response to a pesticide may hinge on how its internal systems "process" the chemical.

Evaluation of the effect of test animals on the pesticide involves consideration of how readily the chemical is absorbed into the animals' system and how the system distributes, breaks down, and eventually eliminates the pesticide. Animals whose systems retain a pesticide for a long time may exhibit effects not seen in animals whose systems eliminate the chemical more rapidly. In some cases, a chemical may appear toxic only after being converted by an animal's system into a more reactive form.

Test animals whose skin does not readily absorb a given pesticide may exhibit only minimal effects, but the same animals might react seriously when the contaminant is administered orally or through inhalation.

The fate of a pesticide from its point of contact with and elimination from the body of laboratory animals is studied to gain an understanding of how humans might respond to the same chemical. A pesticide may gain access to the circulatory system through dermal, inhalation, or oral exposure and subsequently be carried in blood from the point of entry to various organs and tissues. The body's physiological processes may store or excrete the toxin or metabolize (alter) its structure, thus modifying its toxicological effects. Body tissue might absorb the pesticide from the blood and store it, or it might release the contaminant back into the bloodstream for elimination in urine or feces, or through exhalation.

These processes are complex and interactive. The mechanisms by which biological systems handle pesticides determine whether toxic effects will be observed. Following are biological and physiological factors that influence the interaction between pesticides and humans.

Chemical Properties

Each pesticide is unique; pesticide molecules differ in their chemical structure, size, shape, stability, and electronic charge. These differences determine how pesticides function in the human body. For example, the chemical structures of some pesticides make them fat soluble, while those of others dictate water solubil-

ity; the significance is that the former would be stored for various lengths of time in fatty tissue, while the latter might be rapidly eliminated through urination. The shape and electronic charge of some pesticide molecules allow binding to critical site receptors in cells or on cell membranes. These and other variables often lead to significant differences in biological response to pesticides.
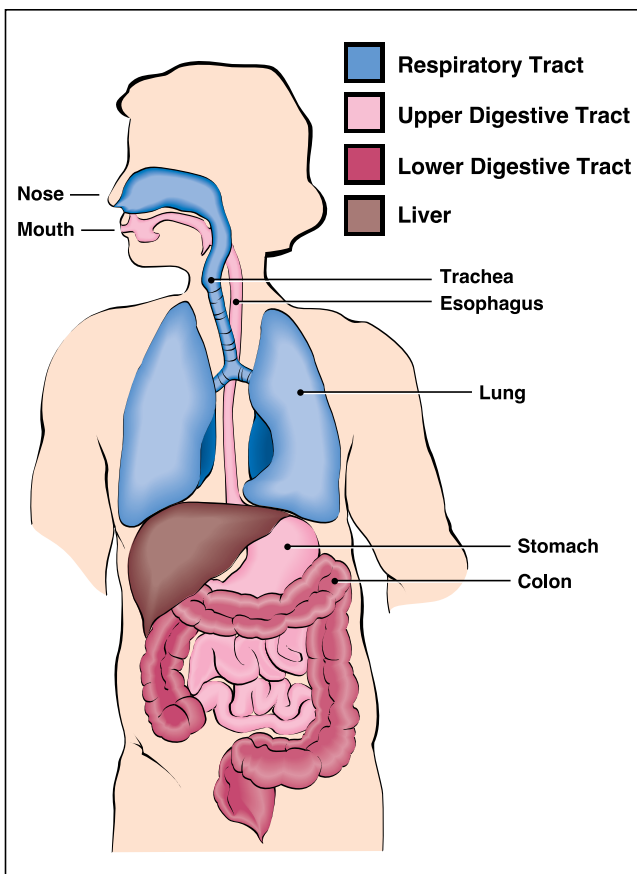
Absorption Into the Body

Pesticides may enter the human body through the skin (dermal exposure), mouth (oral exposure), and lungs (respiratory exposure). The site of exposure to the pesticide impacts the rate of absorption into the bloodstream, as well as its distribution pattern.

Skin is a natural barrier to many pesticides, but penetration can occur, especially if the skin is disrupted by cuts and abrasions.

Each region of the gastrointestinal (GI) tract—mouth, esophagus, stomach, small and large intestines, colon, and rectum—has its own internal environment that dramatically affects the absorption of a pesticide. Some compounds readily absorb in the mouth, while others are absorbed only in the intestines. Some pesticides simply pass through the organism (man or animal) without being absorbed at all.

Pesticides inhaled into the lungs need only to cross the thin barrier separating lung tissue from the blood supply to gain rapid access into the bloodstream.

Respiratory Tract

Upper Digestive Tract

Lower Digestive Tract

Liver

Nose
Mouth
Trachea
Esophagus
Lung
Stomach
Colon

Movement Within the Bloodstream

The transport of a pesticide within the body depends on whether the pesticide is absorbed through the skin, lungs, or GI tract. Pesticides absorbed in the GI tract enter the bloodstream flowing directly to the liver—the major site of pesticide metabolism—where they are broken down soon after absorption. Those that are absorbed into the bloodstream through the skin or through inhalation to the lungs actually circulate throughout the body before reaching the liver to be broken down. Therefore, the same pesticide dose may be more toxic through inhalation than ingestion.

Uptake by Organs, Tissues, and Cells

Blood is the medium through which the pesticide is transported to organs, tissues, and cells. Uptake by cells is dependent on a pesticide's physical and chemical properties and the type of cell involved. Therefore, different pesticides may be distributed to different tissues in the body. Pesticides can enter a tissue passively by simple diffusion; that is, the pesticide can move from a high concentration in blood to a lower concentration in body tissue. Pesticides may be actively transported by reactions that use energy to "pull" the chemical into the cell.

Metabolism Within Cells

Pesticides are subjected to chemical alterations by enzymes in the body via a process known as metabolism. Metabolism refers to chemical reactions that alter the structure and the physiochemical properties of the pesticide by adding, removing, or substituting various chemical components.

Metabolism takes place primarily in the liver, where cells usually change the original pesticide molecule to a less toxic, more water soluble form which makes the chemical easier to excrete. However, some molecules are converted to more toxic forms.

Pesticide Storage Sites Within the Body

Pesticides may accumulate in body tissues, proteins, fat, and bone. It is fat-soluble pesticides, primarily, that are stored in the body for long periods of time. The depletion of body fat can release them into the bloodstream.
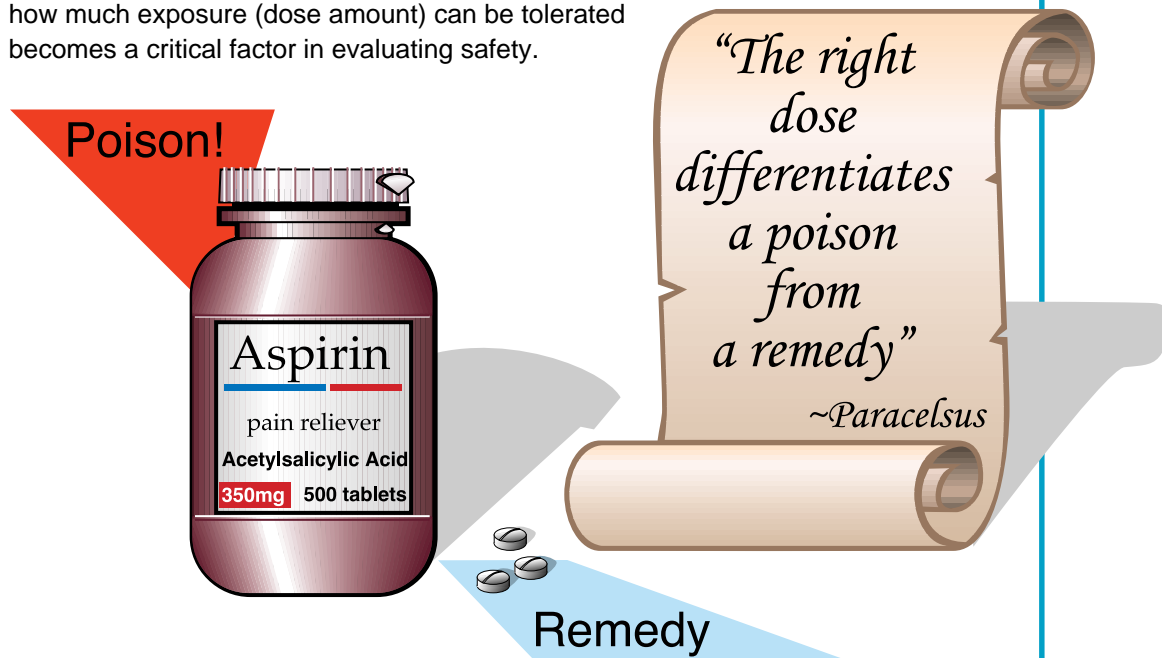
Metabolized chemicals in the body ultimately are eliminated by the kidneys, in urine, or carried to the intestine in bile from the liver. Pesticides may be reabsorbed into the bloodstream from the intestinal tract or excreted in fecal material. Pesticides also can be eliminated through the lungs in expired air or in body secretions such as tears, saliva, and milk.

The Relationship Between Dose and Response

The Swiss physician Paracelsus (1493-1541), the father of toxicology, believed the relationship between dose and response to be inseparable. Paracelsus asked, "What is it that is not poison? All things are poison and nothing is without poison. The right dose differentiates a poison and a remedy."

These principles, having withstood the test of time, serve as the basis of toxicology. We all have some understanding of this basic tenet. For instance, two aspirins may remedy a headache, but a much larger dose—a whole bottle of aspirin—may be poisonous. Therefore, the question of how much exposure (dose amount) can be tolerated becomes a critical factor in evaluating safety.

Poison!

Aspirin

pain reliever

Acetylsalicylic Acid

350mg **500 tablets**

Remedy

"The right dose differentiates a poison from a remedy"

~Paracelsus

It is important to discuss sensitivity differences among individuals of a single species. Just as people do not look alike, they also do not respond in exactly

the same manner to medicines, pesticides, or other chemicals. We know, for example, that penicillin is a life saving antibiotic for most people; however, some hypersensitive individuals may exhibit serious side effects from it. Certain individuals become intoxicated after a few alcoholic drinks, while others can consume considerable quantities of the same drink without adverse effects.

People and all living organisms exhibit a broad spectrum of reactions and sensitivities to chemicals. Some people are very resistant to high doses while others are very susceptible even to low doses; most individuals are somewhere between very resistant and very susceptible. In fact, when the sensitivities of a large number of people (or rats) are graphed against increasing doses of a drug, a pesticide, or some other chemical, the resulting distribution curve is shaped like a bell. The far left and right sides of the bell lip represent the small number of people that are either very susceptible or very resistant to the chemical, and the sensitivities of the remaining majority of people fall between those extremes. If a certain dose of a toxic chemical is given to a large number of animals, some show no effect, some get sick, and some die. Thus, in any toxicological test, we do not expect all animals to be affected, nor do we expect all animals affected to exhibit the same degree of severity.
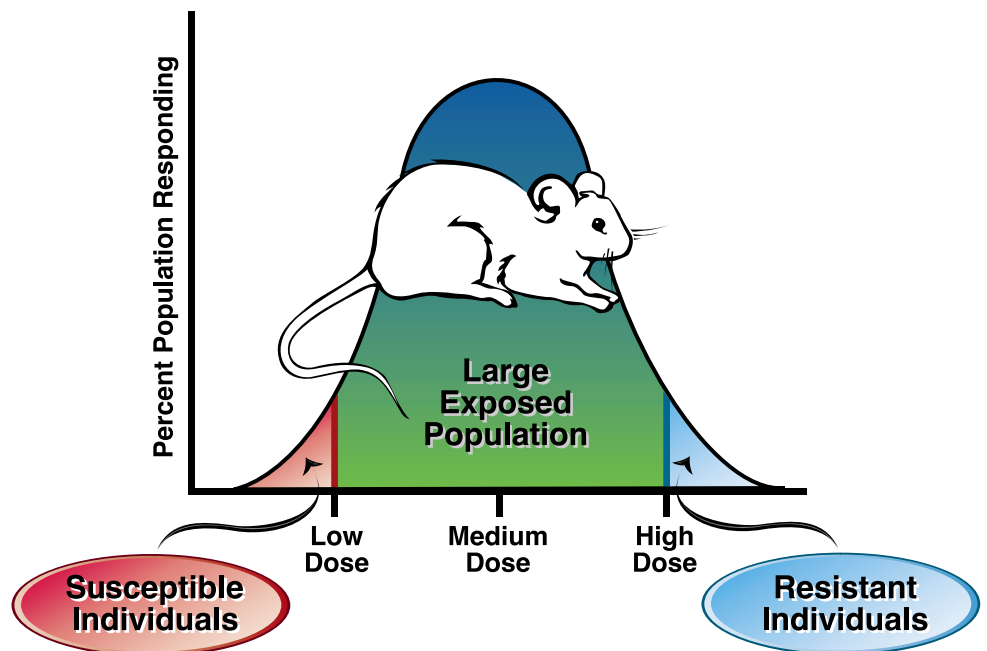
Understanding the Concept of Dose-Response

The toxicity of a pesticide is determined by quantifying the response of laboratory animals to a series of increasing doses. This relationship between administered dose and animal response is graphically depicted as the dose-response curve. The graph includes the measured *response* (e.g., the number or percent of animals affected, or the severity of the response) on the vertical axis and increasing doses of the test chemical on the horizontal axis. For a measured response such as death, the percentage of animals that die increases proportionally as the dose increases. A common measure used to define toxicity when about one-half of the animals die at a certain dose is the LD_{50}—the lethal dose for 50 percent of the animals tested. More than 50 percent of the animals die at doses higher than the LD_{50}, while fewer or no animals die at lower doses. Thus, the higher the LD_{50} dose, the *less* acutely toxic the pesticide.

A generalized dose-response curve has three distinct regions:

- no detectable response
- increasing linear response
- plateau (maximum) response

Animals exposed to low doses exhibit no signs of toxicity. The specific point on the dose-response curve where the more susceptible animals are first affected
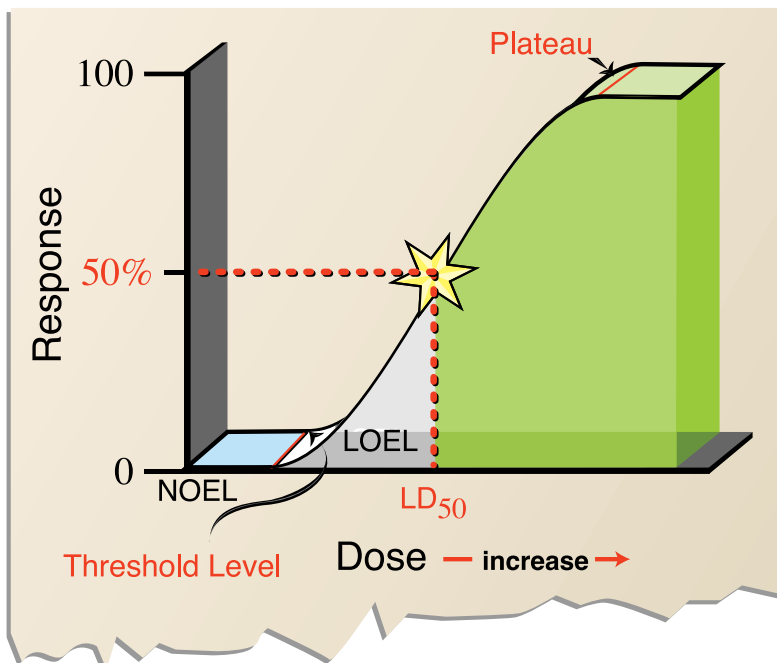
by a pesticide dose is termed the *threshold level:* the lowest dose that produces a measurable response in the most sensitive animals. The threshold level is the beginning of the linear response region of the curve and is the demarcation between the "no observed effect level" (NOEL) and the "lowest observed effect level" (LOEL). Increasing the dose beyond the threshold level increases not only the proportion of animals that show response, but the severity of the effect, as well. It is this linear region of the dose-response curve—increased dose, increased response—that is used to measure, describe, and predict the toxicological properties of a pesticide.

The third part of the curve, the plateau, begins at the point where an increase in dose no longer produces an increase in response. The point at which the response levels off at the upper end of the dose range is known as the maximum effect level. The response may plateau at 100 percent because all of the animals tested are affected, or it may plateau at a lower response level because the remaining unaffected animals are resistant to even the highest dose tested.

The following should be considered when reviewing toxicology studies and interpreting dose-response relationships.

• No single dose-response curve can describe the entire range of toxicological responses exhibited by an experimental animal. Each response (death, cell injury, etc.) is a separate end point and can have a different dose-response curve.

• End points may be actual observations, such as changes in animal behavior or food consumption patterns; they also may be an indirect indicator of toxicity. Toxicologists can use measurements of blood and urine constituents as indicators of toxicity, disease, or deterioration in test animals without having to resort to surgery, x-ray, or whole body scanning. For instance, a toxicologist might use changes in blood enzymes as an indicator of damaged liver cells.

• The dose required to produce a given effect (end point) may vary, depending on the pesticide.

• The data used to develop each dose-response curve are unique to the organism (test animal) that received the pesticide dose.

• Dose-response curves may differ dramatically, depending on the route of exposure to the pesticide: oral, dermal, or inhalation.

Using Dose-Response Curves

Dose-response curves provide valuable information to the toxicologist. Examination of these curves and their supporting data provides a basis for comparing pesticide toxicity threshold levels as well as medium (LD_{50}) and maximum effective doses. For example, regulatory agencies categorize pesticides according to LD_{50} values which, in turn, are useful in determining label language for precautionary statements, first aid directions, packaging restrictions, and transport recommendations.

The slope of the dose-response curve is of critical importance. A steep curve indicates only a slight difference between a nontoxic dose and a toxic dose; in such a case, even a small increase in dose produces a significant change of effect. Conversely, a somewhat flat dose-response curve indicates that a relatively large increase in dose has little effect. A flat dose-response curve indicates a larger margin of safety between a nontoxic dose and a toxic dose. Clearly, correct interpretation of toxicological data and valid conclusions on the toxicity of a pesticide require an in-depth understanding of the dose-response curve.

Describing Adverse Toxicological Effects

Toxicity Described by Exposure Duration

The effects of a pesticide vary with duration of exposure:

• acute (short-term exposure; a single exposure or multiple exposures within a very short period of time)

• subchronic (intermediate-term exposure; repeated exposure over a longer period of time)

• chronic (long-term exposure; repeated exposure over a very long time)

For many pesticides, the response to acute exposure can be very different than the response to subchronic or chronic exposure; that is, a dose administered once may evoke little or no response, while multiple exposures (at the same dose) over an intermediate or long period of time might generate a significant response.

A pesticide is said to be acutely toxicity when adverse effects result from a single exposure, usually at a relatively high dose. But it should be noted that

exposure to the same or lesser doses multiple times within a very short period of time (e.g., 24 hours) also is termed *acute.* Acute effects in humans often result from accidents, such as a child ingesting a pesticide. Suicide attempts and, in some cases, the blatant misuse of pesticide products, may constitute acute exposure. Anytime a pesticide causes adverse effects following acute exposure, it is said to exhibit *acute toxicity.*

Subchronic toxic effects manifest after frequently repeated (e.g., daily) exposure, over weeks or months, to pesticide doses which might produce only minimal or no response to a single, acute exposure. The body is not allowed time to eliminate the pesticide before successive exposure, thus resulting in a buildup that triggers adverse *subchronic* effects.

Chronic
Low Dose
Repeated Exposure
Years
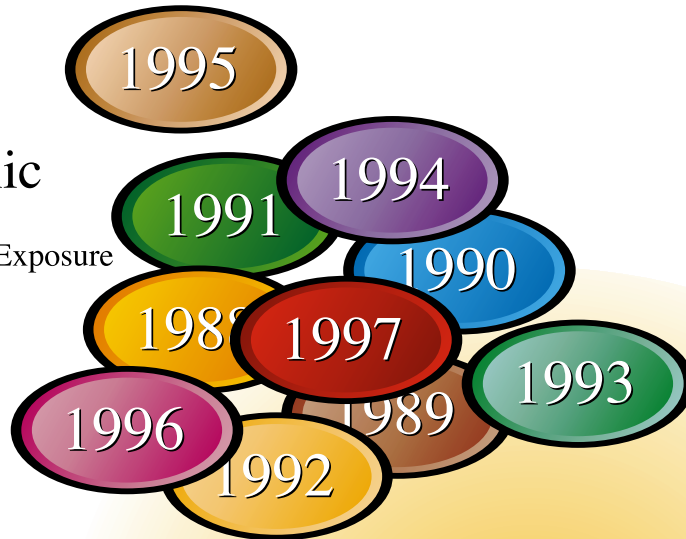
1995
1994
1991
1990
198?
1997
1993
1996
1989
1992

Acute
High Dose
Single Exposure
Days

1997

Subchronic
Medium Dose
Repeated Exposure
Weeks or Months

Chronic effects result from continual exposure over a long period of time—a lifetime, for example. Pesticides can have cumulative effects on the body, even at doses so low that no immediate or short-term effects are apparent. While the body might be able to recover from minimal effects that a single dose or a few low doses might cause, it may not be able to recoup totally between repeated exposures over a long period of time. The buildup of the chemical in the host system eventually becomes recognizable as a *chronic* effect; and the resulting, cumulative damage can be permanent.

Toxicity Characterized as Reversible or Irreversible

The toxicity of a pesticide is described as *reversible* if its effects subside or disappear when exposure ends. But in situations where adverse pesticidal effects persist even when exposure is eliminated, the toxicity is considered *irreversible.*

The toxic effects of some pesticides are reversible when exposure is eliminated, regardless of the dose, while the effects of others may be reversible at low-dose exposures but irreversible at high doses. Toxic effects sometimes are reversible, initially, but with continued exposure become irreversible, the dose notwithstanding.

Toxicity Characterized by Alterations at the Subcellular Level

Following are examples of pesticide interaction at the subcellular level.

• Enzymes are proteins that speed up chemical reactions of specific molecules. A pesticide that interferes with an enzymatic process can prevent, slow down, or speed up a chemical reaction within a cell. Enzymatic interference can lead to a toxic response by the cell, tissue, organ, or system. For example, acetylcholinesterase is an enzyme essential to proper function of the nervous system; it can be inhibited by organophosphorous insecticides, leading to nervous system toxicity.

• Critical cellular components (e.g., DNA, hormone receptors, energy producing chemicals, nerve impulse-transmitting chemicals, and cell membrane transport proteins) can interact with pesticides to produce harmful effects. Pesticides may interfere with molecules that serve specific purposes. For example: Hemoglobin

is a special molecule whose primary function is to transport oxygen in red blood cells. Interfering with hemoglobin so that it does not perform effectively can result in injury stemming from changes in oxygen transport.

Toxicity Characterized by Effect

Toxicity often can be described according to the observable or measurable effect it causes.

• *Death* is the ultimate toxic effect, occurring when critical bodily functions are altered or inhibited.

• *Irritation* is observed when a pesticide affects cells of the skin or eye; corrosion occurs when the integrity of the outer layer of cells is destroyed. The effect frequently is referred to as a "burn." Less severe irritation might appear as redness, swelling, or inflammation of the skin. Irritation/corrosion can result from a single or cumulative exposure.

• *Skin sensitization* is an allergic reaction; sensitization requires multiple exposures over a period of time. The initial exposure "sensitizes" the person, and subsequent exposures cause the individual to react to the chemical by developing a "rash." Poison ivy is a familiar example of a skin sensitizing, natural chemical.

• *Mutagenicity* (also called genotoxicity) results from a change in the genetic material of a cell. There are two general types: a gene mutation that changes the DNA genetic code; and a structural mutation that causes structural chromosome damage.

A mutagenic compound may produce chromosomal aberrations by modifying the physical structure or number of chromosomes; the result is chromosomes that are fragmented or mismatched, or chromosomes that fail to undergo cell division.

Gene mutations include the deletion, addition, or substitution of the chemical components of DNA, which contains all the coded information that allows organisms to function. Disruptions in genes or chromosomes can lead to diseases (including cancer) and birth defects. A mutagen is of concern when it damages egg or sperm cells, enabling the defect to be passed on to successive generations.

• *Tumors*—also called neoplasms—are abnormal growths of tissue; they can be either benign or malignant. Most benign tumors are not life-threatening because cell division usually is slow and the cells are noninvasive: They will not spread to surrounding tissue. Malignant tumors divide rapidly, in an uncontrolled

fashion, and spread to other body tissues; this, coupled with their tendency to intercept nutrients needed by healthy tissue, thereby destroying it, renders them life-threatening.

Malignant tumors may be one of four cancer types:

• *Leukemias* are cancers of red blood cells, certain white blood cells, and the tissues that produce these cells.

• *Lymphomas* are cancers that affect organs of the lymphatic system, such as lymph nodes.

• *Sarcomas* are cancers of connective tissues such as bone, muscle, and cartilage.

• *Carcinomas* are cancers of the internal or external epithelial tissues.

Toxicity Described by Target Organ/System Effects

Toxicological effects often are described according to the organ or system that they impact: cardiovascular, respiratory, gastrointestinal, urinary, muscular, skeletal, and dermal effects; central or peripheral nervous system and sense organ effects; immune system effects; endocrine gland effects; and reproductive system effects. Organ effects in experimental animals often, though not always, are predictive of the effects expected from human exposure to the same pesticide. A toxicological insult to one organ may have indirect repercussions on other parts of the same system, or on multiple systems, due to the complex interaction and coordination of various systems of the body.

Animal Testing Crucial to Safety Evaluation

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) requires pesticide manufacturers to conduct and submit to the U.S. Environmental Protection Agency (EPA) an extensive battery of toxicological studies to evaluate the pesticide product. Toxicological studies, in combination with other required tests (e.g., ecotoxicology, crop residues, and environmental fate), are used by pesticide manufacturers to judge whether to proceed with the development of a pesticide. EPA scientists use the data to evaluate whether a registration should be granted and, if so, to determine its

conditions of use; the data also are useful in developing appropriate label language (e.g., permitted uses, use restrictions, warnings, precautions).

The Use of Animals in Toxicity Studies

Biomedical research has for decades relied on experimental animals as human surrogates. The development of new medicines and the characterization of safe and effective doses are impossible without the use of laboratory animals specifically bred for this purpose. The use of animal models for describing the toxicological properties of pesticides was prescribed by EPA at its inception in the 1970s, and by the Food and Drug Administration (FDA) which preceded it. Regulatory entities around the world require similar testing.

The use of specially bred research animals in toxicological research is not without controversy. Views on the ethics of animal use and welfare issues are polarized, as is scientific dialogue on the relevance of data derived from animal models. Animal rights organizations contend that experimental use of laboratory animals is cruel, unethical, and indefensible. Although, at present, animals cannot be totally eliminated from the testing process, pesticide manufacturers and government agencies worldwide have made great strides in reducing the number of animals used, eliminating unnecessary experimentation, and ensuring that animals are housed properly and treated humanely.

The reliability of predicting human hazards from animal data has long been debated. Many argue that drawing conclusions about human safety from animal models is fraught with uncertainty. For example, experimental results can be influenced by the choice of species and strain; an animal species that absorbs, metabolizes, or eliminates a pesticide differently from humans makes extrapolation to humans less relevant. It is essential that toxicologists use all available knowledge, data, and expertise to select the appropriate animal species, to evaluate the results of experimentation to reach reliable conclusions.

While physiological differences exist between humans and experimental animals, most scientists agree that the similarities outweigh the differences and that animals are the only alternative to direct human testing. There is overall agreement that animal models

generally provide reliable information that helps to safeguard public health.

The inclusion of laboratory animals as models in pesticide testing programs provides many advantages to scientists and regulators:

- Animals are the only alternative to human testing.

- Disease-free animals bred for uniformity are available commercially at a reasonable cost.

- Laboratory animals can be produced in numbers sufficient for toxicological investigations. They can be housed in a relatively small space, have reasonable food requirements, and are amenable to frequent physical examinations.

- The historical information background on a particular animal strain or species—normal responses, disease rates, and tumor frequencies—provides accuracy in toxicological data interpretation.

- The use of genetically similar individuals (inbred strains) can lead to more consistent results.

- The relatively short life span of laboratory animals facilitates the observation of effects and diseases associated with lifetime exposures.

- Certain diseases such as cancer can be predictably observed through the use of animals bred specifically for their susceptibility to a disease or for an anticipated response.

- Experimental animals reach sexual maturity at an early age, have relatively short pregnancies, and produce large litters; these characteristics facilitate the study and evaluation of pesticidal impact on reproduction over multiple generations.

- Test animal routes of entry—oral, dermal, or inhalation—simulate human exposure, yielding a relevant understanding of the chemical fate and properties of a pesticide inside the body.

- Experiments can be standardized by monitoring and controlling "uncertainties." For instance, food and water quality can be monitored and adjusted as required to achieve standardization, and environmental factors such as temperature and light can be manipulated, as well.

- Historically, regulatory agencies around the world make pesticide registration decisions based on their established familiarity with certain test animal species. This standardization of test species, along with experimental methodology, forms a database by which to judge the toxicity of the pesticide being tested.

Species Commonly Used in Pesticide Testing Programs

Researchers and regulators do not rely on any one animal species in conducting safety assessments. Human responses to a pesticide cannot be mimicked exactly or modeled by a single animal species; therefore, toxicologists must use multiple species—rats, mice, rabbits, guinea pigs, dogs—to predict pesticide toxicity to humans. Hamsters, monkeys, pigs, chickens, and cats are used less frequently.

Toxicologists repeatedly test the same strain of animals to facilitate toxicity comparisons between new and existing pesticides. Animals are purchased from sources that document the history and purity of the genetic strain and guarantee the animals to be healthy and disease-free.

Mouse

The mouse (*Mus musculus)* is commonly used in pesticide and pharmaceutical testing; in fact, current estimates indicate that 70 percent of all animals used in testing programs are mice. Mice are used for pesticide carcinogenincity tests, predominantly, offering these advantages: They are small; they are easy to maintain; and they have relatively short life spans.

Rat

Strains derived from the Norway rat *(Rattus norvegicus,* commonly called the laboratory rat) have been used in agricultural and pharmaceutical research since the 1850s. The rat is the second most common experimental animal, comprising 20 percent of all animals used, and it offers many of the same advantages as mice.

Albino Rabbit

Albino rabbits *(Lepus cuniculus)* are used to evaluate skin and eye irritation as well as birth defects. They breed readily, produce large litters, and are easily reared in quantity. Their large bodies and eyes facilitate skin and eye exposure studies.

Guinea Pig

The guinea pig *(Cavia porcellus),* through decades of testing, has been a reliable human surrogate in identifying pesticides that induce skin sensitization— that is, allergies.

Domestic Hen

The nervous system of the domestic hen *(Gallus domesticus)* is sensitive to organophosphorus insecticides; thus, it is used to evaluate nervous system toxicity for this class of pesticides.

Dog

The beagle dog *(Canis familiarus)* is commonly used as the nonrodent species of choice. Dogs share many physiological properties with man and fully complement rodent studies. Their size facilitates difficult surgical procedures, and their ample blood supply allows larger and more frequent samples to be taken without affecting the animals' health. Dogs have longer life spans than laboratory rodents, lending them useful in pesticide toxicology studies that last a few months to a year, or longer. Some studies have lasted eight or more years.

On page 23 is a summary of the comparison of the biological and physiological parameters of animals with those of humans.

Standard Operating Procedures and Protocols

Scientists conducting toxicological studies to support regulatory approval of pesticides in the United States must comply with commonly accepted scientific standards, the guidelines of EPA and other regulatory authorities, and Good Laboratory Practices (GLP, 40 CFR 160). Animal studies also must comply with the Animal Welfare Act Regulations (9 CFR 3) regarding animal use and care.

GLP regulations require that each testing facility develop written procedures that describe all aspects of animal testing, from start to finish, including data development, collection, and security. These procedures are viewed as the codes of experimental conduct and are commonly referred to as Standard Operating

Parameter	Rat	Mouse	Rabbit	Guinea Pig	Hen	Dog	Human
Comparison of Biological and Physiological Parameters							
Life Span (years)	2–3	2	6	6	3–5	15	70
Adult Weight (kilograms)	.2–1	.02–.04	2.5	.5–.8	1.5–3.5	8–12	70
Estrus Cycle (days)	4–5	4–5	induced	15–18	daily	21–28	21
Age at Maturity (weeks)	13	7–9	24–35	12–16	22	40–72	15–18 yr.
Gestation Period (days)	21	19–21	29–35	58–70	21	56–58	270
Litter Size	12–16	10–12	5–10	2-4	270/yr.	4–8	1–2
Birth Weight (grams)	4–6	1–2	30–70	80	50	300–500	3200
Eye Opening (days)	12–14	10–12	7	0	0	10	0
Weaning Age (days)	21	21	28	18–24	hatched	42–56	250
Weaning Weight (grams)	40–50	10–12	1800	250	50	1500–2500	8000
Body Temperature (F)	99.5	99.0	103.0	103.0	103.0	101.3	98.6
Heart Rate (beats/minute)	330–480	320–780	205–220	230–380	280	130–150	72
Blood Volume (percent body weight)	6–7	5	5.5	7–7.5	9	8	8
Respiratory Rate (breaths/minute)	100	163	35–65	84	12–30	20–25	10–13

Procedures (SOPs). GLP requirements also include the following:

• Develop study protocols prior to testing.

• Ensure that the test pesticide is appropriately characterized as to composition, purity, and stability.

• Train, allocate, and schedule personnel.

• Provide adequate resources and facilities, instrumentation and equipment, animal care, and archives.

• Establish an independent quality assurance unit to inspect and ensure the quality and reliability of all study data.

Animal Husbandry Procedures

Newly purchased animals brought into the testing facility are quarantined away from all animals already housed there. They are acclimated to their new quarters, food, and environment for two weeks prior to testing. Animals showing signs of illness or disease during the quarantine period are not used for study. The animals' diet and water are monitored and analyzed for impurities. The sterilized, absorbent bedding placed in the animals' cages is changed regularly. Cages, racks, and other equipment are cleaned thoroughly on a regular basis.

Housing Conditions

Individual studies are isolated from all other studies. Animals are housed in well ventilated rooms where their environment remains constant; lighting, temperature, and humidity are preset and monitored to prevent significant fluctuation. All instruments used during each experiment are calibrated routinely to ensure accurate measurements.

Data Collection Procedures and Reviews

GLP regulations require laboratories to accurately document every step of an experiment. All data and observations are recorded in study notebooks or electronically recorded by computers interfaced with data-generating instruments such as weight scales and blood analysis instruments. Changes in procedures or corrections of errors must be recorded, dated, and initialed; and reasons for changes in data or text must be provided.

EPA's Laboratory Data Integrity Assurance Division inspectors audit the accuracy and integrity of data generated by pesticide research facilities. EPA inspectors look at employee education and training records, check calibration of analytical equipment, and review compliance with written SOPs. Inspectors also examine the integrity of the data; the housing, feeding, handling, and care of test animals; the handling of test, control, and reference substances; and the accuracy of the study reports. Collectively, the procedures established by the manufacturer and the inspections by EPA

assure that testing is accurate, scientifically sound, and properly documented, and that the experiments are generating meaningful, reliable results.

Administration of Pesticides to Animals

Typically, the pesticide being tested is administered in the animals' food or water, or in the air they breathe; dermal effects are studied by placing the chemical on the test animals' skin or in their eyes. The dose is calculated on each individual animal's weight and is expressed in milligrams of administered chemical per kilogram of body weight (mg/kg). As an example, two rabbits weighing 4 and 6 kilograms are to be administered the same pesticide *dose* of 5 mg/kg. The smaller rabbit should be administered 20 milligrams, while the larger should receive 30 milligrams.

Animals are typically administered a pesticide dose via the predominant route of expected human exposure: oral; dermal; inhalation.

Oral Administration

The method chosen for administering an oral dose often depends on the chemical, the animal species, and the duration of the study. In a short-term study, the pesticide might be administered to dogs as a gelatin capsule, or to rodents through a stomach tube; these methods place the entire dose directly into the stomach. In longer-term studies, the pesticide usually is incorporated into the animals' feed or water, allowing their access to small amounts each time they eat or drink.

A syringe with a tube is used to insert a pesticide into the stomach of a rat.

Dermal Administration

The animals' fur is clipped prior to placing a pesticide dose directly on the skin. Solid materials are crushed and mixed with a liquid to form a paste, slurry, or solution, then applied to the skin. The site of application is bandaged to keep the animals from licking the treated area and ingesting the chemical. Another method employed to deter licking is the placement of a large "collar" around the neck to restrict the animals' access to the application site.

Animals are confined in air-tight chambers into which pesticide vapor, aerosol mists, or dusts are introduced. It is critical that the test substance be uniformly distributed throughout the chamber for the time period during which animals are obliged to breathe the treated air. The pesticide concentration and particle size in the air is monitored regularly. If the particles are too large, they are ground to assure accessibility to the lungs. Placing animals in chambers exposes not only the respiratory system, but all external body surfaces, as well. Alternative testing systems are available for limiting exposure to the animals' nose or face.

Establishing Dose Levels

Most tests require four groups of animals, each receiving a different dose level of the pesticide:

• none (control group—no pesticide whatsoever)

• low (an amount of pesticide estimated to produce no toxic response)

• medium (enough pesticide to evoke a moderate response, between those at low and high doses)

• high (in acute studies, enough pesticide to cause death; in chronic studies, enough to produce significant signs of toxicity, but not death)

Making Comparisons Between Treated and Untreated Animals

An essential part of any toxicological program is the response comparison between the animals exposed to a pesticide and those not exposed. The animal groups exposed to a pesticide are known as "treatment groups" and those left untreated are referred to as "controls." Control groups are handled exactly like the treatment groups, except that they are not administered a pesticide. Toxicologists use controls to demonstrate normal growth and development and to provide valuable information on the occurrence, type, and frequency of background disease in untreated animals. Data from both groups are evaluated to differentiate abnormal from normal response.

The type of control group used in a toxicological study may be specified by regulatory protocol, or the

manufacturer may choose control groups known to ensure levels of performance and reliability of the testing program.

Untreated Control Group

The most common control is the *untreated control group,* often called the negative group. Control animals are housed in the same room with those being treated, and they are fed an identical diet—minus the pesticide.

Vehicle Control Group

Some pesticides are incorporated into a capsule or dissolved in a solvent such as corn oil prior to administration to the test animals. Animals assigned to a *vehicle control group* receive the capsule or the solvent without the pesticide, which helps differentiate effects caused by the vehicle.

Positive Control Group

The *positive control group* also is treated with a substance known to produce a specific effect; thus, treated control groups ensure that the animal test system is appropriately sensitive to the end point of interest. For instance, tri-ortho-tolylphosphate (TOTP) is a chemical known to produce certain neurotoxic symptoms; so neurotoxic effects observed in test groups treated with TOTP demonstrate those which might be expected in groups treated with a similarly neurotoxic pesticide.

Historical Control Group

Facilities that supply animals to toxicological testing laboratories typically maintain *historical control* records, and information from those records is used to assess changes in diseases, over time. Testing laboratories also maintain *historical control group* records of animal parameters and disease rates for the testing facility. The large numbers of animals represented in historical records yield better estimates of normal disease rates than do the relatively small numbers of control animals in individual studies.

Self Control Group

An animal, under some circumstances, can serve as both a treated and untreated control. *Self control groups* are especially useful in eye and skin irritation studies; a response to a pesticide in one eye or on one area of skin on an albino rabbit, for instance, is compared to the untreated (control) eye or skin of the same animal.

Methods Used for Measuring Toxicological Effects

Many observations in toxicological studies are used to determine if pesticides impact animal health. In general, effects can be observed as

- changes in appearance and behavior,
- findings from routine physical examinations,
- changes in body weight,
- shifts in food consumption,
- alterations in blood chemistry,
- physical and chemical changes in urine,
- changes in appearance and weight of internal organs, and
- microscopic changes of internal organs and tissues.

Observations of General Behavior

Experimental animals are typically observed twice daily for mortality and signs of toxicity. The observer needs extensive, on-the-job training and experience to become proficient in recognizing differences between normal and abnormal behavior and symptoms. Examples of observable end points are listed on the following pages.

Routine Physical Examinations

Animals are handled and their reactions and reflexes observed during detailed physical examinations. Each animal also is examined for unusual growths or lumps; abnormalities are recorded each time they are observed.

Scientist examining a rat, looking for abnormalities and preparing to enter the information into the computer.

Observable End Points ....

Activity

- hyperactivity
- hypoactivity
- nonresponsiveness
- prostrate position
- aggressiveness

Ears

- discharge
- tears/lacerations
- pallor
- redness
- swelling
- scabs
- encrustation

Eyes

- red discharge
- blood-like discharge
- pus-like discharge
- lacrimation (tearing)
- wetness around the eyes
- excessive blinking
- partially/completely closed eyelids
- opacity (cloudy eyes)
- pupil contraction
- pupil dilation (expansion)
- pallor
- yellow/brown conjunctival discoloration
- diffuse conjunctival redness
- dilation of conjunctival blood vessels
- pitted/raised corneal surface
- protrusion
- necrosis/rupture of globe
- loss of eye
- periorbital encrustation

Excreta

- unusual urine color
- blood-like urine color
- decreased urination
- excessive urination
- unusual fecal color
- blood-like fecal color
- black stool
- soft stool
- diarrhea
- decreased defecation
- hard stool
- bright yellow urine

Feet and Limbs

- focal loss of hair
- swelling
- abrasions
- sores on feet
- torn toenails
- loss of limb/paw/toes
- cysts

General Appearance

- emaciation
- dehydration
- distended abdomen
- self-mutilation
- intra-abdominal swellings
- protrusion of tissue from rectum

Genitalia

- urogenital discharge
- vaginal discharge
- red discharge
- yellow/brown discharge
- clear discharge
- blood-like discharge
- swelling
- protrusion of tissue
- greenish discharge
- abnormal penile erection

Mouth

- emesis (vomiting)
- salivation
- wetness around the mouth
- swollen mouth
- bleeding gums
- ulceration of lips
- ulceration of oral mucosa
- blood-like discharge
- broken teeth
- missing teeth
- mismatched teeth
- overgrown teeth
- pale mucosa

Movement/Posture

- head tilt
- ataxia (incoordination)
- convulsions
- twitching
- tremors
- misuse of limbs
- arching of back
- circling movements
- somersaulting
- lateral rolling movements
- side-to-side head motion
- up-and-down head motion
- backward walking

Nose

- red/pink discharge
- clear discharge
- yellow/brown discharge
- blood-like discharge
- frothy discharge
- perinasal encrustation

Respiration

- labored breathing
- gasping
- rapid breathing
- slow breathing
- shallow breathing
- sneezing
- high-pitched sounds
- rattling sounds
- intermittent apnea (stopped breathing)

Skin

- hair loss
- abrasions
- scabs
- scars
- swelling
- flaking/peeling
- sloughing
- necrosis
- ulceration
- discoloration
- pallor
- blood-stained appearance
- red/brown encrustation
- urine-stained hair
- feces-stained hair
- anogenital staining
- mammary gland staining
- mammary gland secretion
- cysts

Changes in Body Weight

Body weight is an important end point in assessing the toxicological effects of a pesticide. Animals in short-term studies are weighed at least weekly. Those in longer studies are weighed weekly for 12 weeks and monthly, thereafter, as the rate of weight change slows. Decreased body weight may correlate to toxicity, in that an animal that does not feel well likely will not eat well; another correlation might be that the toxin inhibits the animal's ability to utilize the food consumed.

Shifts in Food Consumption

A measured amount of food is offered to each animal. At specified intervals, uneaten food is weighed. The difference between the amount offered and the amount uneaten represents food consumption for that time interval. Comparisons of what is consumed by controls and by treated animals reveal early indications of toxicity. Water consumption also may be recorded.

Changes in Hematology

Hematology is the study of cellular components in whole blood. Small amounts of blood are taken from experimental animals for hematological studies. The following cellular constituents are recognized as key measurements in describing toxicity: hemoglobin concentration and platelet, erythrocyte (red cell) , and leukocyte (white cell) counts.
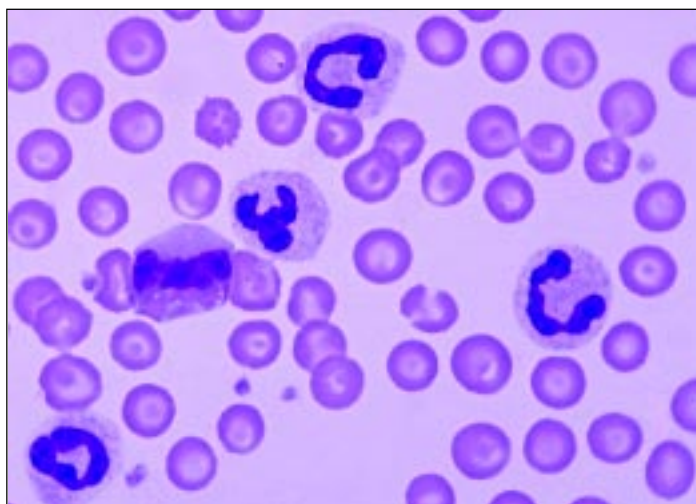
Red blood cells—large cell, no nucleus; white blood cells—large cell, blue nucleus; platelets—small cells.

Alterations in Blood Chemistry

When cells are removed from blood, what remains is the liquid portion known as plasma. Blood chemistry measurements are important because cells, tissues, and organs are in close contact with circulating blood. Damaged organs often release enzymes and other substances into the bloodstream, which are recognizable as indicators of toxicity. The measurement of enzymes, electrolytes, and biochemical changes in plasma facilitates indirect detection of organ damage.

The following chemicals are typically measured in blood:

Acid/base balance

Alanine aminotransferase

Albumen

Alkaline phosphatase

Aspartate aminotransferase

Bilirubin

Blood creatinine

Calcium

Chloride

Cholesterol

Cholinesterase activity

Glucose (blood sugar)

Hormones

Lipids

Methemoglobin

Phosphorus

Potassium

Protein

Sodium

Total bilirubin

Urea nitrogen

Alterations in Urine

The following parameters in urine provide clues to toxic effects from pesticides:

Appearance	pH
Bilirubin	Protein
Cast kidney cells	Specific gravity
Glucose	Urobilinogen
Ketones	Volume
Occult blood	White blood cells

Measurements of hematology, blood chemistry, and urinalysis are similar to analyses performed during human medical examinations.

Gross Observations of Internal Tissues

Autopsies are performed on animals that have died or been put to sleep during or at the conclusion of studies. Internal body cavities are examined. The testes, thyroid, thymus, spleen, heart, lungs, ovaries, brain, liver, and kidneys are individually weighed and examined for changes in size and color, and for lesions. Organs are observed to verify presence and proper location within the body cavity. Once all body tissues have been evaluated and removed, the focus turns to muscles and bones.

Microscopic Evaluation of Tissues

As many as fifty tissues from each animal are examined. Each is sliced into thin sections—a process which can take as long as six months—for microscopic observation to detect the presence of lesions and abnormal growth. Pathologists look at each tissue from each animal and examine cell structure, organization, size, and shape; some chronic studies require examination of as many as 20,000 tissue slices. Organs and tissues are subject to routine microscopic evaluation; there is no limitation on the types or numbers of tissues examined.

Adrenals	Muscle
Aorta	Nose
Bone marrow	Ovaries
Brain	Pancreas
Caecum	Peripheral nerve
Colon	Pituitary
Duodenum	Rectum
Esophagus	Salivary glands
Eye	Skin
Femur (bone)	Spinal cord
Gall bladder	Spleen
Gross lesions	Sternum
Heart	Stomach
Ileum	Testes
Jejunum	Thymus
Kidneys	Thyroid
Liver	Tongue
Lungs	Trachea
Lymph nodes	Urinary bladder
Mammary glands	Uterus

liver

size ——
color ——
position ——
structure ——
lesions ——
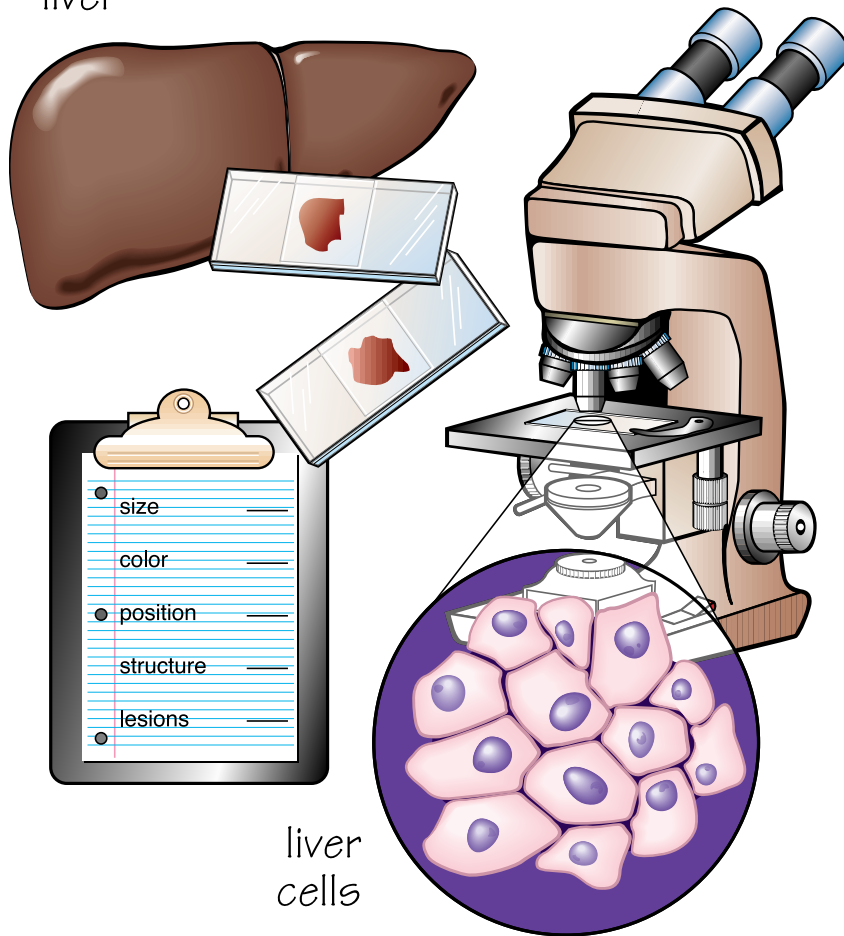
liver
cells

Pesticide Testing: Legal Requirements and Experimental Designs

Data Requirements for Pesticide Registration

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA) authorize the United States Environmental Protection Agency (EPA) to regulate the registration, manufacture, sale, and use of pesticides. EPA is charged with assuring that pesticides perform their intended functions without causing unreasonable adverse effects on man or the environment. In carrying out these responsibilities, EPA requires all pesticides to undergo a rigorous testing process and a comprehensive regulatory review to determine if unacceptable adverse effects are likely to occur as a result of their use. All pesticides require an EPA registration, which is a revocable license to sell and use a pesticide product.

Regulations dealing with pesticide registration are part of the Code of Federal Regulations (CFR) Title 40 part 158. The 40 CFR 158 regulations specify the types of data required by EPA as a basis on which to make regulatory judgments on the risks associated with pesticides. The following examples illustrate the kinds of information contained in 40 CFR 158 and its accompanying Appendix A.

Pesticide Use Sites

The pesticide registrant (in most cases, the manufacturer) needs to first identify the intended use sites for the pesticide being tested; there are fourteen site categories:
- Agricultural Crops
- Ornamental Plants and Forest Trees
- General Soil Treatment and Composting
- Processed or Manufactured Products and Food or Feed Containers or Dispensers
- Pets and Domestic Animals
- Agricultural Premises and Equipment

- Household
- Wood or Wood Structure Protection Treatments
- Aquatic Sites
- Noncrop, Wide Area, and General Indoor/Outdoor Treatments
- Antifouling Treatments
- Commercial and Industrial Uses
- Domestic and Human Uses
- Miscellaneous Indoor Uses

Specific Use Patterns

Once the site group has been identified, the registrant must place the pesticide into one or more subgroups which more clearly identify proposed uses. The following examples represent subgroups listed for agricultural crop, aquatic, and household site groups.

- Agricultural Crop Uses—small fruits, tropical and subtropical fruits, vegetables, commercial greenhouses, fiber crops, forage crops, grain and edible seed crops.
- Aquatic Uses—food processing water systems, pulp and paper mill systems, swimming pool water, agricultural irrigation water and ditches, and estuaries.
- Household Uses—nonfood areas, food handling and food storage areas, household contents and space.

General Use Patterns

Specific use patterns and their subgroups are consolidated into nine general use patterns:
- terrestrial food crop
- terrestrial nonfood
- aquatic food crop
- aquatic nonfood
- greenhouse food crop
- greenhouse nonfood
- forestry
- domestic outdoor
- indoor

It is these general use patterns that determine the data requirements for new product registration. For example, an agricultural crop use demands an exceptionally rigorous testing program because of food safety issues and the general proximity of application sites to fish and wildlife.

Data Requirement Tables

The types of data that must be submitted with an application for pesticide registration are listed by general use patterns. The Code of Federal Regulations (Subpart D of 40 CFR 158) lists the types of information required by major data subheadings:

- Environmental Fate
- Insect
- Nontarget
- Physical and Chemical Characteristics
- Plant Protection
- Product Performance
- Reentry Protection
- Residue Chemistry
- Spray Drift
- Toxicology
- Wildlife and Aquatic Organisms

Each general use pattern is listed as a headline on each data table, and the studies required for registration appear in the column directly underneath. For instance, a pesticide classified for use on a terrestrial food crop would list the following required studies under the toxicology table: acute oral toxicity, acute dermal toxicity, acute inhalation toxicity, primary eye irritation, primary dermal irritation, dermal sensitization, acute delayed neurotoxicity, 90-day feeding, 21-day dermal, 90-day dermal, 90-day inhalation, 90-day neurotoxicity, chronic feeding, oncogenicity, teratogenicity, reproduction, gene mutation, structural chromosomal aberration, other genotoxic effects, general metabolism, and domestic animal safety. The total number of studies

listed constitutes the minimum data package needed to support a pesticide registration. Additional studies are listed in the data tables as "conditionally required"; their necessity is dependent upon results of the "required" studies and other product use considerations. Data requirements for agricultural food crop pesticides are rigorous, whereas the requirements for a pesticide intended for use as a household disinfectant are less stringent.

Pesticide Assessment Guidelines

The regulations in 40 CFR 158 do not specify what methodologies and procedures are to be used in toxicological research. The specific study designs and measurements are provided to the registrant through an EPA publication series called Pesticide Assessment Guidelines. These guidelines also specify how tests are to be evaluated, analyzed, and reported. For example, step-by-step information for performing a chronic feeding study is found in the Pesticide Assessment Guidelines Subdivision F, 83-1. Specifications include study design; animal selection parameters such as age, species, weight range, and numbers; dose administration route, frequency, and duration; and observations, measurements, analyses, and procedures that must be performed and recorded for each animal. Similar guidance is provided for each required study. The Pesticide Assessment Guidelines are updated as scientific knowledge expands. Studies that do not conform to these rigorous guidelines are not acceptable for regulatory purposes and must be repeated.

Toxicological Testing and Evaluation of Pesticides

Describing the acute and chronic effects of a pesticide on experimental animals requires a thoroughly planned program. The tests are designed to determine dose-response.

Acute tests are conducted, first, to measure observable effects at relatively high doses administered over short periods of time. Longer-term testing generally necessitates the use of lower doses administered regularly for extended periods, allowing the animals to survive for the duration. This is especially important for chemicals that accumulate in the body. High doses can

be used in short-term studies because the animals need not survive for long periods of time. The identification of subtle effects on animals exposed to pesticides in longer-term studies requires sophisticated analysis.

Toxicological conclusions are dependent on the complete compilation of data and the integration of all studies. This is critical because no single species of laboratory animal nor any single study is necessarily predictive of human toxicity.

The studies listed in the following table are commonly conducted for most pesticides. Some studies (e.g., delayed neurotoxicity in hens and subchronic inhalation studies in rats) are conducted only for certain pesticides or use conditions.

Toxicology Studies for Human Health Assessments

Acute Testing

End Point	Typical Species
oral mortality	rat
dermal mortality	rabbit, rat
inhalation mortality	rat
eye irritation	rabbit
dermal irritation	rabbit
dermal sensitization	guinea pig
acute, nondelayed neurotoxicity	domestic hen
delayed neurotoxicity	rat

Subchronic Testing

End Point	Typical Species
subchronic oral	rat, mouse, dog
subchronic dermal	rabbit, rat
subchronic inhalation	rat
subchronic neurotoxicity	rat

Chronic Testing

End Point	Typical Species
chronic toxicity	rat, dog
oncogenicity	rat, mouse

ReproductiveTesting

End Point	Typical Species
developmental toxicity	rat, rabbit
reproduction/fertility	rat

Genetic Testing

End Point	Typical Species
chromosome damage	cells, organisms
gene mutation	cells, organisms

Pharmacokinetics

End Point	Typical Species
absorption	rat, mouse, dog, monkey
distribution	rat, mouse, dog, monkey
excretion	rat, mouse, dog, monkey
metabolism	rat, mouse, dog, monkey

Acute Tests: Single Exposure, High Dose, Short Duration

Six tests are used for acute testing. Three measure lethal and sublethal effects (oral LD_{50}, dermal LD_{50}, inhalation LD_{50}) while the remaining three examine eye irritation, skin irritation, and dermal sensitization (allergy). Not only do all pesticide active ingredients undergo this battery of testing, but so does each *product formulation* containing the active ingredient. These tests are critical in assessing the potential impact of accidental exposure to pesticides.

Mortality Studies

Single-dose mortality studies assess the consequences of short term, high dose pesticide exposure on animal health; and subsequent autopsies allow valuable observation of organ damage.

Young adult males and nonpregnant females are randomly assigned to treatment groups for acute toxicity studies, with treatment of the groups differing primarily by route of exposure and dose.

Rats in acute oral studies are given a specific pesticide dose administered through a stomach tube. Dermal studies use rabbits or rats, with the pesticide applied to a shaven area of skin (roughly 10 percent of the body) on the back. The pesticide is held next to the skin with a gauze dressing for 24 hours. Inhalation studies expose groups of rats to various pesticide concentrations in air in tightly sealed chambers.

Animals in all three lethality studies are observed for fourteen days after exposure; signs of illness or changes in behavior are noted. Changes in weight, length of recovery period, and time of death are also recorded. Finally, each animal is autopsied, allowing careful visual inspection of external body parts, internal organs, and body cavities.

The primary objective of the three lethality studies is to estimate the relative toxicity of the pesticide. The number of deaths occurring in each group is noted, as is the dose or concentration level which is lethal to 50 percent of the treated animals: the LD_{50} (oral or dermal exposure) or LC_{50} (inhalation). Information collected from single-dose acute toxicity studies is useful in developing dosage regimens for subchronic studies. Acute lethality data is useful in comparing the acute

A rabbit is weighed on an automatic scale system that simultaneously enters the weight into a computer.

toxicity of one pesticide to that of another; in placing the pesticide into relative toxicity categories; and in developing label language, warnings, and precautions for the proper handling and use of the product.

Irritation and Sensitization Testing

Data gathered from eye irritation, dermal irritation, and dermal sensitization studies are used to estimate the ability of a pesticide to produce temporary or permanent damage to eyes and skin. The type of damage (if any), its duration, and the severity of the injury are recorded for each pesticide.

Eye Irritation Study

Rabbits are used for eye irritation studies. The lower eyelid of one eye is pulled down and the pesticide applied. The eyelids are held together for a few seconds to ensure that the pesticide remains in place, and the other eye is left untreated to serve as a control. The pesticide may be washed from the treated eye only after 24 hours.

Effects on the treated eye are recorded one hour after treatment, then at 24, 48, and 72 hours. If irritation is not observed at 72 hours, recording ceases; if irritation persists, however, daily recording may continue for up to 21 days. Eyes are scored relative to the opacity of the cornea and any redness, swelling, or discharge observed. The pesticide is classified as corrosive if the eye injury is permanent.

Rabbit eye being examined for irritation.

Acute Mortality Protocols

Parameter	Oral	Dermal	Inhalation
Preferred test species	rat	rabbit or rat	rat
Age of animals	young adult	young adult	young adult
Pregnancy status	not pregnant	not pregnant	not pregnant
Substance tested	active ingredient, formulation	active ingredient, formulation	active ingredient, formulation
Number of dose levels	1–6	1–4	1–4
Number of animals/dose	10	10	10
Male/female ratio	50/50	50/50	50/50
Type of control group	none	none	none
Route of administration	stomach	skin	air
Exposure	single dose	single dose	single dose
Duration of exposure	single dose	24 hours	4 hours
Observation period (days)	14	14	14

Skin Irritation Study

A section of fur on the back of each rabbit is shaved one day prior to the start of skin irritation studies. A single dose of pesticide is applied to a 1-inch square of skin which is then covered with a gauze patch. The patch is removed after four hours and the treated area washed. Observations are recorded after one hour and again at 24, 48, and 72 hours, then daily for up to 14 days. Observations on skin damage, redness, and swelling are scored.

Dermal Sensitization Study

Dermal sensitization studies are used to evaluate the potential of a pesticide to produce allergic contact dermatitis after repeated skin contact. Guinea pigs' fur is clipped or shaved from their backs, then a minimally irritating pesticide concentration is applied once a week, for six hours, for three consecutive weeks (induction phase). At that point, the animals are given a two-week rest period after which a challenge dose—at a concentration previously determined to be non-irritating—is applied to a previously untreated area of skin. The challenge dose site is examined for redness and swelling 24 and 48 hours after exposure. The pesticide is considered a sensitizer if the challenge dose produces a skin reaction.

Acute Irritation and Sensitization Protocols

Parameter	Eye Irritation	Dermal Irritation	Dermal Sensitization
Preferred test species	rabbit	rabbit	guinea pig
Age of animals	adult	adult	adult
Pregnancy status	not pregnant	not pregnant	not pregnant
Substance tested	active ingredient, formulation	active ingredient, formulation	active ingredient, formulation
Dose	0.1 ml	0.5 ml (liquid) 0.5 g (solid)	(depends on skin reaction) (depends on skin reaction)
Number of animals/dose	3–6	3–6	10–20
Male/female ratio	50/50	50/50	50/50
Type of control group	self	self	positive, negative
Route of administration	eye	skin	skin
Exposure	single dose	single dose	multiple doses
Duration of exposure	24 hours	4 hours	6 hours x 4 weeks
Observation period (days)	3–21	3–14	35

Acute Nondelayed Neurotoxicity Testing

Rats are good models for detecting *nondelayed* neurotoxicity. Testing consists of a functional observational battery (FOB), a motor activity test, and microscopic neuropathology. The FOB uses predefined scoring scales to quantify and describe gross functional changes observed in the home cage, during handling, and while the animal is allowed to move freely in an open space. The motor activity test involves an automated device that measures the movement activity in individual animals; and the neuropathological assessment is achieved by microscopic examination of nerve tissue throughout the animals' central and peripheral nervous systems.

Acute Delayed Neurotoxicity Testing

Some organophosphate insecticides are known to produce a form of neurotoxicity that is delayed relative to initial exposure to the pesticide. Scientific studies have determined that this delayed neurotoxicity can be readily detected in hen chickens but not in mice or rats. Acute delayed neurotoxicity testing is required for organophosphorus and carbamate insecticides or other pesticides suspected of causing delayed neurotoxicity. The pesticide is administered orally as a single dose to domestic hens. Behavioral and locomotor abnormalities

Acute Neurotoxicity Protocols

Parameter	Delayed Neurotoxicity	Nondelayed Neurotoxicity
Preferred test species	domestic hen	rat
Age of animals	adult	7 weeks
Substance tested	active ingredient	active ingredient
Number of dose levels	3	3
Number of animals per dose	10	20
Male/female ratio	0/100	50/50
Type of control	positive, untreated	positive, untreated
Route of administration	oral	oral
Exposure	single dose	single dose
Observation period (days)	21 or 42	21
Microscopic pathology	none	nervous system of 5 rats/sex/dose

(e.g., splayed, waddling gait; circling; somersaults; paralysis) are observed for 21 days. If no neurotoxic responses are observed, the hens are redosed after 21 days. The hens are then evaluated for 21 additional days. Nervous system tissues—brain, spinal cord, and nerves—are examined for microscopic changes.

Subchronic Tests: Repeated Exposure, Intermediate Dose, Moderate Duration

Subchronic *exposure* occurs repeatedly over weeks or months and subchronic *effects* are those resulting from such exposure. Subchronic studies assess *sublethal* toxic effects, generating a portfolio of toxicological information distinctly different from that of acute testing. Acute studies involve high doses administered during a very short period of time, the result of which often is death of the test subjects. Subchronic testing, on the other hand, employs lower doses to facilitate keeping test animals alive for long periods of time; this is essential in identifying any subtle effects resulting from cumulative exposure.

Subchronic testing programs must utilize toxic and nontoxic dose levels. High doses must elicit sublethal effects, middle doses must evoke only minimal adverse effects, and low doses should trigger no toxic effects whatsoever. Generally, 3 to 5 dose levels are tested. Typical subchronic studies expose animals for a period of time equal to 10 percent of their normal life span. Exposure routes are identical to those of acute testing programs: oral, dermal, inhalation.

Each animal is observed twice daily for signs of toxic effects such as abnormal appearance and altered behavior (see list on pages 30–31). Thorough weekly examinations are conducted to track growth (including weight) and development. Blood is examined, periodically, for hematolotical and chemical parameters. Animals that die are autopsied, and those that survive are euthanized and autopsied at termination of the study. Tissues are examined, then evaluated microscopically for signs of disease or toxic effects; major organs are weighed. In some studies, the persistence or reversibility of pesticidal effects may be tested upon cessation of exposure.

Subchronic Protocols

Parameter	Oral	Dermal	Inhalation	Neurotoxicity
Preferred test species	rat, mouse, dog	rat, rabbit, or guinea pig	rat	rat
Age of animals	mouse/rat, 6–8 wk.; dog, 4–6 mo.	adult	6–8 weeks	6–8 weeks
Pregnancy status	not pregnant	not pregnant	not pregnant	not pregnant
Substance tested	active ingredient	active ingredient	active ingredient	active ingredient
Number of dose levels	3	3	3	3
Number of animals per dose	rat, 20; beagle, 8	10	10	20
Male/female ratio	50/50	50/50	50/50	50/50
Type of control group	untreated	untreated	untreated	untreated, positive
Route of administration	diet/stomach tube	skin	air	diet/stomach tube
Exposure (day)	continuous (diet)	6 hours	6 hours	diet/stomach
Duration of exposure(days)	90	21 or 90	90	90
Observation period (days)	90	21 or 90	90	90

Subchronic Oral Studies

Young rats and mice (6–8 weeks old) are fed diets containing a pesticide for 90 days. Beagle dogs (4–6 months old) may be tested likewise, although they may receive the pesticide in a gelatin capsule rather than in their food.

Subchronic Dermal Studies

The pesticide is applied to the shaved skin of rats, rabbits, or guinea pigs and immediately covered with gauze to hold the material next to the skin. The dose is readministered 5 days a week over the 21- or 90-day duration of each study.

Subchronic Inhalation Studies

Groups of rats are exposed to various air concentrations of the pesticide in tightly sealed chambers 6 hours each day, 5 days per week, for 3 months.

47

Rats are given an oral dose daily for 90 days. Administration may be by stomach tube, by capsule, or by a pesticide-laced diet. Subchronic neurotoxicity testing consists of a functional observational battery (FOB), a motor activity test, and microscopic neuropathology. The FOB uses predefined scoring scales to quantify and describe gross functional changes observed in the home cage, during handling, and while the animal is allowed to move freely in an open space. The motor activity test involves the use of an automated device to measure the movement of individual animals. The neuropathological assessment is accomplished via microscopic examination of nerve tissue taken from the central and peripheral nervous systems of the test animals.

Chronic Tests: Multiple Exposure, Low Dose, Long Duration

Chronic studies measure the effects of daily exposure to a pesticide over the majority of the test animals' life span. Long-term studies are conducted with two rodent species (mice and rats) and one nonrodent species (dogs). The chronic rodent toxicity studies might involve 400 or more animals and require three to five years, from the start of the experiment to compilation of the data into a final report; the cost could total $400,000 to $800,000 *for each species tested.*

Chronic studies assess specific toxic effects on body organs and identify cumulative effects resulting from repeated exposure. While chronic toxicity studies are similar to those conducted for subchronic effects, there are major differences, as well. For instance:

• Animals used in chronic studies are exposed over a longer period of time—typically one to two years, depending on the species.

• Longer exposure extends observation periods, allowing latent symptoms to develop.

• Daily dose levels generally are lower in chronic studies; however, the total cumulative dose may be greater due to the longer duration.

• Microscopic tissue and organ evaluations are key measurements in determining long-term effects of pesticide exposure; in a cancer study, about fifty tissues per animal—15,000 to 20,000 tissue *sections*— are examined.
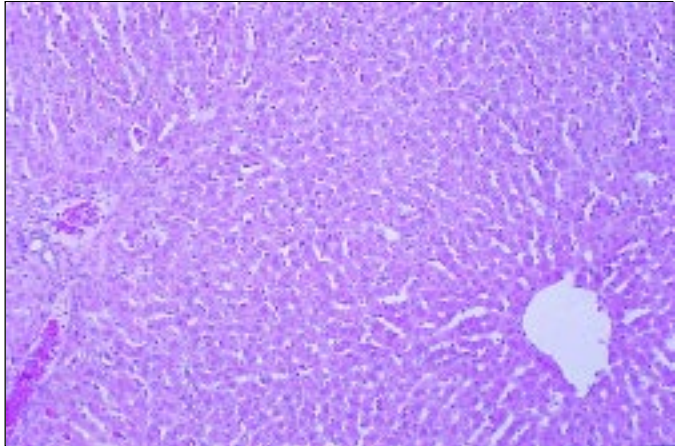
Normal tissue from a rat liver is stained and magnified. The large, clear area is a blood vessel.

• An organized data management system is required for chronic studies because of the number of animals involved, and because the amount and type of information collected, stored, and preserved for data analysis are greatly increased.

Chronic toxicity research (typically performed on rats and dogs) examines cumulative effects of a pesticide on body organs: the lungs, kidneys, liver, etc. Results are interpreted as indicators of the potential for chronic exposure to result in illnesses such as kidney or liver disease, or cancer.

Carcinogenicity studies are conducted specifically to examine the potential of a pesticide to cause cancer. Scientists look for growths of new or abnormal tissues and determine whether they are benign or malignant.

In determining what routes of exposure should be studied, preference is given to those through which human exposure is most likely. Dietary administration is generally preferred for chronic toxicity and carcinogenicity studies because chronic effects in humans usually result from ingesting small amounts of the pesticide, over time.

Testing Procedures, Dose Administration, and Measured Responses

Alternative methods of exposure may be used when animals refuse to eat a pesticide-treated diet or when exposure is negated by rapid degradation of the pesticide in the diet. Options might include dissolving the pesticide in drinking water, administering the pesticide in capsules, or using a stomach tube.

A study may be designed to maintain a constant exposure level in the animals' diet, or exposure might be based on the weight of the animals, i.e., milligrams of pesticide per kilogram of body weight. The latter approach requires periodic adjustment of the individual dose to account for the growth of each animal. Regardless, it is preferable that animals consuming the low-dose pesticide diet exhibit no adverse physical of behavioral effects. A mid-level dose regimen should produce only minimal signs of toxicity, while animals on the high-dose regimen should display significant signs of toxicity (but not death).

EPA defines the Maximum Tolerated Dose (MTD) as the highest dose that causes no more than a 10 percent decrease in body weight of the test population (compared to control groups) without imposing life threatening diseases (other than a cancer response) or producing mortality. The definition and interpretation of the MTD differ among international regulatory agencies, and there is considerable debate among toxicologists on the ramifications of testing at doses so high that they alter the physiology of the test animals.

Chronic studies require intense observation and time-consuming measurements of each animal. Test animals are examined weekly, using visual inspection and observation to assess the general condition and overall behavior of each animal. The animals are weighed weekly for the first 13 weeks and monthly, thereafter. Food consumption is monitored on a similar schedule.

Blood composition and urine composition are indicators of the general health of the test animals. Each is collected and analyzed at six-month intervals and at the conclusion of the study. The analysis is much the same as for humans during physical checkups and for the diagnosis of disease. Blood is drawn and analyzed more frequently if subchronic testing of the pesticide has indicated toxic tendencies.

All animals that die during the course of study are autopsied, and those remaining are euthanized and autopsied at the conclusion of study. All organs and tissues within the body cavity are carefully examined, and specific organs such as the liver, kidneys, brain, and testes are removed and weighed. Approximately 50 organs and tissues are removed, cut into thin slices, and mounted onto glass slides; the samples then are stained for microscopic examination to determine whether the pesticide causes changes in cell numbers, size, type, or structure. Microscopic abnormalities are
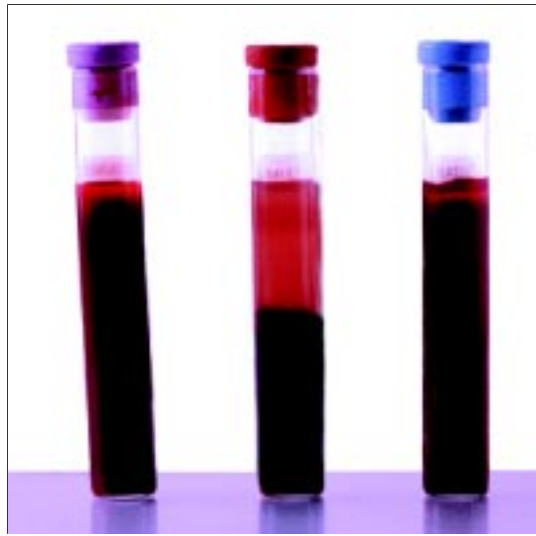
Clotting assays: purple cap, unclotted blood (blood counts/smears); red cap, clotted blood; blue cap, unclotted blood.

Urine samples: Yellow, normal; red, blood; brown, liver disease.

Parameter	Chronic Protocols	
	Chronic Toxicity	*Oncogenicity*
Preferred test species	rat, mouse, and beagle dog	rat and mouse
Age of animals	mouse and rat, 6 to 8 weeks; dog, 4-6 months	6 to 8 weeks
Pregnancy status	not pregnant	not pregnant
Substance tested	active ingredient	active ingredient
Number of dose levels	3	3
Number of animals/dose	rat, 100 animals; beagle, 8 animals	100
Male/female ratio	50/50	50/50
Type of control group	untreated or vehicle	untreated or vehicle
Route of administration	diet, inhalation, stomach tube	diet, inhalation, stomach tube
Exposure (day)	daily	daily
Duration of exposure (days)	12–24 months	18–30 months
Observation period	rodents, 12–24 mo.; dog, 12 months	rats, 24–30 months; mice, 18–24 months

documented for each organ and each animal. The findings are summarized by sex and dose and compared to control groups to ascertain whether the pesticide has influenced the prevalence or severity of effects.

Reproductive Toxicology

The toxicological evaluation of any pesticide is incomplete without a thorough assessment of the chemical's potential to interfere with reproductive processes. Adverse effects on reproduction include, for example, infertility, the tendency to abort, and birth defects. Toxic "insults" to reproduction processes can cause diseases or behavioral abnormalities, as well as learning and functional deficits in offspring. Toxicological studies on reproduction address the pesticide's influence on the fetus and the chemical's ability to interfere with normal reproduction processes. Two types of studies are routine: developmental/teratological (e.g., birth defects) and reproduction/fertility.

Developmental Toxicity Studies

These studies assess pesticidal toxicity to pregnant females; fetal growth and development and physical birth defects. Abnormalities include spontaneous abortion (miscarriage), embryo death, reduced birth weights, and birth defects. The latter may manifest as obvious physical deformities such as cleft palate or missing apendages, or they may occur more subtly as internal organ or skeletal damage.

Developmental toxicity studies evaluate chemical effects on the mother and fetus from the time of implantation of the fertilized egg into the wall of the uterus through birth. The highest dose should produce some toxic effects to the mother, such as weight loss, but it should not be lethal; this ensures the opportunity to evaluate fetal and birth defects, where present. Ideally, a medium dose should not produce maternal toxicity but should stress the developing fetus. And a low dose should have no adverse effect on the mother or the developing fetus.

Generally, one rodent species (usually rats) and one nonrodent species (usually rabbits) are used to assess developmental toxicity. Females are mated or artificially inseminated and the pesticide administered daily, by stomach tube, from the time of fertilized egg implantation to one day prior to delivery. The mothers are

Developmental Biology

Male

Sperm

Female

Egg

Mating

Fertilized Egg

Pregnancy

Developmental
Toxicology

Prenatal Development

Birth

Nursing

Postnatal Development
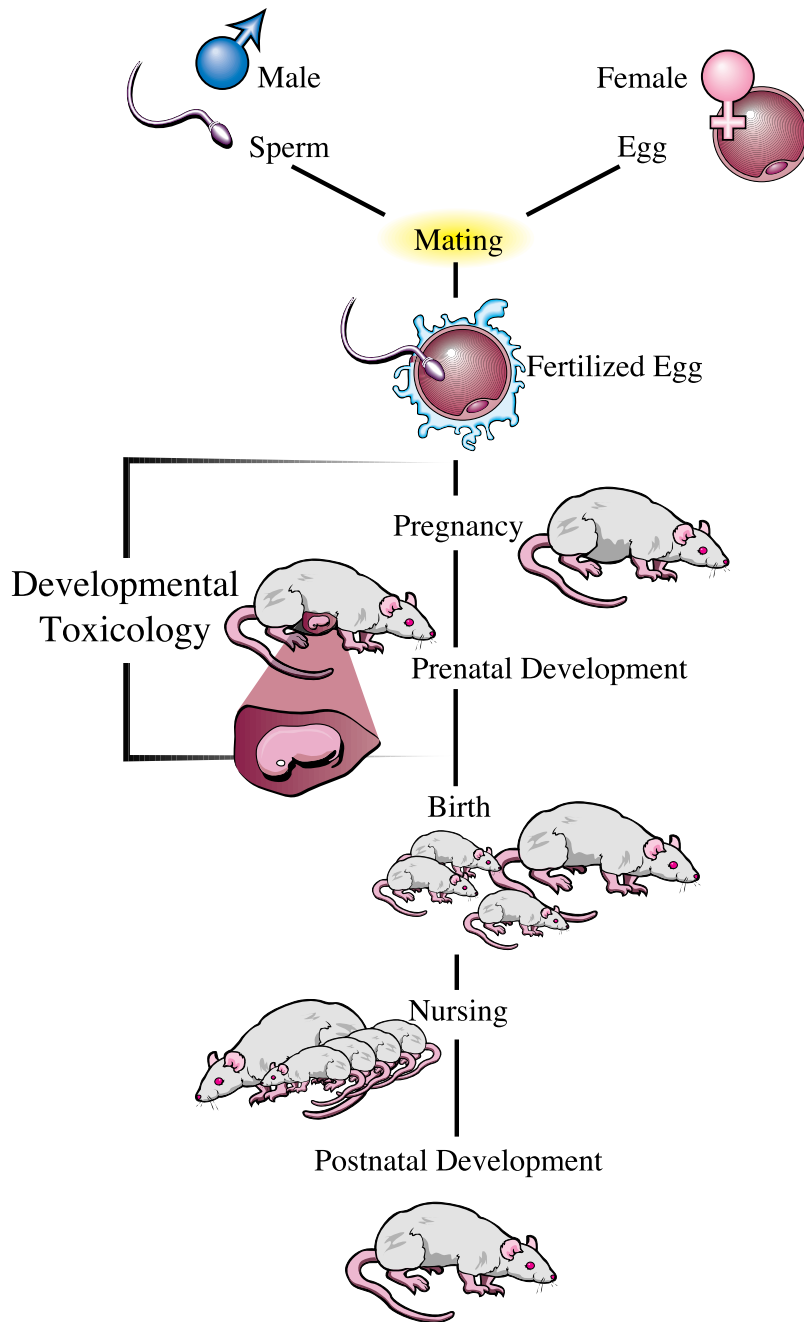
euthanized one day prior to their projected delivery date because they sometimes will eat their abnormal young—which, of course, are critical to the study. The uterus is removed from the euthanized female and its contents examined for embryonic and fetal death and live fetuses. Mean litter weights are recorded for each mother. Uterine implantation sites are counted and correlated with the number of live and dead fetuses to determine if any fertilized eggs implanted in the uterus failed to mature and were reabsorbed.

Each fetus is weighed and measured and its sex determined. Each is examined for external and internal anomalies, including all major organs. The soft tissue is removed from some or all of the fetuses to facilitate skeletal examination, and all bones—particularly the vertebrae, long and short bones, and head bones—are checked for size, shape, position, and hardening.

Reproduction/Fertility Studies

Reproduction studies address the effects of the pesticide on male and female reproductive processes, from egg and sperm production and mating through pregnancy, birth, nursing, growth and development, and maturation. The studies are conducted through two generations of offspring—that is, three generations including the parents. Groups of young adult rats, both male and female, are fed diets containing various concentrations of the pesticide.

The animals are fed the treated diet continually for approximately ten weeks prior to mating and through pregnancy, birth, and nursing of the young. Potentially, the fetuses are exposed to the pesticide in the womb, through the mother's blood, and later when nursing the mother. At about 21 days of age the pups are weaned and thereafter fed the same treated diet as the mother. At sexual maturity, they are mated with other pups from the same treatment groups; e.g, females on the high dose treatment regimen are mated to males also on the high dose. The males are then euthanized and the females fed the high-dose diet throughout pregnancy. Thus, the cycle is repeated as the females deliver second generation pups, which are allowed to nurse and grow for 21 days. The adult females are then euthanized.

All animals are observed daily; behavioral changes, signs of toxicity, food consumption, body weight, and mortality are recorded for each animal. Litters are examined for number of pups, stillbirths, and live births.

Two Generation Reproduction Study
## With Daily Dosing of Adults and Offspring

Young Male Rat　　**Young Female Rat**

Parents

10 weeks' growth

S M T W T F S

Mate

Autopsy Males　　　**3-Week Pregnancy**

S M T W T F S

1st Generation

Deliver and Nurse Young

Autopsy Mothers　**Young Male Rat**　**Young Female Rat**

10 weeks' growth

S M T W T F S

Mate

Autopsy Males　　　**3-Week Pregnancy**

S M T W T F S

2nd Generation

Deliver and Nurse Young

Autopsy Mothers　　　**Examine Offspring**
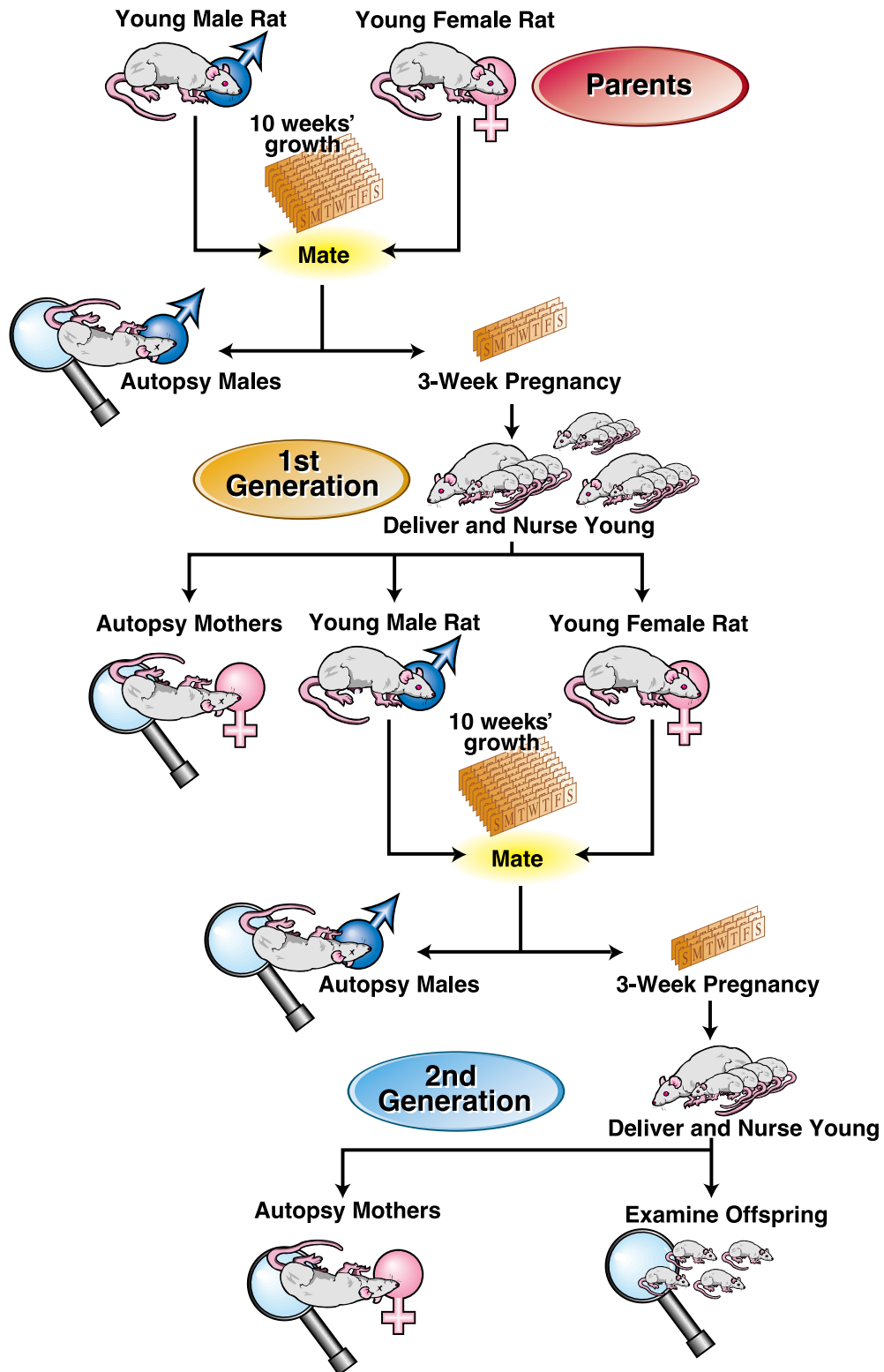
Live pups are weighed at birth and thereafter on days 4, 7, 14, and 21. All animals are autopsied, with special attention directed to the organs of the reproductive system.

Records are kept on

- egg and sperm formation and viability;

- estrus cycles;

- mating, conception, and pregnancy;

- fetal development and survival;

- birthing and nurturing;

- growth, development, and survival of offspring through two generations; and

- toxic effects in mothers or offspring.
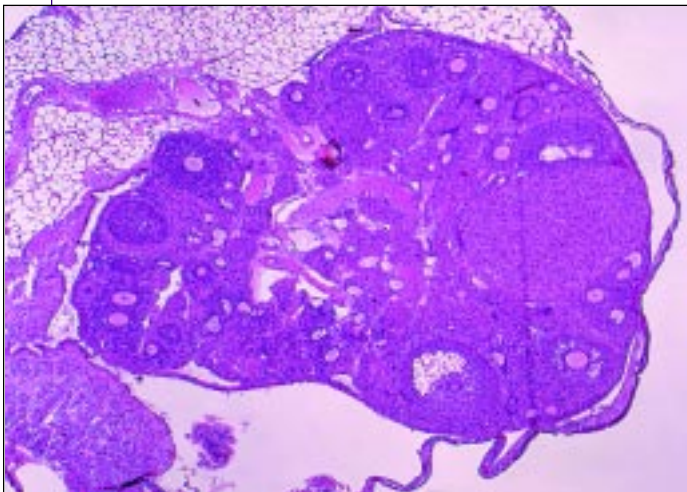
Normal ovary from a mouse (magnified). Numerous eggs at various stages of development are present.
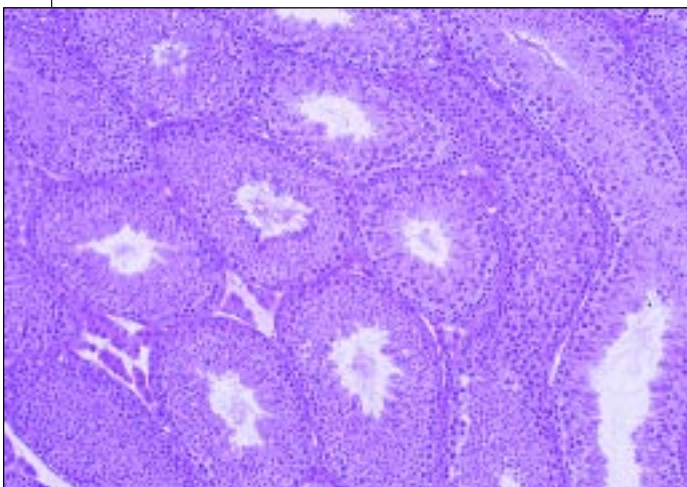
Normal testes from a mouse (magnified). Spermatozoa can be seen in the middle of the tubules.

	Reproductive Protocols	
Parameter	Development	Reproduction
Preferred test species	rat and rabbit	rat
Age of animals	young adults	6–8 weeks old at start of dosing
Pregnancy status	pregnant	not pregnant at start of dosing
Substance tested	active ingredient	active ingredient
Number of dose levels	3	3
Number of animals/dose	rat, 20; rabbit, 20	60
Male/female ratio	females only	50/50
Type of control group	untreated or vehicle	untreated
Route of administration	stomach	oral (in diet or water)
Exposure (days)	daily through pregnancy	continuous through two generations

Genetic Toxicology

Genetic toxicology is the study of how a chemical interferes with the genetic material of a cell. It is studied in two ways:

• One is by visual inspection, through a microscope, of the damage to whole chromosomes. Damage at this level is referred to as chromosome damage, or aberrations.

• The second is by determination of any damage to genes (DNA sequences) that are too small to view under a microscope. Damage to genetic material inside the chromosome is referred to as gene mutation.

The body often can repair genetic damage, but unrepaired damage can result in mutation. In general, a mutagen is defined as a substance that permanently changes the genetic material of a cell. Mutations in germ cells (eggs or sperm) can cause heritable effects (those that can be passed on to future generations). Mutations in somatic cells, i.e., all cells but germ cells, may result in health problems, including cancer.

In order to determine if a pesticide is a mutagen, a battery of genotoxicity assays is conducted. This battery evaluates the potential of the chemical to cause damage to both chromosomes and genes both in cell cultures and in animals. Although the goal of this testing program is to identify mutagens, especially those that could affect future generations, the tests also are valuable as screens for potential carcinogens. The various individual tests which comprise the battery detect different kinds of genetic damage. Genetic toxicology uses *in vitro* (cell culture tests in bacteria or yeast and mammalian cells) and *in vivo* (whole animal) tests. Because a chemical may have the potential to cause different kinds of damage, a tiered approach to assessing each end point (gene or chromosome) is used. A positive response in a lower-tier test leads to more informative and complicated, higher-tier testing.

Chromosome Damage

DNA

Gene Mutation

normal chromosome
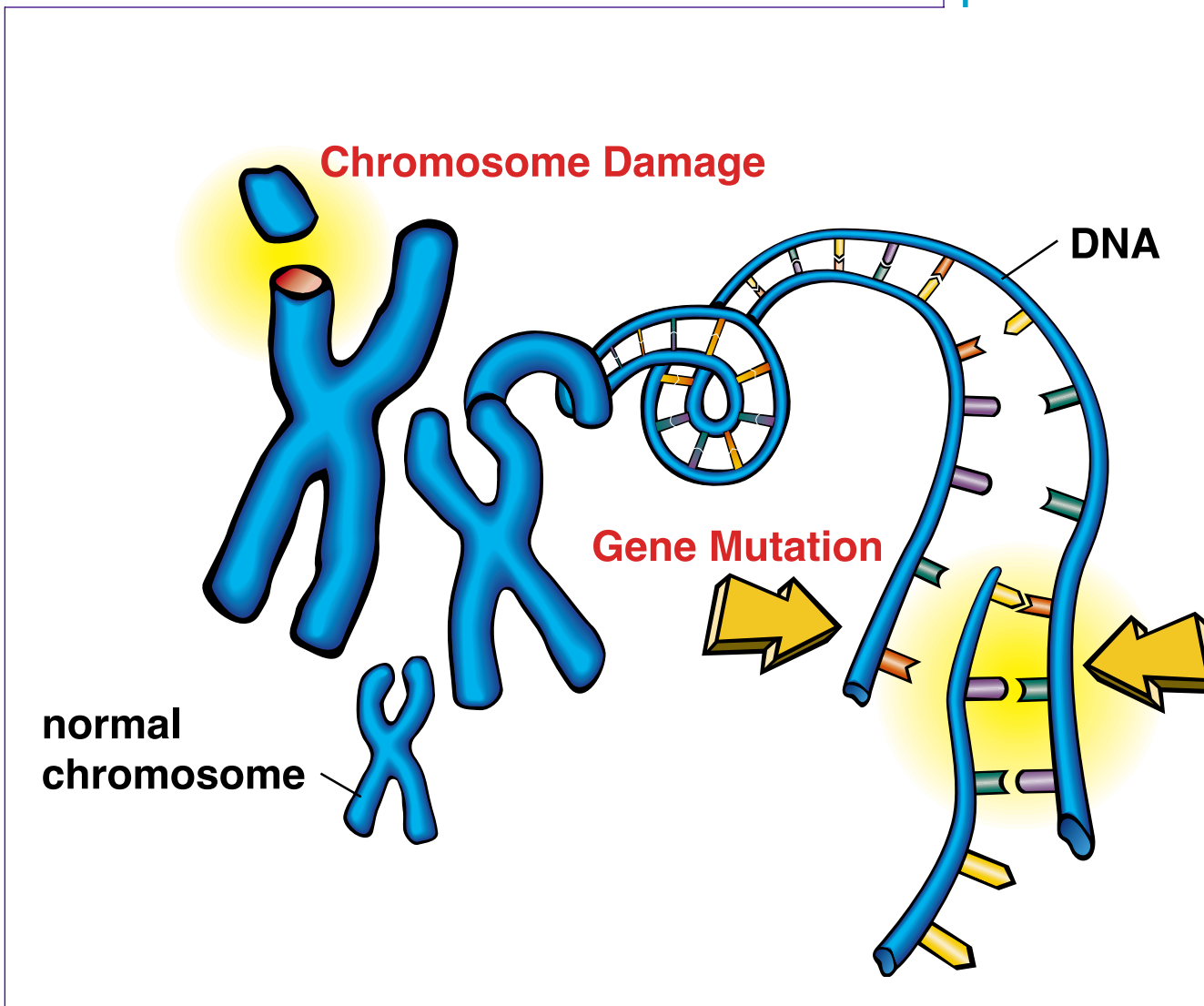
An *in vitro* test battery provides the foundation of genotoxicity screening. It is the first tier of testing and often the most sensitive. Because these tests are so sensitive, many positive responses that need further characterization are obtained.

The second tier generally is performed in the whole animal. These tests are not as sensitive but are more relevant. Like other toxicological tests in animals, the chemical must be absorbed and metabolized and must reach the target organ. These tests allow complex physiological factors to be examined.

The third tier focuses on germ cells. A positive result in this higher-order test is a serious indication that the chemical can cause effects in future generations. A positive genotoxic response in somatic cell assays requires investigation into the potential of the chemical to cause germ cell mutation. In almost all cases, a germ cell mutagen is also a somatic cell mutagen; but most somatic cell mutagens are *not* germ cell mutagens. Most chemicals do not reach the germ cell; and of those that do, most tend not to interact with the DNA.

Interpretation of test results can be challenging. Because a battery of tests is used, not all tests will be positive, nor all negative. A chemical is assessed as a mutagen by first evaluating individual test end points. A single positive response in a test does not necessarily determine that a chemical is a mutagen. The overall assessment takes into consideration whether the test is *in vitro* or *in vivo,* whether it involves mammalian or nonmammalian cells, and whether somatic or germ cells are used.

in vitro

in vivo

germ cells

Tier I

Tier II

Tier III
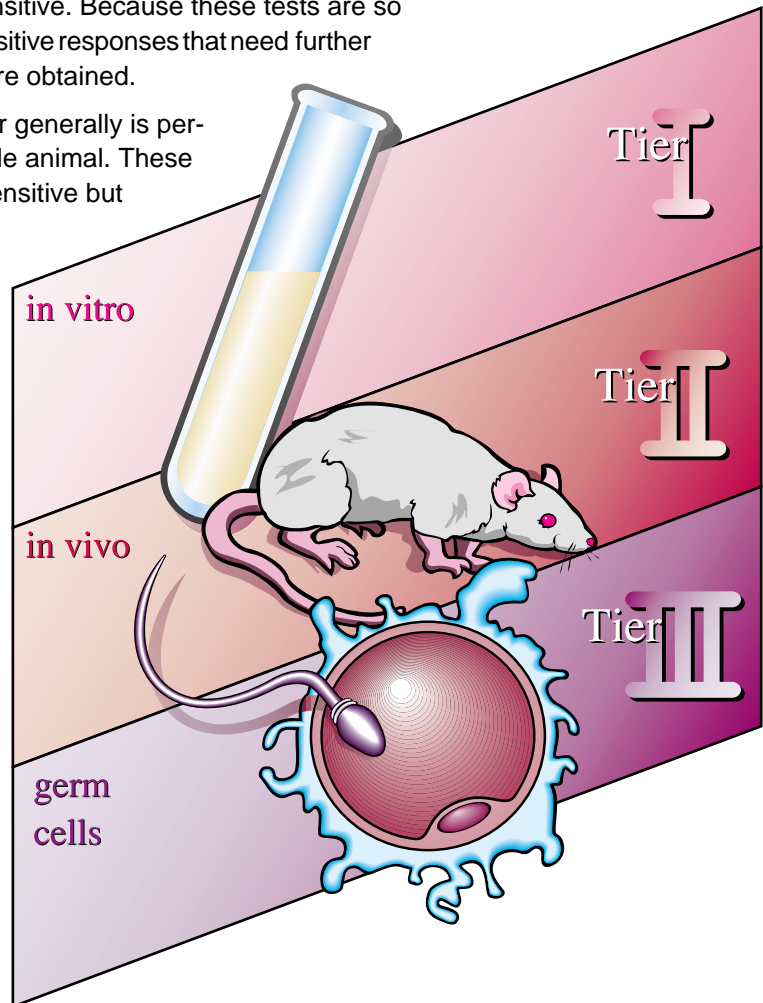
Questionable results generally are resolved by advancing to a higher level in the hierarchy of testing. For example, a positive bacterial cell test may be confirmed by an *in vitro* test in mammalian cells or in the whole animal, and a negative mammalian test normally supersedes a positive response in a bacterial test. A negative germ cell test, however, does not negate mutagenicity in somatic cells. Generally, a mutagenic response for a new pesticide will halt further development of that chemical.

Regulatory Testing for Mutagenicity

Numerous tests have been developed to detect chemical induction of both gene and chromosome damage. A battery of tests is performed, initially, to assess a chemical's genotoxic potential. EPA requires that the battery include a bacterial gene mutation test, an *in vitro* mammalian gene mutation test, an *in vitro* mammalian chromosomal aberration test, and an *in vivo* mammalial chromosomal aberration test.

Bacterial Gene Mutation Test

The *Salmonella typhimurium* bacteria gene mutation assay was developed in 1973 by Dr. Bruce Ames and is commonly referred to as the Ames test. It has proven an efficient and reliable method for testing large numbers of chemicals and has enhanced the detection of potentially mutagenic and/or carcinogenic compounds.

There are a number of *Salmonella* strains used in this assay. Each is engineered to detect different types of gene mutations, such as a frame shift or base pair. To make the system sensitive for the detection of potential mutagens, the bacterial cell walls are altered to be more permeable to chemicals otherwise excluded, and the bacteria are modified to *not* repair some types of DNA damage. Because bacteria do not metabolize chemicals as do animals, rat liver enzymes are added to the bacteria cultures to approximate the metabolism of mammalian systems and allow the detection of mutagenic chemicals that are only mutagenic in their metabolized form.

The procedures for conducting the Ames test are quite simple. Bacteria are treated with the chemical, with and without metabolic activation, and grown on agar plates in such a way that only the mutated bacteria will grow. Bacterial colonies are counted after two

days and compared to the number of colonies growing that were not treated with the pesticide. A positive mutagenic response occurs when the number of colonies with the pesticide treatment is 2–3 times higher than the number of colonies from untreated bacteria cultures.

In Vitro Mammalian Gene Mutation

This group of tests is very similar in concept to the *Salmonella*/Ames bacteria test. Mammalian cell lines that are sensitive to gene mutation are selected. The most commonly used mammalian cells are cultured from Chinese hamster ovary (CHO), Syrian hamster embryo (SHE), V79 hamster, and mouse lymphoma cells. One of the most common mammalian in vitro gene mutation tests is the CHO/HGPRT assay, so named because it uses CHO cells and monitors gene mutation in the HGPRT gene. Metabolic activity is achieved by introduction of rat liver enzymes. Cells are treated for 4 to 18 hours, after which the chemical is removed and the cells grown for a short time. The selective agent 6-thioguanine that kills all normal cells is then added, and only mutant cells will grow. Growing cell colonies are counted and mutant frequencies determined.

In Vitro Mammalian Chromosomal Aberration

In this type of assay, CHO cell lines or cultured human cells (lymphocytes) are most commonly used. Cells are exposed to the chemical in the presence and absence of rat liver enzymes. The cells are stained to facilitate microscopic examination to count the number of chromosomes present and identify chromosome abnormalities. Scientists look for deformed and extra chromosomes, and for the loss of chromo- somes. The findings are compared to those of untreated control cells.
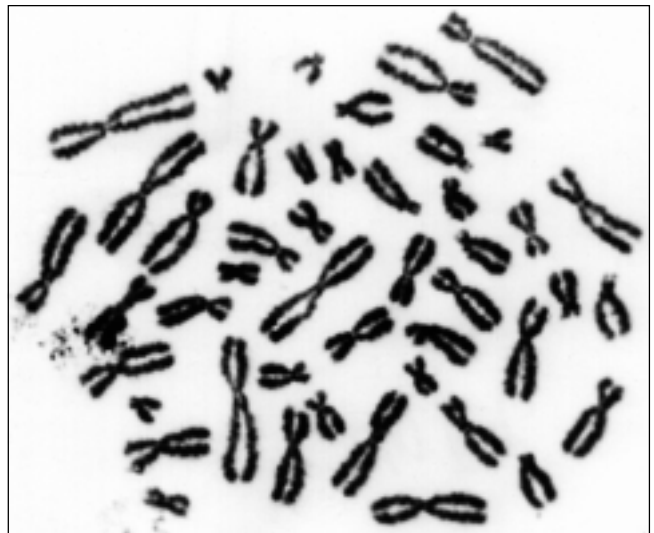
Chromosomes have different sizes and shapes, thus making them easy to identify.

In Vivo Mammalian Chromosomal Aberration

The two most commonly used tests for this end point are the mouse micronucleus test and the rat bone marrow chromosomal aberration assay. Animals are administered the chemical by stomach tube or injection. After 24 hours, they are sacrificed and their bone marrow cells removed. As with in vitro, scientists look for deformed and extra chromosomes, and for the loss of chromosomes. The findings are compared to those of untreated control cells.

In the micronucleus test, bone marrow is removed from treated and untreated mice and stained for the presence of DNA. Normal red blood cells (RBC) do not have a nucleus and therefore do not have chromosomes or DNA; i.e., normal red blood cells do not stain. The DNA of mutated cells, on the other hand, does stain. Evaluations are conducted under the microscope.

Germ Cell Mutations

There are two basic tests for germ cell effects: a *Drosophila* (fruit fly) sex-linked recessive lethal assay, and a rat dominant lethal test. In rare cases, a mouse spot (specific locus) test or a mouse heritable gene translocation assay is required. The mouse spot and the heritable gene translocation tests require a large number of animals and are costly and cumbersome to perform. Pesticides that test positive in the *Drosophila* or mouse dominant lethal test generally are withdrawn from development and marketing without additional germ cell testing.

Pharmacokinetics: Absorption, Distribution, Excretion, and Metabolism

Pharmacokinetic studies determine how a pesticide moves into, gets distributed within, and finally leaves the body. The studies are designed to address several major areas of interest:

• The quantity of pesticide absorbed;

• The distribution of the pesticide in tissues, organs, blood, and urine;

• The identity, quantity, and location of the major metabolites;

• The ability of the pesticide to be stored in tissues and organs;

• The routes of excretion; and

• The differences in the absorption, metabolism, excretion, and distribution of a pesticide when animals are administered single doses versus repeated doses, or small doses versus large doses.

Special studies may be required to better define the pharmacokinetic properties of a pesticide. These studies might address tissue residues, placental transfer, occurrence in breast milk, and metabolism by specific organs, tissues, and cells. Comparative studies with different animal species are useful, also, to explain species differences in toxicity.

Testing Protocols

A radioactive tracer (Carbon[14], sulfur[35], tritium) is incorporated into the pesticide molecule, making it possible to track the pesticide or its by-products as they move within and are expelled from the body. Young adult rats are assigned to several groups for which the dose differs in level, method of administration, and frequency. The adult rats are housed in metabolism cages equipped to collect urine and feces. Animals are observed for 7 days after the administration of the dose or until 90 percent of the dose has been excreted in urine and feces, whichever comes first. Four typical groups are summarized below.

Group A: A low, single dose of the tracer pesticide is administered by intravenous injection.

Group B: A low, single dose of the tracer pesticide is administered orally by capsule or stomach tube.

Group C: A low dose of the pesticide, without the tracer, is administered by capsule or stomach tube daily for 14 days. The animals then receive a low, single dose of the tracer pesticide 24 hours after the last dose of the nonlabeled pesticide.

Group D: A high, single dose of the radiolabeled pesticide is administered orally by capsule or stomach tube.

Urine, feces, and various animal tissues are analyzed at various points in time following pesticide administration to determine levels of pesticide and metabolites.

Pharmacokinetic studies can be subdivided conceptually into three distinct phases:

• The *distribution phase* determines where the pesticide goes in the body.

• The *metabolism phase* chemically analyzes the identity and amount of metabolites in urine, feces, and tissues.

• The *excretion phase* determines the extent to which the pesticide and its metabolites are eliminated from the body, and how long it takes.

Conclusions

The public hears a steady barrage of pesticide issues: endocrine disruptions, multiple chemical sensitivity, reproduction problems, cancer, and others. No longer a bystander, the public asks why pesticides are used, how we know that pesticides are not contributing to health problems, and countless "what if" questions. The public clearly expects solid data to support government decisions on pesticides.

"What if" questions drive regulatory agencies, producers, application industries, the media, and public interest groups. The pesticide debate subsides and intensifies in cadence with controversial issues addressed in the public forum. Discourse on some issues spans years as opposing points of view shape public opinion and regulatory policy. The issues take old knowledge to the fringes of contemporary science. Government agencies must adjust their registration requirements to keep pace with scientific capabilities. Pesticide manufacturers must adapt their research to reflect the latest, most sophisticated procedures available, thereby lending credence to the data supporting registration. The public must base inquiries on scientific fact, therein trusting science and regulation to measure and control the potential benefits and risks associated with the use of pesticides.

An effective pesticide policy requires public confidence in the regulatory, scientific, and business communities. Such trust can be gained only through open, balanced, fact-based knowledge and effective communication of pesticide safety evaluations. Research, education, and cooperation are the critical elements of pesticide risk management.
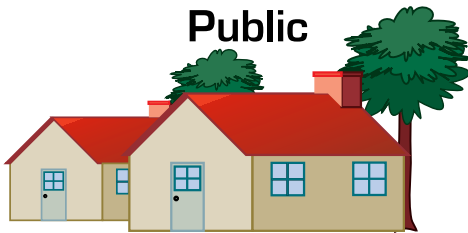
EPA, through two decades of public policy decisions, has guided pesticide manufacturers concerning what kinds of data and what steps are necessary for pesticide registration. EPA's message to the pesticide manufacturer is clear: Provide beneficial products with minimal risks—a policy with strong influence on the kinds of pesticides being developed. The shift is toward the development of reduced-risk, yet effective pesticide products to benefit the end user.

Society at-large—manufacturers, government, health professionals, users, educators, the media, and the public—shares responsibility for understanding and reducing pesticide risks to human health. Manufacturers are developing reduced-risk pesticide products with increased benefits, and they are promoting good stewardship of products in the marketplace. State and federal agencies must continue to require comprehensive testing of all pesticide active ingredients and require new tests when needed to answer emerging questions. Government must balance its oversight of

Government

Research

Health

Public

Cooperation

Manufacturing

ACME PESTICIDE CO.

Media

Daily Times

Education

PURDUE UNIVERSITY

Educators

X + Y = Z
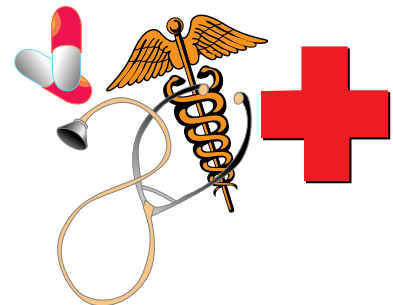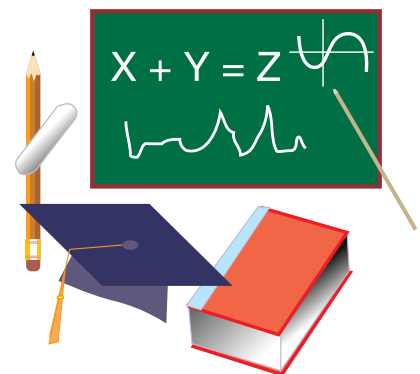
pesticide product registration and regulation with the benefits of modern pest management technology. It is imperative to guard against unacceptable risk to the public and the environment and simultaneously award the benefits of prudent, chemical pest management. Scientists must continue to channel energy and resources into research to address questions on adverse health effects resulting from pesticide exposure. All pesticide users—homeowners as well as private and commercial applicators—must shoulder the responsibility for safety: Use precautions stated on pesticide labels *must* be heeded. Universities must take the lead in enhancing educational methods for delivering pesticide-related information. And the media must accept responsibility for accurate, balanced reporting of *factual information* on which the public can base informed decisions.

Society should expect maximum benefit and minimum risk from the use of pesticides. As the preventive philosophy of current regulatory policy merges with new and exciting technologies, the development of low-risk, effective pest management strategies will enhance protection of human health and the environment.

~ *Acknowledgments* ~