Chapter 8

Alternatives to Animal Use in Testing

Queen: ***I will try the forces***
Of these compounds on such creatures as
We count not worth the hanging, but none human . . .
Cornelius: ***Your Highness***
Shall from this practice but make hard your heart.

Shakespeare, ***Cymbeline***
Act I, Scene VI

The experimental means to be used for safety evaluations is left open to suggestion. As unorthodox as this might sound, leaving such means open for consideration is the best solution. *Safety evaluations* ***should not be based on standard, specified series of tests. They*** *are best* ***approached by first raising all pertinent safety questions and*** then ***searching for the experimental means to provide the best answers. Under such circumstances, even the standard LD test might on occasion be the best experimental means to resolve outstanding satfety questions.***

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D. Homburger (cd.) (Base]: Karger, 1985)

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Alternatives to using animals in testing serve the same purposes that using whole animals does—protecting and improving human health and comfort. The technologies on which alternatives are based result primarily from biomedical and biochemical research. Several of them are reviewed in this chapter, though they are discussed in greater detail in chapter 6. Some alternatives that might eventually replace the tests covered in chapter 7 are also described here.

Notable progress in the move to alternatives has been achieved in certain areas (78). For example, biochemical tests to diagnose pregnancy have replaced those using rabbits, and the ***Limulus*** amebocyte lysate test, which relies on the coagulation of a small amount of blood from a horseshoe crab, has replaced rabbits in testing for the presence of bacterial endotoxins that would cause fever (25,117). Many companies have modified the widely used LD_{50} test to use fewer animals (22) and have otherwise refined the methods used to test for toxicity (100). Mammalian cell culture assays are used extensively in industrial laboratories for safety testing of medical devices (52,53) and pharmaceutical substances (1,84) and as immune response assays (97,98).

The development of alternatives to animals in testing has accelerated in recent years with the establishment of programs having development and implementation of alternatives as their goal (see ch. 12). However, the barriers to adoption of these tests are more than the technical barrier of developing and validating anew technology. Testing is an integral part of many regulatory schemes and product liability law, and validation ultimately rests on acceptance by the scientific, regulatory, and legal communities.

Public concern over animal use in testing appears to be increasing in tandem with public concern for product and drug safety . Ironically, the public's-increasing concern for safety could lead to more testing. Yet it also provides an incentive to develop new techniques, particularly those that promise to be cheaper and faster than current whole-animal methods. A further irony is that developing alternatives, as well as validating them, sometimes requires animal use.

CONTINUED, BUT MODIFIED, USE OF ANIMALS IN TESTING

It has been suggested that many more animals are used for testing than are needed (90) and that changes in experimental design or improved methods of data analysis could substantially reduce the number of animals used. Each experiment has unique requirements (see ch. 7), and the ways in which the number of animals might be reduced will vary accordingly.

Many of the methods discussed in chapter 6 for the modified use of animals in research are also applicable to testing, such as gathering more data from each animal or improving the analysis of results by using random block design or covariance analysis. In random block design, animals with a particular characteristic, such as litter mates or animals of a certain size, are randomly assigned to different groups to balance whatever effect these variables might have. If the groups being distributed are sufficiently large, the results can also be analyzed to determine the effect of the masking variable (47). Covariance can be used to analyze results when some of the experimental variables are uncontrolled but known, thus estimating their effect on the results.

As in research, the number of animals needed as controls can be reduced by using the same group as a control for several simultaneous experiments. A laboratory's ability to do this will be limited by its size and the amount of lead time available to allow testing to be coordinated. Another difficulty is that environmental conditions must be exactly the same and the tests must start and finish at exactly the same times. The reduction in animal use that simultaneous experiments brings about is

modest because the control group should be larger if it is being used in several simultaneous experiments (34),

The use of historical data for control groups is constrained by the difficulty of exactly duplicating the conditions of a study. However, the size of the groups and other controlled variables can be better planned if historical data are used to discover the background incidence of specific tumors or other diseases before testing begins. This use of historic controls has been recognized by the National Cancer Institute, the world Health Organization, the Canadian Government, and the now-defunct Interagency Regulatory Liaison Group (104). The Federation of American Societies for Experimental Biology has developed a data book containing such information based on the Laboratory Animal Data Bank (see ch. 10) (2).

Avoiding Duplicative Testing

Animal use in testing can and has been reduced by industry and others through improved communication and cooperation in the planning and execution of testing, thereby avoiding unintentional duplication. Trade groups such as the Chemical Manufacturers Association, the Pharmaceutical Manufacturers Association, and the Soap and Detergent Association play important roles in this coordination.

The sharing of data after testing has occurred is often done for pesticides (see chs. 10 and 11). And in 1978, the Food and Drug Administration implemented a policy of permitting approval of new drug applications solely on the basis of published scientific papers (113). The possibility of an unintentional repetition of an experiment is also avoided through the work of organizations such as the Chemical Industry Institute of Toxicology (CIIT) (Research Triangle Park, NC). Using contributions from member companies, CIIT conducts toxicological tests and distributes the results widely.

Governments contribute greatly to information sharing, which allows duplicative testing to be avoided, by providing both access to test results and information about their own planned and ongoing tests. The International Agency for Research on Cancer makes it easy for duplicative carcinogenicity testing to be avoided by informing testing facilities and governments about planned and ongoing testing, Federal and international databases and publications also contain information about planned tests and those under way (see ch. 10).

Reducing Pain and Distress

As with research, testing can be modified to reduce animal pain or distress in two ways: by providing relief with drugs or by changing the procedures so that less pain or distress is produced (see ch. 6). A third alternative might be to use a less sensitive species, but there is no method by which relative distress among species can be discerned. Relief from pain and distress is accomplished through analgesics, anesthetics, tranquilizers, or sedatives and modification of the test itself.

Few pain-relieving drugs have been developed and marketed for animals. Little information is available on recommended doses (122) or on the likely effect on test results. Thus, before pain relief could be incorporated into a test, it would be necessary to determine the needed dose and the effect on the toxic response, thus using additional animals as well as subjecting them to pain.

Several small changes that do not interfere with the experimental design can be made by an investigator. Small needles can be substituted for large. Animals can be comforted by petting. Social animals can be caged in groups, although there are often reasons that multiple housing cannot be used. Smaller doses can be used and tests can be ended at the earliest feasible time. Sometimes, smaller doses will actually result in increased sensitivity of the test (38). Making such changes sometimes depends on the attitude and expertise of individual researchers rather than the contents of testing guidelines, which may not be sufficiently detailed.

USE OF LIVING SYSTEMS IN TESTING

As detailed in chapter 6, two kinds of living systems can reduce whole-animal use—in vitro systems based on animal or human components (cell, tissue, and organ cultures) and systems based on organisms not considered animals for purposes of this report (micro-organisms and invertebrates). (Some people consider both of these in vitro system s.)

In Vitro Systems

Cells, tissues, and organs can be kept alive outside a living organism and used for testing. Although animals are still required as a source for these in vitro systems, the animal would experience distress for a much shorter time, and perhaps less distress overall, than occurs with whole-animal testing because it would be killed before any experimental manipulations were carried out. Occasionally, different cells, tissues, or organs from the same animals can be used for different investigations. In addition, many fewer animals would be required for a given test, in part because variability in the toxic response is smaller than it is with whole-animal tests and in part because one animal can be used for multiple data points, further reducing variability. The fact that human tissues sometimes can be used confers an additional advantage because the need for extrapolation from animal data is obviated.

These isolated components also have disadvantages. They are usually unable to produce the complete physiologic responses of a whole organism. The components often become undifferentiated and lose their ability to perform their special functions when isolated from the organism, particularly when the sample is broken up into its constituent cells, and even more so when the cells replicate. Another disadvantage is that the effect of the route of exposure, a variable that can have profound effects on test results, is often impossible to determine.

There are many measures of damage to differentiated or undifferentiated cells—the rate of reproduction, the rate of synthesis of certain substances,

Microscopic View of Cell Culture From Rabbit Corneal Epitheliums

Photo credit: Kwan Y. Chan, University of Washington

changes in membrane permeability, and damage to some part of the cell structure. Those functions having to do with viability and growth are most frequently measured because they require an integration of many physiologic events within the cell, are sensitive, and lend themselves to automation (73).

Quantifiable tests are preferred over subjective ones, and a wide variety of quantitative approaches are available to measure irritation, including the release of prostaglandins (35); the production of enzymes (46), proteins (57), antigens, antibodies, or hormones (73); and the migration of certain white blood cells (macrophages) to the area of irritation (12,101). Irritation can also be measured by the extent to which cells exfoliate from the surface of the tissue. The extent of damage can be determined by counting cells and by examining the nuclei (1O2). Another indicator of irritation, the integrity of cell membranes, can be monitored through the uptake of nutrients through the cell wall. Where the nutrient uptake is active (that is, when the cell is required to expend energy for transport), uptake can also be used to indicate changes in metabolism (86,102).

Liver cells have been the subject of considerable research, in part because they play such an important role in an organism's removal of toxic substances and in part because they retain most of their special functions when cultured. The response of liver ceils to toxic substances may be measured in many ways: the use of sugar as an indication of metabolic activity; the production of proteins or other substances that have been correlated with toxicity; uptake of amino acids as an indication of protein synthesis; changes in appearance that parallel those observed in livers of whole animals (106); and morphological changes and reductions in viability (75). Other promising techniques in this rapidly expanding field include culturing:

- beating heart cells to detect the effect of certain vapors on irregularities in heartbeat (68);
- rabbit kidney tubules to detect substances that can cause acute renal failure, and rat vaginal tissue to test vaginal irritancy of contraceptives (27);
- various kinds of cells to test for biocompatibility of implants (15,52,53); and
- nerve cells to test for the synthesis of neuro-

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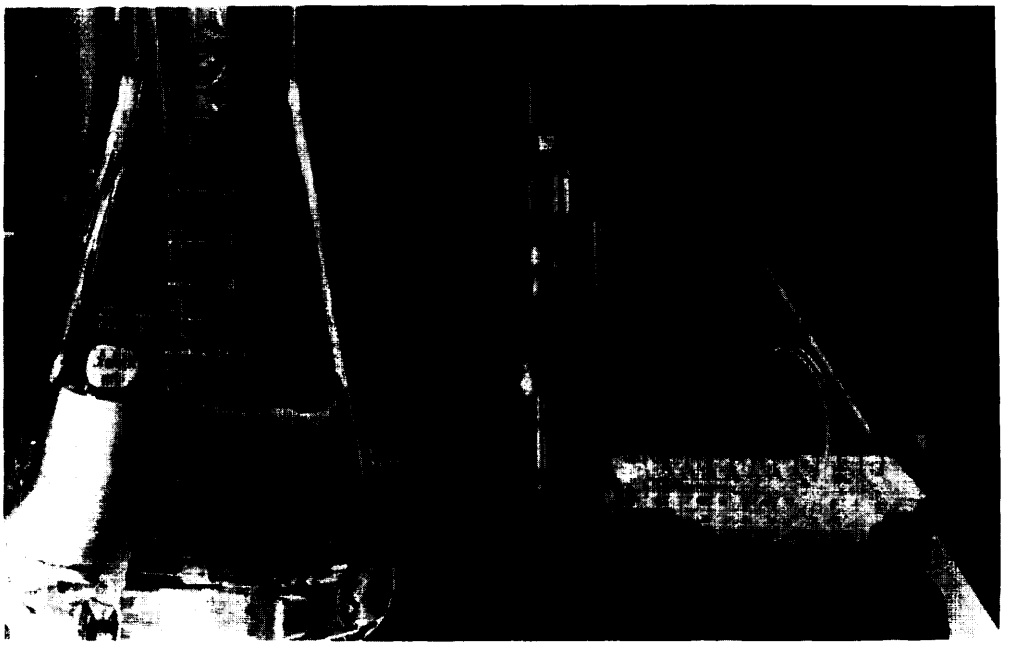


Photo credit: The Johns Hopkins University

transmitter chemicals, the formation of synapses, and the conduction of impulses (7).

Although tissue and organ cultures may approximate more closely the physiology of the human or whole-animal model, they are more difficult to manipulate than cell cultures (see ch. 6). Sophisticated equipment must be used to monitor and control the environment and to perfuse the sample with nutrients. Where the sample is more than a few cell layers thick, uniform delivery of the test substance, nutrients, and oxygen is difficult, as is the removal of waste products. Cell differentiation can usually be maintained in tissue and organ cultures, albeit with some difficulty (50).

Human placentas have proved quite useful in testing the ability of a drug to cross the placenta from mother to fetus. There are certain logistical problems with this method, however. The placenta must be transferred to the perfusion apparatus within 5 minutes after it is eliminated from the uterus, and it is only useful for about 3 hours afterward (77).

Nonanimal Organisms

There are a variety of nonanimal organisms that can replace some animals in testing, ranging from plants to single-celled organisms to invertebrates. All of these can respond to certain noxious stimuli, and some may experience pain. However, many commentators believe that they do not experience pain or suffering in the same way that animals do, particularly in those cases where there is no brain or neural tissue (90). The use of such organisms, which has never been controlled under any Federal or State law, is regarded as a replacement for animals in this report.

Micro-organisms

In recent years, increased emphasis has been placed on the use of bacteria and fungi to measure certain genotoxic effects. A major advantage of these organisms is that they can be cultivated much more easily and quickly than most animal or human cells. Their genetic makeup is simple compared with that of animals and humans and the fact that a great deal is known about it facilitates their use, particularly in toxicological research leading to new methods (74). A change in genetic material is relatively easy to detect and characterize. Fungal systems have been shown to be especially useful in mutagenicity testing and seem to be more sensitive than bacteria (126), perhaps at the expense of falsely indicating a hazard. Other species that have proved useful include slime molds, algae, and protozoa (74).

Protozoa, although rather primitive overall, frequently have specialized functions that mimic those of humans. For example, the cilia of protozoa respond to smoke or phenols as do the cilia in the human bronchial tube (5). Various protozoans have been used in toxicity testing of cigarette smoke. protozoans are currently being evaluated for use in screening tests for carcinogenesis, mutagenesis, and reproductive toxicity (93).

Invertebrates

Invertebrates have made major contributions in biomedical research because certain aspects of their physiology are sufficiently similar to that of mammals (74). Although models for toxicity testing require greater similarity to animals or more thorough characterization of differences than models for research, invertebrates offer exciting possibilities.

Of the invertebrates, insects offer the greatest selection of models, there being over 2 million species from which to choose (74). Among them, the fruit fly, ***Drosophila rnelanogaster,*** is the best understood. procedures have been developed for detecting mutagenicity (18), as well as teratogenicity (11) and reproductive toxicity (93).

The sea urchin has long been a favored test organism for basic reproductive research (74). Consequently, the mechanisms and procedures of testing this invertebrate can easily be developed and performed. The sea urchin model for fertilization and development can be used in screening for reproductive toxicity, teratogenicity, and mutagenicity. Nematodes, annelids, and mollusks are also used for alternative mutagenesis testing regimes and, additionally, mollusks are used in the area of reproductive toxicology. Sponges, mollusks, crustaceans, and echinoderms are being used in metabolism studies, as understanding metabolize formation in nonmammalian species can lend insight to interspecies variation (93).

USE OF NONLIVING SYSTEMS IN TESTING

Animal use can sometimes be avoided altogether with nonliving biochemical or physiochemical systems, although most such systems currently require animal derived components. Computer simulation can also be used when there are sufficient data available for substances related to the one of interest and when the mechanisms of toxicity are at least partially understood.

Chemical Systems

Whole animals have been replaced with analytical chemistry for tests involving detection of a substance or measurement of potency or concentration, such as for vaccines, anticancer drugs, and vitamins (10). However, toxicity testing in nonliving systems is quite limited at this time.

Recently developed methods of detection or measurement are based on the selective binding that occurs between a particular substance and the antibodies to it. In an assay for botulism toxin (which traditionally required up to 200 mice), antibodies obtained from rabbits are modified so that the binding of the toxin can be detected easily. The rabbits are initially injected with a small, harmless dose of the botulism toxin. Small amounts of blood are then removed from the rabbits at regular intervals. In 4 weeks, a rabbit can produce enough antibody, with little discomfort, to perform tests that would otherwise require thousands of mice (32).

Chemical systems that test for toxicity are based on determining whether a substance undergoes a specific reaction. For example, it is well known that carbon monoxide binds to hemoglobin in the blood, thus greatly reducing the blood's ability to carry oxygen. The extent to which a substance would displace oxygen in hemoglobin can be a measure of its ability to produce asphyxiation. Substances can also be tested in isolation for their effects on enzymes crucial to certain bodily functions.

An important limit of chemical systems is that they do not indicate the extent to which an organism can recover from or prevent these reactions. For example, a substance that binds strongly to hemoglobin may not be a problem because it is not absorbed. A substance will not have a significant effect on an enzyme of interest if it is excreted before it has an effect.

Physiochemical systems have some ability to determine whether a substance will be absorbed and what will happen to it. The tendency of a substance to accumulate in a biological system can be roughly estimated by the relative proportions that dissolve in equal volumes of water and the organic solvent octanol (34,55). Artificial skin made with filter paper and fats is being tried as a means of mimicking absorption of cosmetics and drugs (45). Reactivity and other toxicity-related properties can be deduced from chemical structure alone (109).

Mathematical and Computer Models

Advances in computer technology during the past 20 years have contributed to the development of sophisticated mathematical models of quantitative structure activity relationships (QSAR). These models are used to predict biological responses on the basis of physical and chemical properties, structure, and available toxicological data. The limitations of such models are due in part to a lack of understanding of the mechanisms by which toxic effects occur.

In applying QSAR, the biological effects of chemicals are expressed in quantitative terms. These effects can be correlated with physiochemical properties, composition, and/or structure. Frequently used properties include an affinity for fats versus water (octanol/water partition coefficient), the presence of certain reactive groups, the size and shape of molecules, and the way reactive fragments are linked together.

The simplest extrapolation is for a series of closely related chemicals. The several characteristics they have in common need not be incorporated into the model as variables. This type of analysis has been performed for several hundred families of chemicals and has established that relationships within a series are fairly predictable (64).

Another approach, more broadly applicable, is to examine the contributions of various portions of a molecule. In more elaborate computer pro-

grams, it is possible to identify likely reactions and cascading physiological events in various species, techniques first developed for pharmacology (54). A similar approach is the use of multitiered classification schemes that use large databases to draw semiempirical conclusions (36).

Epidemiologic Data on Humans

Perhaps the most useful alternative to animal testing is epidemiologic studies on humans. Such studies were used to detect carcinogenicity in humans as early as the 18th century (49,85,87). The most well known study detected scrotal cancer in chimney sweeps (85). A more recent example in which epidemiologic evidence was used to detect a human carcinogen was the finding that vinyl chloride causes a rare liver cancer in humans (26). A major disadvantage of epidemiologic studies is that considerable human exposure can take place before a toxic effect is detectable, particularly in the case of diseases that take many years to develop. Another disadvantage is that they can be quite expensive to conduct. Privacy must also be considered (112), preventing many data that would be useful from being collected or analyzed.

Epidemiologic studies may be divided into three general types: experimental, descriptive, and observational. Experimental epidemiology is the human equivalent of animal testing—providing or withholding a substance to determine its toxic or beneficial effects. Such studies are greatly limited by ethical and legal considerations, as well as the difficulties involved in securing the cooperation of a large number of people.

Descriptive epidemiology analyzes data on the distribution and extent of health problems or other conditions in various populations, trying to find correlations among characteristics such as diet, air quality, and occupation. Such comparisons are frequently done between countries or smaller geographic regions, as is the case for cancer statistics collected and analyzed by the National Cancer Institute (9).

observational epidemiology uses data derived from individuals or small groups. Data would be evaluated statistically to determine the strength of the association between the variable of interest and the disease. In cohort studies, a well-characterized and homogeneous group is studied over time. In case-control studies, a control group is selected retrospectively based on variables thought to be relevant to the effect, Both methods rely on an accurate prediction of the variables that are important and are subject to various selection biases (62)112).

THE LD_{50} TEST

The LD_{50} testis one of the most widely used toxicity tests, and the development of alternatives to it is regarded by many as a high priority. As described in chapter 7, this acute toxicity test measures the amount of a substance needed to kill half the population of the test species. The LD_{50} is used as a rough indicator of the acute toxicity of a chemical,

The LD_{50} is useful for testing biological therapeutics, although there remain few such substances for which the LD_{50} is the only available means of standardization (13)90). Other applications, perhaps not so well justified (90), are determining doses for other toxicological tests and set - ting regulatory priorities.

There has been political pressure to abolish the LD_{50} and it has been criticized by many toxicologists on scientific grounds. It has poor reproducibility and the results are difficult to extrapolate to humans because there are so many mechanisms by which death could occur (70,90,125).

Despite the many criticisms of the LD_{50}, most toxicologists agree that acute toxicity information has valid uses, and that measurements of lethality also are important. Nevertheless, the precision with which the LD_{50} is measured is often unjustified for several reasons. First, most applications of the information do not require precision. Second, even if the information were precise for a given species, the LD_{50} varies so much from species to spe-

cies that extrapolation to humans is only rough. Third, the LD_{50} of a given substance varies significantly from laboratory to laboratory, and even in the same laboratory.

Various regulatory classification schemes make distinctions between levels of toxicity ("highly toxic" versus "toxic, " versus "moderately toxic, " versus "nontoxic"). The LD_{50} for two neighboring levels typically differs by a factor of 4 to 10. Yet, the reproducibility of test results does not justify even these distinctions. A recent study, though not necessarily typical, indicates the magnitude of the problem. A series of LD_{50} tests were performed in 60 European laboratories for five substances on one species. The LD_{50} for one substance ranged from 46 mg/kg body weight to 522 mg/kg, possibly ranging over three toxicity levels in some classification schemes. Although the variations were not this large for the four other chemicals tested, the smallest variation was 350 to 1,280 mg/kg. Each test was done with 50 or more animals so that the results would be precise (61).

Using Fewer Animals

The standard LD_{50} requires at least three groups of 10 animals or more each. An alternative procedure for determining the Approximate Lethal Dose (ALD) was developed as early as the *1940s (29),* in which individual animals are administered doses that increase by 50 percent over the previous dose. Depending on the initial dose level, the total number of animals needed is usually 4 to 10. Because the test substance might not be cleared between doses or because there maybe cumulative effects, the ALD can be lower than the LD_{50}, perhaps by 70 percent, though more typically by less than 20 percent (29).

Many other acute toxicity tests that require fewer animals than the LD_{50} have been developed (14, 17,33,61,69,71,94,105,107). Most require that the doses increase sequentially, thereby allowing the experiment to stop when a certain limit is reached. Thus, fewer animals die in the conduct of a test, but its duration could increase from 2 weeks to a month or more. Although many investigators are moving to less precise LD_{50} tests, no generally accepted alternative seems to have emerged.

The Limit Test and Other Refinements

If a substance is not lethal at high doses, its precise LD_{50} is not very important. In the limit test (80), a small number of animals is given a single oral dose, e.g., 5 g/kg body weight. If no animals die and no major ill effects occur, no further testing is needed. However, this limit is so high that this approach may have little practical value in reducing animal use (24).

Rather than determining the dose that is lethal, studies can also be done to detect toxic effects at doses that are not lethal. As with the LD_{50}, increasing doses can be administered to a small number of animals, perhaps stopping when some limit is reached. This approach can be further refined so that animals that are in distress could be sacrificed without affecting the outcome of the test (14).

In Vitro and Nonanimal Methods

Cell toxicity—changes in cell function or death of cells-can sometimes be used to detect acute toxicity. However, cell toxicity cannot be expected to function as a replacement for the LD_{50} because lethality can occur by so many mechanisms that are supercellular. Cell toxicity is particularly useful in comparing members of chemical families, such as alcohols and alkaloids (79).

At present, mathematical modeling has limitations, although it may have some utility in range-finding and in screening substances for testing (109). Modeling of acute toxicity fails to meet one of the criteria suggested by a working party on quantitative structure activity relationships, namely that the mechanism by which the response occurs should involve a common rate determining step (88). Nonetheless, in a large study involving thousands of substances, a computer program was developed that predicted LD_{50} values within a factor of 2.5 for 50 percent of the substances and within a factor of 6 for 80 percent. Considering

the reproducibility of the test itself, this might be satisfactory for some purposes, and it certainly warrants further investigation. Furthermore, many of the larger deviations in this study, upon further examination, were found to involve reporting errors. This program relied on a multi-tiered classification scheme based on chemical structure (36).

SKIN AND EYE IRRITATION

The widely used Draize eye irritation test and, to a somewhat lesser extent, the skin irritation test have been criticized because of the amount of pain inflicted and because they are unsatisfactory models for human irritation (91,95). First, the rabbit eye has structural differences, such as a thinner cornea and differing tearing apparatus (103), and animal skin is much less sensitive and discriminating than human skin (56,63). Second, both of these tests are sensitive to too many variables, making reproducibility poor (83,118).

As with most tests, the number of animals used can sometimes be reduced. Several refinements have also been proposed. For example, screening tests based on pH or skin irritancy might also serve as alternatives to eye irritancy tests in limited circumstances, although preliminary studies indicate that this approach is frequently misleading (119). Other refinements involve local anesthetics (51,65, 110), applying smaller (43) or more dilute (120) doses, and testing whole eyes in vitro (20). The latter method has particular appeal when cow eyes are used because they are so readily available from slaughterhouses. In the case of smaller doses, a recent comparison with over 500 accidental human exposures showed that doses smaller than those now in use yielded results more predictive of the human response while causing less severe irritation (38).

Skin and eye irritation are similar in many respects. Thus, even though little work has been done to develop alternatives to skin irritation tests, the many approaches just summarized for eye irritation may eventually be applied to skin testing as well (91).

In Vitro Tests

Several in vitro alternatives have been examined, and it appears to some commentators that no single alternative will be adequate, but that a battery of in vitro tests might be a useful replacement (67). Several types of cell cultures have been used in developing an in vitro test for eye irritation. The cells used are rabbit and human corneal cells (72), mouse and hamster fibroblasts, human hepatoma cells, and mouse macrophages (96).

A variety of effects have been used as surrogates for eye irritation, such as the rate of uptake of uridine as an indication of cell functioning and recovery, visible changes in cell structure, decreases in the concentration of cell protein (96), and release of plasminogen activator from the injured cells (21). Some techniques appear promising, particularly in their ability to rank substances based on irritancy, Rapid progress is being made in the development of techniques, but none can be considered validated at this time (91).

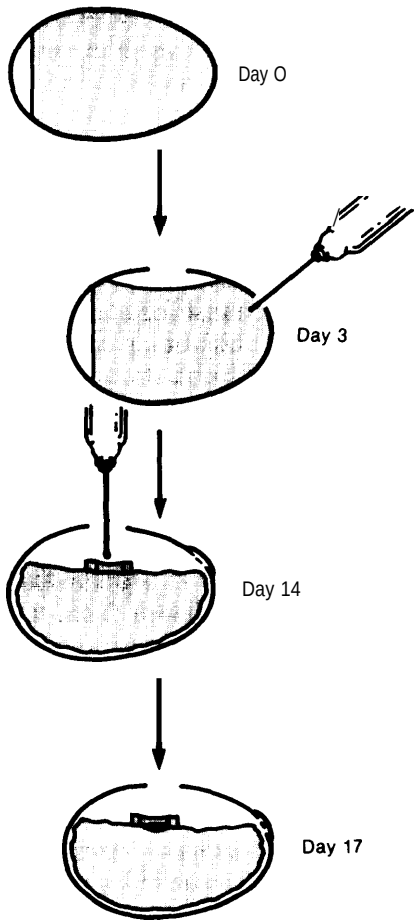
To date, little work has been done on in vitro replacements for skin irritancy testing. However, the growth of skin in tissue culture is of interest for treating burn victims, and it is expected that culture techniques currently being developed for that purpose can be used in testing methods. In addition, it has also been suggested that suitable specimens can be obtained from cadavers and surgery and from judicious use of human volunteers (63).

Chick Embryo

One test system receiving considerable attention is the fertilized chicken egg. A part of the eggshell is removed and the test substance applied to the chorioallantoic membrane surrounding the developing embryo (see fig. 8-l). This test has the potential for assessing both eye and skin irritancy.

The chorioallantoic membrane of the chick embryo is a complete tissue, including arteries, capil-

Figure 8-1.—Chronological Sequence of Chick Embryo Chorioallantoic Membrane Assay



Day O. Fertile eggs are incubated at 37" C. Day 3. The shell is penetrated in two places: A window is cut at the top, and 1.5 to 2 milliliters of albumin is removed with a needle and discarded. The chorioallantoic membrane forms on the floor of the air space, on top of the embryo. The window is taped. Day 14. A test sample is placed on the embryonic membrane and contained within a plastic ring. Day 17. The chorioallantoic membrane is evaluated for its response to the test substance, and the embryo is discarded.

SOURCE: J. Leighton, J. Nassauer, and R. Tchao, "The Chick Embryo in Toxicology: An Alternative to the Rabbit Eye," *Food Cherry. Toxicol.* 23:293-298. Copyright 19S5, Pergamon Press, Ltd.

laries, and veins, and is technically easy to study. An embryonic membrane tested after 14 days of incubation responds to injury with a complete inflammatory reaction, a process similar to that induced in the conjunctival tissue of the rabbit eye. The embryonic membrane can show a variety of signs of irritation and has capabilities for recovery (59,60).

Assessment of toxicity is made and the embryo is discarded on about day 17 of incubation. The criteria used for macroscopic rating of lesions on the chorioallantoic membrane are listed below (59):

- size,
- contours and surface,
- color,
- retraction of surrounding chorioallantoic membrane,
- spokewheel pattern of vessels,
- overall grade of severity, and
- necrosis (confirmed microscopically).

Although this is, strictly speaking, an in vivo test, the chorioallantoic membrane does not have nerve cells, and thus it is unlikely that the organism experiences any discomfort. In addition, fertile eggs are inexpensive and do not require elaborate animal room facilities.

REPEATED-DOSE TOXICITY TESTS

Repeated-dose toxicity testing involves the repeated application of a substance to a biological assay system and subsequent measurement of many different effects of the substance. In repeated-dose testing, the long-term effects of repeated, sublethal exposure to a substance are of interest, rather than acute, lethal effects. Cell cultures may be useful adjuncts for suspected tar-

Chick Embryo Chorioallantoic Membrane Assay

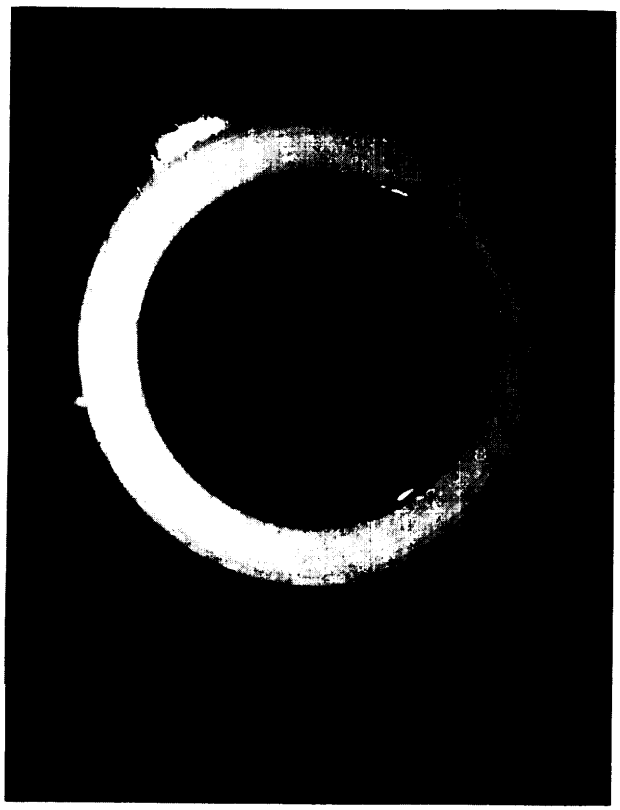


Photo credit: Joseph Leighton, Medical College of Pennsylvania

Typical react ion seen *3* days after certain concentrations of household products have been placed on the 14-day-old chorioallantoic membrane. The thin white plastic ring has an internal diameter of 10 millimeters. The area of injury within the ring is well defined with a distinct edge. All of the cells in the injured area are degenerating or dead. The severity of this positive lesion is quantified by measuring its diameter.

get organs or tissues, but they are not a replacement for whole-animal testing. The most promising alternatives in the near future involve modifications of animal use (for example, by combining tests), and the use of screening tests and computer simulation for improved experimental design. The screening tests with the greatest promise are for hepatotoxicity and neurotoxicity.

Hepatotoxicity

Several in vitro alternatives for hepatotoxicity have been developed, including perfused liver (108), liver cell suspensions (39), and liver cell cultures (39)44). Liver perfusions can only be maintained for a few hours, and with some difficulty. Cell cultures can retain the special functions of liver cells with specially prepared culture media (76,81). However, the cells are viable for only a limited period of time and do not replicate in a reproducible manner. Although these techniques have been used to study mechanisms of liver toxicity, only limited attention has been given to their use in screening or as alternatives (91).

Neurotoxicity

The development of alternatives for neurotoxicity is more difficult than for hepatotoxicity. The nervous system is the most complex organ in the body, both in terms of structure and its function. Because many neurotoxins affect only one kind of cell, a battery of in vitro tests would probably be required to replace whole-animal testing–if anything could. Substances can also affect various areas differently, partly because of distribution factors, For example, very few substances are able to enter the brain because of the ‘(blood-brain barrier.” Thus, pharmacokinetic studies will continue to be very important.

Some in vitro tests (41) and tests using invertebrates (8) seem useful, at least for screening. As yet, however, the primary use of in vitro techniques has been the elucidation of mechanisms of known toxic effects (31). Many toxic effects to neural tissue have been correlated with concentrations of specific chemicals in or around the cells, thus offering the means for developing in vitro tests (31).

MUTAGENICITY

Mutation, the change in the DNA sequence of genes, is a mechanism by which toxic effects may be initiated. If the DNA replicates, the mutation is passed from the mutated cell to its descendants. Mutation can lead to cell death or the gain or loss of certain functions. When it occurs in germ cells,

the gene pool is affected, even if the mutation is not expressed in the progeny. The mutations that occur in somatic cells that are of greatest concern are those that lead to cancer (18).

Recent advances in the techniques of cell biology have led to an increase in the types and sophistication of mutagenicity tests available. Mutations can be detected by analyzing DNA or its fragments or by observing changes in the size, shape, or number of the chromosomes (which contain DNA), as well as by observing changes in a whole organism (34). Mutation can also be detected by measuring the amount of DNA repair.

Micro-organism Tests

The most commonly used test for mutagenicity is the Ames test for "reverse mutation" in ***Salmonella typhimurium*** **(3).** Mutagenicity is detected by exposing an already mutated strain to potential mutagens. If the mutation is reversed, the bacteria regain their ability to produce the amino acid histidine and will proliferate in a histidine-deficient culture medium.

The Ames test, as well as most other mutagenicity tests involving micro-organisms, does not avoid animal use entirely, To determine whether the metabolic products of a substance might be mutagenic even if the substance itself is not, liver preparations from rats or other rodents are used to produce at least some of the likely metabolic products.

Microorganism systems may fail to detector may overpredict mutagenic changes that could occur in whole animals or humans. For example, the system provided for metabolism may not be capable of reproducing conditions in vivo, or in the case of screening for carcinogenicity, mutation may not be the initiating event. on the other hand, such systems may indicate mutagenicity when the DNA repair system of mammals would reverse the mutation.

Other bacterial tests have been developed using S. ***typhimurium, Escherichia coli, and Bacillus subtilis.*** These systems do not seem to offer any particular advantage over the Ames test, although thorough evaluation is hampered by lack of a comparable database of results (28). Tests have also been developed for molds (30), fungi (16), and yeasts (18,82).

In Vitro Tests

In vitro mutagenicity tests maybe done with cultured mammalian cells that are exposed to toxic substances, although many mammalian in vitro tests also have an in vivo variant. Such tests typically measure acquired resistance or lost resistance to the effects of the toxic substance. Most commonly used are a mouse lymphoma ceil line or hamster ovary cells, but almost any well-characterized cell can be used. ovary cells are often used because, as germ cells, they have half the number of chromosomes to be evaluated (18).

A test known as the specific locus test can be done with Chinese hamster ovary cells. They are exposed to a test substance and their response to the normally lethal 8-azaguanine or 6-thioguanine in cell culture determined. The cell's ability to survive, requiring the ability to metabolize the 8-azaguanine or 6-thioguanine, is an indication of the occurrence of mutation as a result of exposure to the test substance. This test can also be done with mouse lymphoma cells exposed to 5-bromodeoxyuridine or trifluorothymidine (23).

The sister chromatid exchange test relies on the fact that certain substances will cause DNA breakage and reunion. This damage can be observed by staining the original chromosomes so that any segments exchanged during replication can be observed. Commonly used cells include human lymphocyte cells and rodent and human fibroblasts (37). Both the specific locus test and the sister chromatid exchange can also be performed as in vivo procedures (see ch. 7).

Although the cells are usually derived from animals, there is a considerable net savings in animal lives when in vitro mutagenicity tests are performed. For example, the rat mast cell assay can be used to screen severe irritants, and one rat can supply enough tissue to replace the use of 48 animals in in-vivo procedures (103).

Tests Using Insects

The most widely used insect for genetic studies is the fruit fly, ***Drosophila melanogaster (114, 115).*** The fruit fly has well-characterized genetics and is similar to mammals in many key reactions, A variety of end points can be detected. The most common, and probably most sensitive, test is the

sex-linked recessive lethal assay (18). Treated males are mated with untreated females, and the progeny are mated to each other. The number and characteristics of the male progeny are evaluated to determine if lethal mutations (that is, mutations that prevent viability) have occurred.

Other tests involving fruit flies also exist or are likely to be developed. End points that can be measured include the loss, gain, or breakage of chromosomes detected by examining germ cells. With the availability of mutant strains, the measurement of reverse mutations can be a valuable tool. Eye color is a popular method of following genetic effects in the fruit fly (18).

CARCINOGENICITY

Many assays meant to replace carcinogenicity testing are designed to detect the initiation of cancer rather than the formation of tumors. First, detecting initiation is faster and easier than detecting cancer. Second, although not all initiation leads to cancer, certain kinds are considered reliable surrogates for the disease.

A major problem with evaluating the predictiveness of alternatives to whole animals for carcinogenicity testing is that very few human carcinogens have been positively identified. Most substances treated as human carcinogens, although documented to be ***known*** animal carcinogens, must be viewed as ***probable*** or ***suspected*** human carcinogens. The development of alternatives is somewhat hampered by a lack of epidemiologic data on humans.

Various molecular and physiochemical properties of substances have been correlated to carcinogenicity. Some structure-activity models developed for families of chemicals have predicted the carcinogenic properties for 75 to 97 percent of them. The chemicals modeled include polycyclic aromatic hydrocarbons (123), nitrosamines (89,99, 121), and aromatic amines (124).

The Ames Test

Because mutation is often the first step in carcinogenesis, the Ames test has been suggested as a possible screen **or** replacement for carcinogenicity testing. It has been evaluated for this purpose, both alone and as one in a battery of tests. Alone, it is less predictive than whole-animal tests. In a battery, it has been shown to be about as predictive as animal testing for certain families of chemicals and substantially less predictive for others for the substances tested. Table 8-1 shows the predictiveness of mouse and rat bioassays and the Ames test for some known human carcinogens.

The Ames test has been performed thousands of times in over 2,000 laboratories throughout the world and has provided results on over 1,000 chemical substances since it was developed less than two decades ago. Portions of this large body of analytical data have been reviewed in over a dozen evaluation studies with the intent of determining the test ability to predict carcinogenicity (6,19,66). These evaluations show that the percentage of human carcinogens that are also mutagens (mutagenic carcinogens) ranges from 50 to 93 percent and is most likely about 80 percent (48). About 20 percent of the human carcinogens were not mutagens (nonmutagenic carcinogens) in the Ames test, and it is believed that cancer associated with these carcinogens is initiated by a mechanism other than mutation.

A critical analysis of several studies (19) identified several sources of variation. These include methods of chemical selection, sample coding, use of a high proportion of chemicals known to work well or poorly with Ames testing, and differences in metabolic activation during the test procedure. The conclusion was that a reasonably careful application of the Ames technique to a nonbiased group of chemicals would be expected to yield a predictive accuracy of approximately 80 percent for mouse and rat carcinogens.

The Ames test tends to be positive for a large proportion (about 40 percent) of substances that have not been identified as carcinogens in rodent bioassays. It should be noted, however, that these

Table 8.1.—The Response of Known Human Carcinogens to Rodent Carcinogenicity and Bacterial Mutagenicity Assays

Chemical	Rat bioassay	Mouse bioassay	Ames test
4-Aminobiphenyl	+	+	+
Arsenic	−	−	−
Asbestos	+	+	−
Benzene	−	+	
Benzidine	+	+	+
Bis(chloromethyl)ether	+	+	+
Chromium; some chromium compounds	+	−	+
Cyclophosphamide	+	+	+
Diethylstilbestrol	+	+	−
Melphalan	+	+	+
Mustard gas	n.d.	+	+
2-Naphthylamine	−	+	+
Soot, tars	−	+	+
Vinyl chloride	+	+	+

KEY: + = Positive results (carcinogenic to rodents or mutagenic to bacteria)
− = Negative results (not carcinogenic or not mutagenic)
n.d. = No data.

SOURCES: From H. Bartsch, L. Tomatis, and C. Malaveille, "Mutagenicity and Carcinogenicity of Environmental Chemicals," *Regul. Toxicol. Pharmacol.* 2:94-105, 1982; D. Brusick, devaluation of Chronic Rodent Bioaasays and Ames Assay Tests as Accurate Modeis for Predicting Human Carcinogens," *Application of Biological Markets to Carcinogen Testing*, H. Milman and S. Sell (ads.) (New York: Plenum Press, 1963); B.D. Goldstein, C.A. Snyder, S. Laskin et al., "Myelogenous Leukemia in Rodents Inhaling Benzene," *Toxicol. Lett.* 13:169-173, 1962; and J.V. Soderman (cd.), *Handbook of identified Carcinogens and Noncarcinogens, Vols. I and II* (Boca Raton, FL: CRC Press, 1982),

substances have not been shown to be noncarcinogenic, and many authorities maintain that the information is insufficient to make any statement about the proportion of noncarcinogens that are also nonmutagens in the Ames test (4,116).

Use of the Ames Test in a Battery of Tests

The predictive value of the Ames test, or other mutagenicity tests, can be improved by combining it with additional short-term assays to form a test battery. Although no US. regulatory agency has yet recommended a specific combination, most authorities recommend that an appropriate battery should include information from a minimum of three types of tests:

- gene mutation (Ames test, mouse Iymphoma test);
- chromosomal mutation (in vivo Chinese hamster ovary cell cytogenetics); and
- DNA damage (sister chromatid exchange, unscheduled DNA repair).

At least one test should include a mammalian in vitro cell, tissue, or organ culture assay (4).

In a recent study, 18 Ames tests averaged 66 percent "accuracy" (number of chemicals correctly identified / number of chemicals tested). Comparative results from six batteries of short-term tests that included the Ames test increased the accuracy to 82 to 90 percent (58,111).

CURRENT TRENDS

As long as toxicological data continue to be required by regulators and by the courts to protect human health, animal testing will continue for the foreseeable future. Even major progress in the development and implementation of alternatives will not necessarily eliminate whole-animal tests. Furthermore, there are several impediments to development and implementation:

. A large number of scientists have been trained to solve health problems and to invent new products using animal models.

- Regulatory schemes, product liability law, and patent law also incorporate notions of animal models.
- A large body of animal testing information already exists that is useful in interpreting new testing data.
- There are substantial costs and delays associated with the development and adoption of alternatives. One study indicated that it takes about 20 years for an in vitro test to be developed, validated, adopted, and implemented (92).

At the same time, there are several factors facilitating the development and implementation of alternatives:

- Rapid progress is being made in techniques for culturing mammalian cells and organs, in instruments for detecting and quantifying cellular and molecular changes, and in the understanding of the cellular and molecular processes underlying toxicity. Improved understanding is leading to the ability to predict long-term effects and carcinogenicity from short-term biochemical and morphological changes.
- As such advances are made, the research laboratories that have developed the expertise are often willing to apply it to the development of new testing methods, and can do so efficiently (42).
- Organizations such as The Johns Hopkins Center for Alternatives to Animal Testing and the Rockefeller University laboratory have been set up to facilitate and coordinate research on alternatives (see ch. 12).
- Many organizations have been established to pressure those who conduct animal testing or use data based on it to adopt alternatives or conduct research that will lead to alternatives.

Strategies to speed the development and adoption of alternatives will depend on the needs and resources of the organization involved. The following recommendations encompass a variety of perspectives. They were promulgated by the Toxicity Committee of the Fund for the Replacement of Animals in Medical Experiments, which met from 1979 through 1982 (40). Some involve reassessment of testing needs and priorities; others involve technical strategies thought to be likely to lead to better methods, both in testing and in evaluating results:

- Provide a mechanism for reviewing the need for a given test.
- Investigate the consequences of not requiring or possessing testing data other than what already exists. Particular attention should be given to widely used tests such as the LD_{50} and skin and eye irritation tests with a view toward eliminating unnecessary requirements.
- Encourage flexible use of testing guidelines and frequent reappraisal of them in light of new knowledge.
- Strive for broader-based international harmonization and mutual recognition of data from other countries so that duplicative testing can be avoided.
- Encourage detailed publication of all testing results, particularly for costly or painful tests or those requiring many animals.
- Investigate the possibility of time limits on the confidentiality of test results.
- Make greater use of studies on absorption, distribution, biotransformation, and excretion in humans, as well as in test animals, to select the most relevant exposure conditions, to aid in extrapolation of results, and to improve the reliability of test results.
- Perform preliminary studies before undertaking long-term studies so that results can be as useful as possible.
- Make greater use of the structural and conformational computer models used in developing drugs for the prediction of toxicity.
- Standardize screening tests based on in vitro and nonanimal tests, both to promote efficient use of testing resources and to evaluate the predictiveness of these tests.
- Try to predict toxic reactions before testing, both as a means for improving prediction techniques and to avoid testing highly irritating substances, particularly in the eye, if possible.
- Conduct research on the mechanisms by which toxic effects occur to facilitate the development of new testing methods.
- Develop more accurate, reproducible instrumentation for measuring toxic effects, avoid-

ing subjective measurements and reducing measurement errors.
- Make greater use of depositories in standardizing cell lines or strains of micro-organisms used for testing.
- Study the relationship between physiochemical properties and pharmacokinetic properties, as well as between physiochemical and toxicologic properties.
- Develop techniques for detecting nonmutagenic carcinogens.
- Develop systematic methods for objectively evaluating new techniques.
- Conduct postmarketing surveillance for adverse effects, noting any discrepancies with test results from animals.
- Substitute very specific tests for the LD_{50} and other general toxicity tests, particularly for substances having specialized uses, such as drugs.
- Use skin irritation testing as a rough screening tool for eye irritation.
- Attempt to describe specific effects in eye irritation studies, rather than reporting only the magnitude of the response.
- Investigate specific effects such as neurotoxicity to the extent possible when conducting general toxicity tests.
- Search for cell lines that retain their special functions upon replication and develop techniques for culturing them.
- Evaluate the statistical precision needed in various circumstances with a view toward using the smallest number of animals likely to be adequate.
- Use statistics to maximize the utility of results. Techniques such as blocking, covariance analysis, and factorial design should be used routinely.
- Improve standards of care and diet to reduce background effects.
- Take care that those conducting tests are qualified to do so, including having been trained in humane handling of animals.
- Combine tests wherever possible and keep them as short as possible, compatible with the nature of the test,
- Place greater emphasis on "no observed effect levels" than on lethal doses when they have greater predictive value.
- Use more than one species only to answer specific questions, and not for general safety assessments.

SUMMARY AND CONCLUSIONS

There has been a small but significant shift away from whole-animal testing to in vitro and nonanimal techniques in recent years, partly as a result of advances in biological techniques and partly in response to political and economic pressures. Many new methods are being developed for commonly used tests. Most of these are not yet validated, but they already have potential uses for screening substances for the animal testing they may eventually replace.

There are several kinds of alternatives. The first entails the continued, but modified, use of animals-changes in experimental design or data analysis so that fewer animals are needed or changes in protocols to reduce pain or distress. Living tissues, organs, and cells derived from humans or animals can sometimes be used instead of whole animals. These systems require a larger investment of time and money to develop than do modifications of whole-animal techniques, but their advantages may also be greater. They are usually faster and often cheaper than the corresponding whole-animal test, and they have scientific advantages as well. However, they almost always are less predictive than whole-animal tests and often fail to provide reliable dose-response data, information that is critical in estimating potential toxicity to humans.

Data, both anecdotal and epidemiologic, on toxic effects in inadvertently exposed humans are sometimes useful. However, these data are often confounded by lifestyle and exposure to other toxic

factors. Another drawback is that human exposure can be great if there are long delays between exposure and observable effects.

The LD_{50}, probably the most common and most criticized toxicity test, is well suited to the limited use for which it was first developed. The biggest obstacle to limiting or eliminating use of the LD_{50} is institutional: Many regulatory schemes rely on it for classifying substances. The most promising alternatives in the short term are testing sequences that require fewer animals. Cell culture techniques and computer modeling show some promise, but they have limited value at this time.

Another common and widely criticized test is the Draize eye irritation test. Several promising in vitro alternatives have been developed with cell cultures. Another technique uses the outer (chorioallantoic) membrane of a 14-day-old chicken embryo. This technique, although it uses a whole animal embryo, is thought to involve no pain because the membrane has no nerves. These alternatives may also apply to skin irritation.

Alternatives to carcinogenicity testing and repeated dose toxicity testing are of special interest, in part because the potential savings in testing costs and time are quite large, and in part because these tests require large numbers of animals. The most promising replacements are batteries of tests involving cell cultures and living, nonanimal organisms. Mutagenicity testing uses many in vitro or nonanimal protocols. Mutagenicity is of particular interest because mutation can be the first event in other kinds of toxicity, including carcinogenicity, and because it can permanently affect the human gene pool. The most well known nonanimal mutagenicity assay is the Ames test. When it is combined with other tests, the Ames shows promise as an alternative to carcinogenicity testing, but it is not yet validated for this use.

In general, the development of alternatives is being facilitated by the rapid development of biological techniques, which are being applied to the search for alternatives in many different laboratories. Major contributions to the coordination of these developments in the United States are being made by Rockefeller University and The Johns Hopkins Center for Alternatives to Animal Testing.

The implementation of alternatives is hindered by various forms of institutional inertia, such as regulatory schemes (see ch. 7), product liability law (see ch. 7), and general resistance to change. Important impediments are the large body of existing information—derived from animals—that is relied on for the interpretation of new data and the lack of sufficient information to support the use of alternatives.

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