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Agency

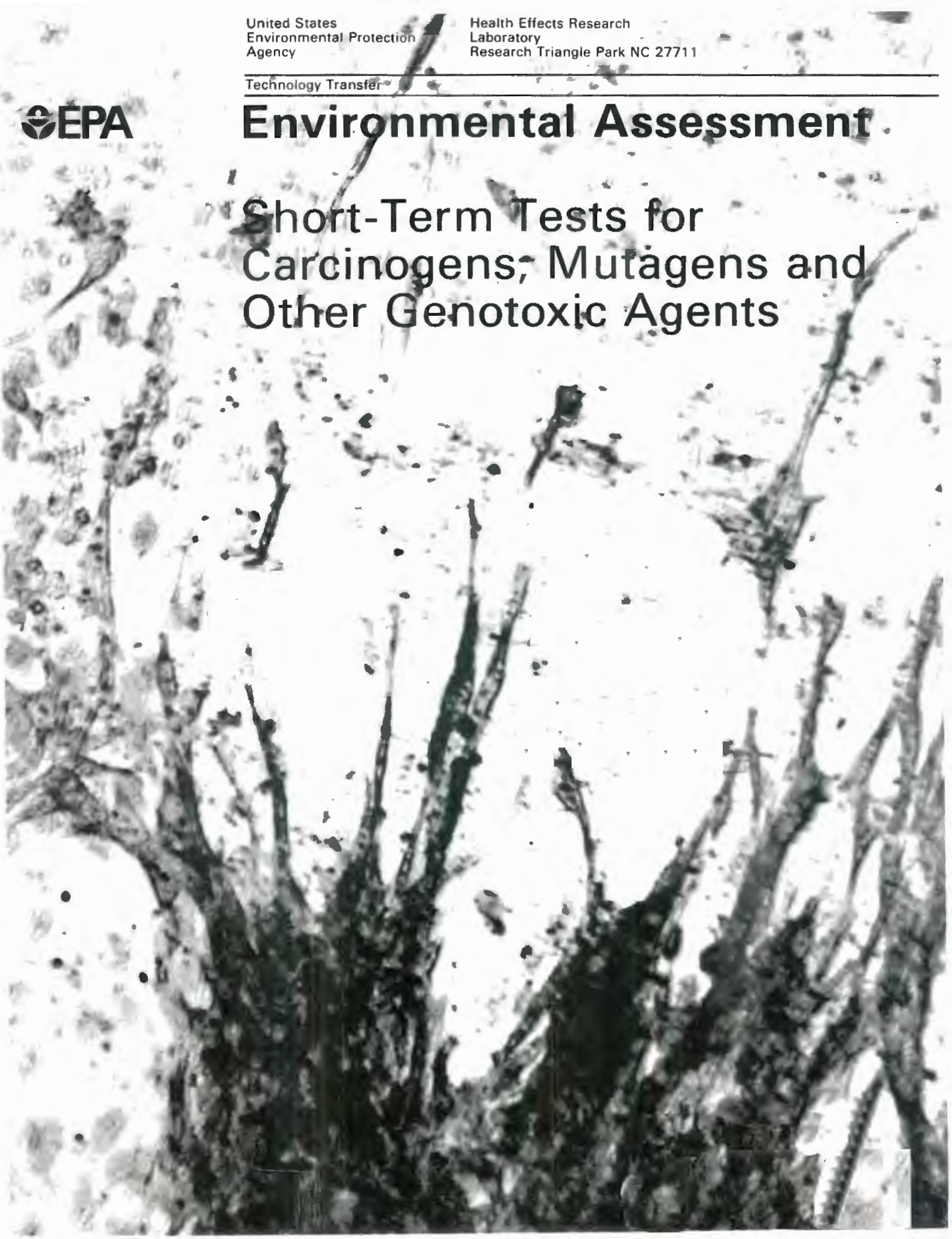
Health Effects Research
Laboratory
Research Triangle Park NC 27711

Technology Transfer

EPA

Environmental Assessment

Short-Term Tests for Carcinogens, Mutagens and Other Genotoxic Agents



Cover—Chemically transformed Balb/3T3, 1-3 mouse fibroblasts (x200, Giemsa stain), showing borderline between normal and malignant cells. The darker, spindle-shaped transformed cells pile up and constitute the foci. Provided by Dr. D. R. Lang, Department of Microbiology, University of Cincinnati, Cincinnati, Ohio.

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Environmental Assessment

Short-Term Tests for Carcinogens, Mutagens and Other Genotoxic Agents

July 1979

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Energy Resources Co. Inc.
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Genetic Toxicology Program
Health Effects Research Laboratory
Research Triangle Park NC 27711
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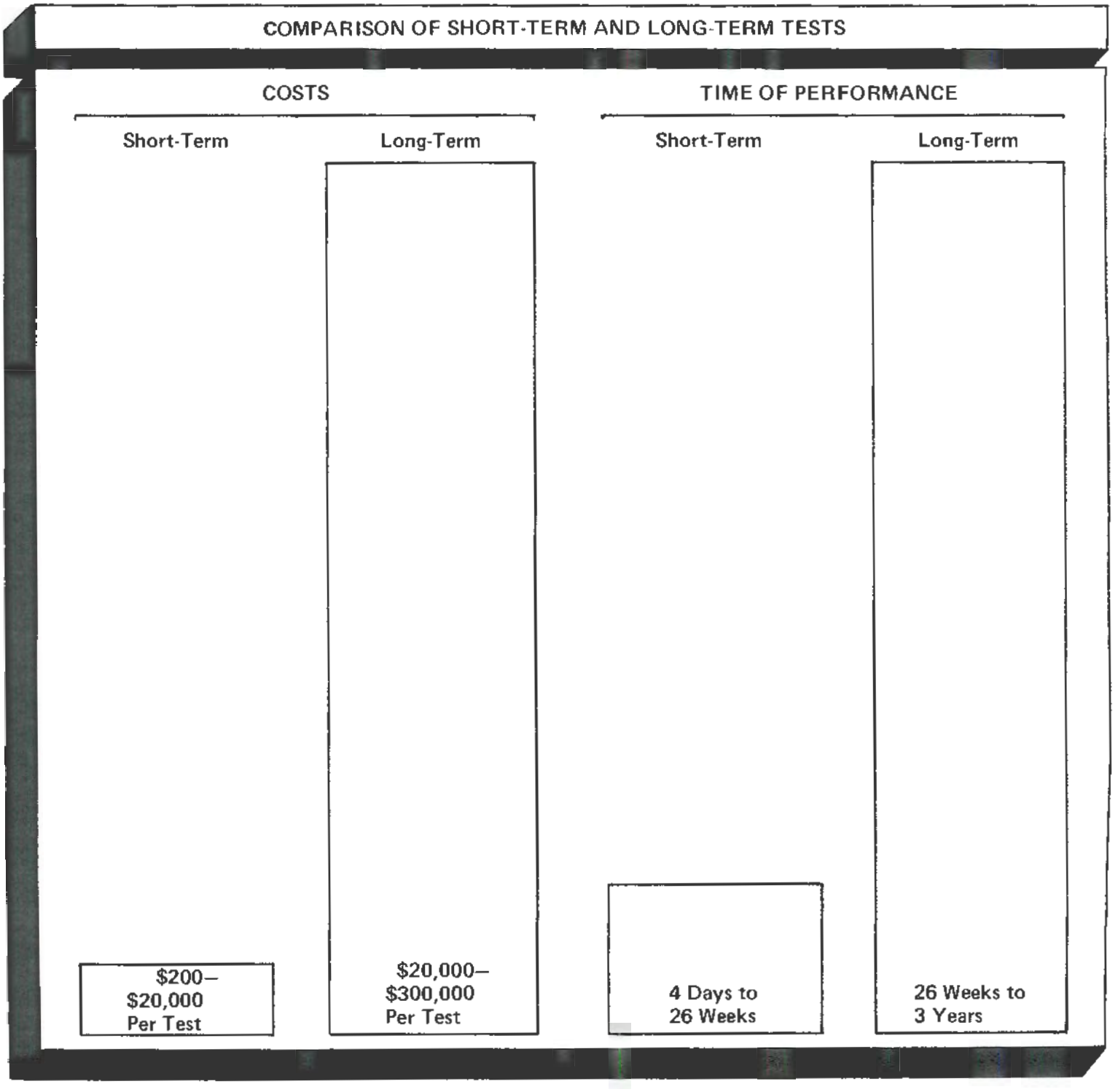
Contents

Introduction

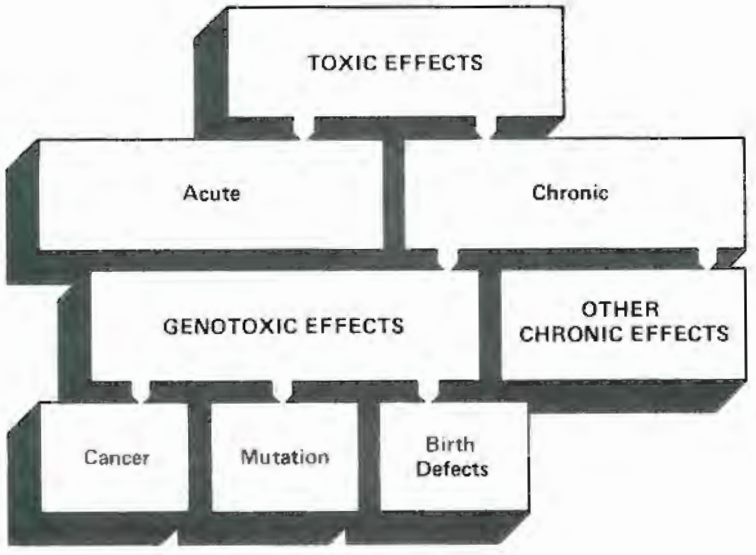


In recent years, federal statutes such as the Toxic Substances Control Act, the Resource Conservation and Recovery Act, and the Clean Air and Clean Water Acts have given the U.S. Environmental Protection Agency (EPA) the responsibility for regulating the release of toxic chemicals into the environment. In order to fulfill this function effectively, the EPA must first determine which of the thousands of chemicals currently in use or proposed for use are toxic.

Detecting a chemical's ability to cause immediate (or acute) toxic effects is a relatively straightforward task. Assessing the long-term (or chronic) toxic effects is much more difficult. Chronic effects such as cancer, birth defects, and genetic disease characteristically appear several years or decades after the initial chemical exposure has occurred, and long-term studies using live animals must be conducted in order to detect these latent effects. Such studies are expensive and time-consuming, and require the use of highly specialized facilities and personnel. A single test for a chemical's carcinogenicity (cancer-causing ability), for instance, may take as long as 3 years and cost $250,000 or more.

The number of compounds whose chronic toxicity has not been determined is overwhelming. Over 50,000 chemicals are currently in commercial production, and most of them have never been examined for chronic effects. The world laboratory capacity for long-term studies has been estimated at only 500 compounds per year, not enough to keep up with the 700 to 1,000 new chemicals that are introduced into commerce annually.

In response to this situation, short-term tests have been developed to serve as rapid and relatively inexpensive predictors of a chemical's potential to cause chronic effects. These tests employ bacteria, yeast, plants, insects, isolated mammalian cells and whole animals. Short-term tests can detect a chemical's genotoxicity, that is, its ability to alter a cell's genetic material (DNA). An increasing amount of evidence exists to indicate that latent diseases such as cancer, birth defects, and genetic disease may be initiated by alterations in the DNA.

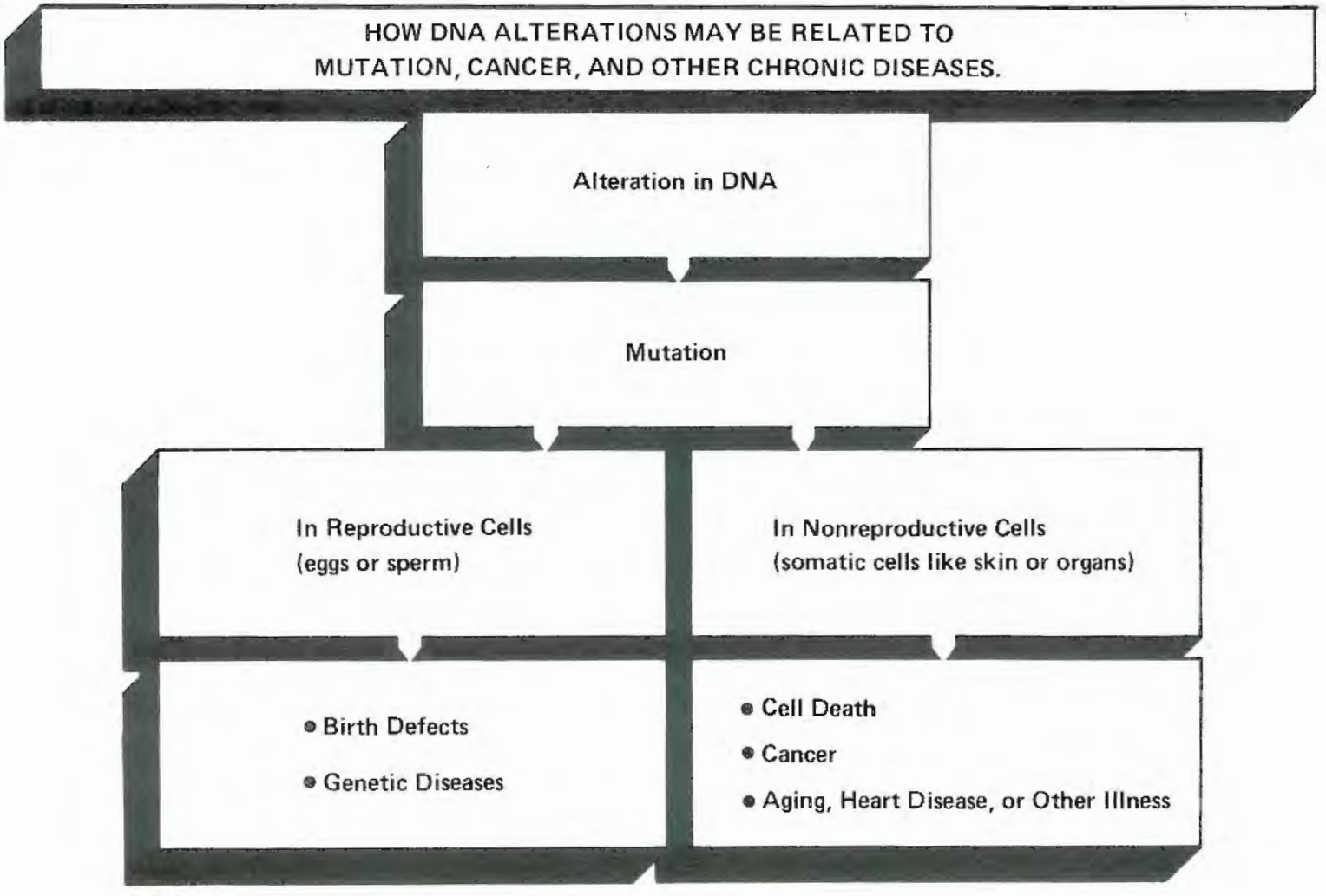


Short-term tests enable a large number of chemicals to be screened for their genotoxic potential at a fraction of the time and cost required for long-term tests. Results from short-term tests can be used to make more informed decisions as to which chemicals should be examined in the limited number of long-term testing facilities available. Several other promising uses for short-term test data include:

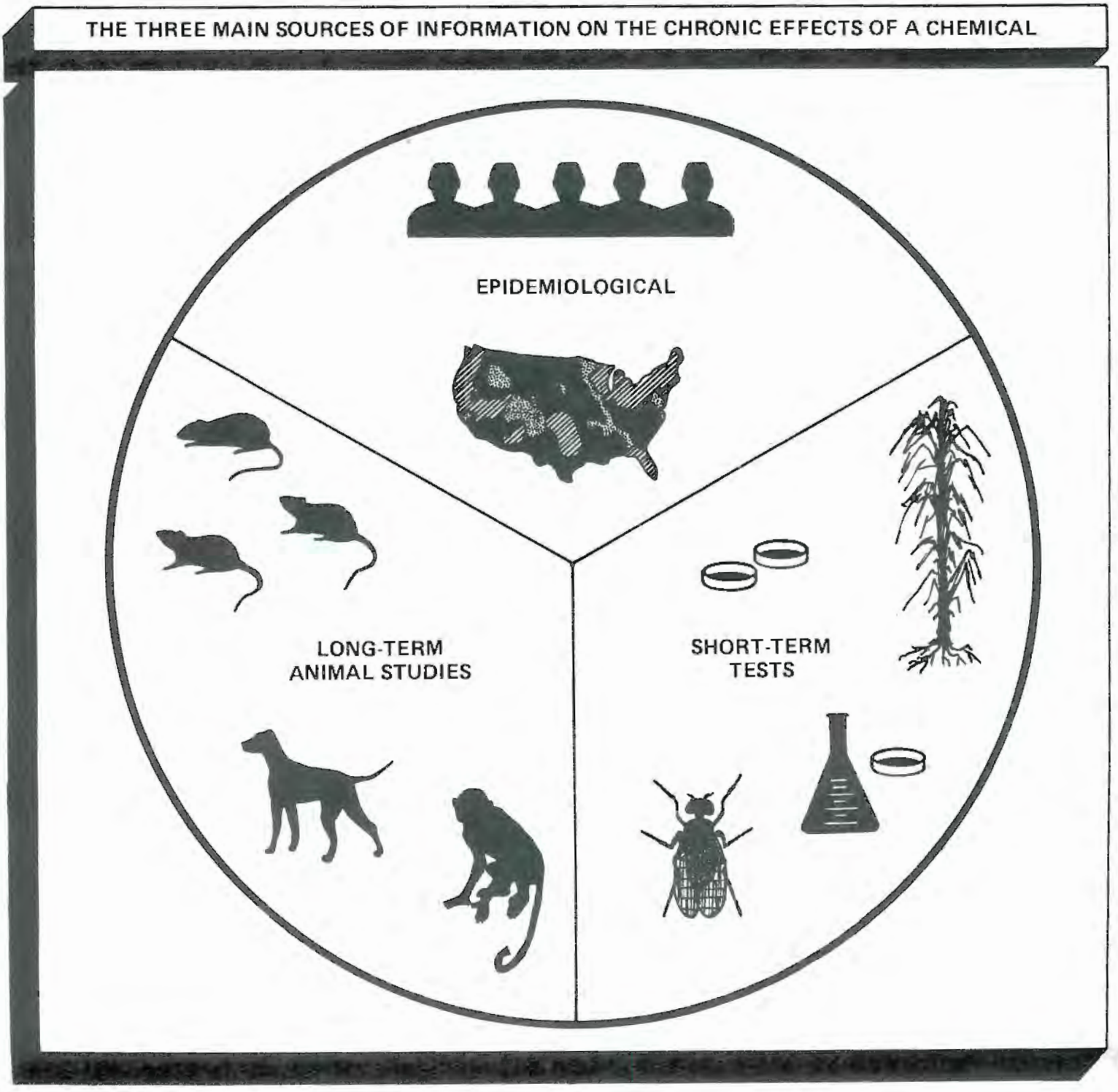
- Determining which of several alternative chemicals under development will be the least hazardous to human health.
- Identifying the toxic components of complex environmental pollutants.
- Monitoring industrial emissions, effluents, and wastes in air, water, and soil.
- Determining which control technologies are most efficient in eliminating toxic chemicals.
- Providing interim guidance for using a chemical when no other data are available.

Because of their rapid and inexpensive nature, short-term tests are extremely valuable in helping the EPA to fulfill its responsibility for identifying and regulating toxic substances. For this reason, significant efforts are being applied to the research and development (R&D) of short-term tests. The purpose of this document is to briefly describe some of EPA's R&D activities in this area.

The document is organized into five sections. The first section discusses how short-term tests can contribute to hazard assessment, while the second describes the scientific basis and techniques of short-term tests. A general strategy for how short-term tests can be used to detect a chemical's potential long-term toxicity is outlined in the third section. Some program applications of short-term test research are presented in the fourth section, and the fifth section describes some of the current research activities. An overall perspective concludes the document. A glossary of technical terms is provided at the end of the document along with an appendix of technical information on specific short-term tests.


HOW DNA ALTERATIONS MAY BE RELATED TO MUTATION, CANCER, AND OTHER CHRONIC DISEASES.
Alteration in DNA
Mutation
In Reproductive Cells (eggs or sperm)
In Nonreproductive Cells (somatic cells like skin or organs)
• Birth Defects
• Genetic Diseases
• Cell Death
• Cancer
• Aging, Heart Disease, or Other Illness

Short-Term Tests and Hazard Assessment



Hazard assessment is the process of evaluating the human health threat associated with a chemical. It involves answering such questions as: Does the chemical have the potential to cause serious human health effects such as increases in cancer levels or birth defects? How great an exposure is necessary to cause an effect? Are any special groups of the population particularly susceptible to the chemical's effects? Is the health risk serious enough to require use restrictions or an outright ban on the chemical? Should more research be done to evaluate the hazard?

Three principal sources of information can be used to evaluate the health risk associated with a chemical:

- Human exposure (or epidemiological) data.
- Long-term tests using various species of animals.
- Short-term tests using microorganisms, plants, insects, and animals.

In a typical hazard assessment, scientists consider all the available data but ascribe different significance or weight to each set of data depending on the type of information and quality of the test or study.

At their present state of development, short-term tests are not considered to be as authoritative as epidemiological data or long-term whole animal studies. For this reason, short-term data are not used to provide "definitive" evidence that a chemical is hazardous. Rather, they are considered to be "suggestive" evidence of a chemical's potential to cause genotoxic effects.

The official EPA policy regarding the use of short-term tests for carcinogens has been expressed by the Interagency Regulatory Liaison Group (IRLG)[1] in their report "Scientific Bases for Identifying Potential Carcinogens and Estimating Their Risks":

Short-term tests for chemical carcinogens presently do not, in the absence of animal bioassay [test] and epidemiology data, constitute definitive evidence as to whether a substance does (or does not) pose a carcinogenic hazard to humans. However, positive responses in these tests are considered suggestive evidence of a carcinogenic hazard.

[1]The Interagency Regulatory Liaison Group is a consortium of four federal agencies, the EPA, the Occupational Safety and Health Administration, the Consumer Product Safety Commission, and the Food and Drug Administration.

Thus, short-term studies can be used to support existing animal data, or as a temporary substitute if these data are lacking In rare instances, short-term tests can call into question adequately conducted long-term animal studies, but this can occu if and only if short-term test data are consistently and clearly positive and long-term findings are negative. In this case, short-term test data are taken as suggestive evidence of hazard until further long-term testing resolves the discrepancy.

Accuracy

A great deal of research is currently being done to determine how accurate short-term tests are in predicting whether or not a chemical has the potential to cause human health effects. The accuracy of short-term tests for carcinogens is usually determined in reference to animal or human data. The more frequently the results of a short-term test concur with what is known about a chemical's carcinogenic potential through long-term tests or epidemiological data, the more accurate that test is considered to be.

For mutagenicity (ability to cause mutations) tests, the issue of accuracy is not so clear-cut. Because of the technical difficulties involved in detecting mutations in the human population, there are no human data that can be used to validate short-term test results. At present, the accuracy of a short-term mutagenicity test must be determined by comparing test results with the findings from other mutagenicity tests. The degree of concordance with other mutagenicity findings is considered to be the best measurement of a test's accuracy.

There are basically two ways a short-term test can give an inaccurate result. It can indicate that a chemical is genotoxic, when in fact it is harmless (this is called a false positive result), or it can indicate that a chemical is harmless when it is actually genotoxic (a false negative result). Different short-term tests vary in their likelihood of making false positive and false negative errors.

False negatives are of great concern in short-term tests, particularly when these tests are used as early warning systems (see section on Phased Testing Strategies). With a false negative, a toxic chemical may not be examined in long-term tests before significant human or environmental exposure occurs. With a false positive, there is a good chance the error will be corrected during follow-up testing. In general, false positives present a cost rather than a public health concern. Ideally, a short-term test should minimize both false positives and false negatives.

Short-Term Tests

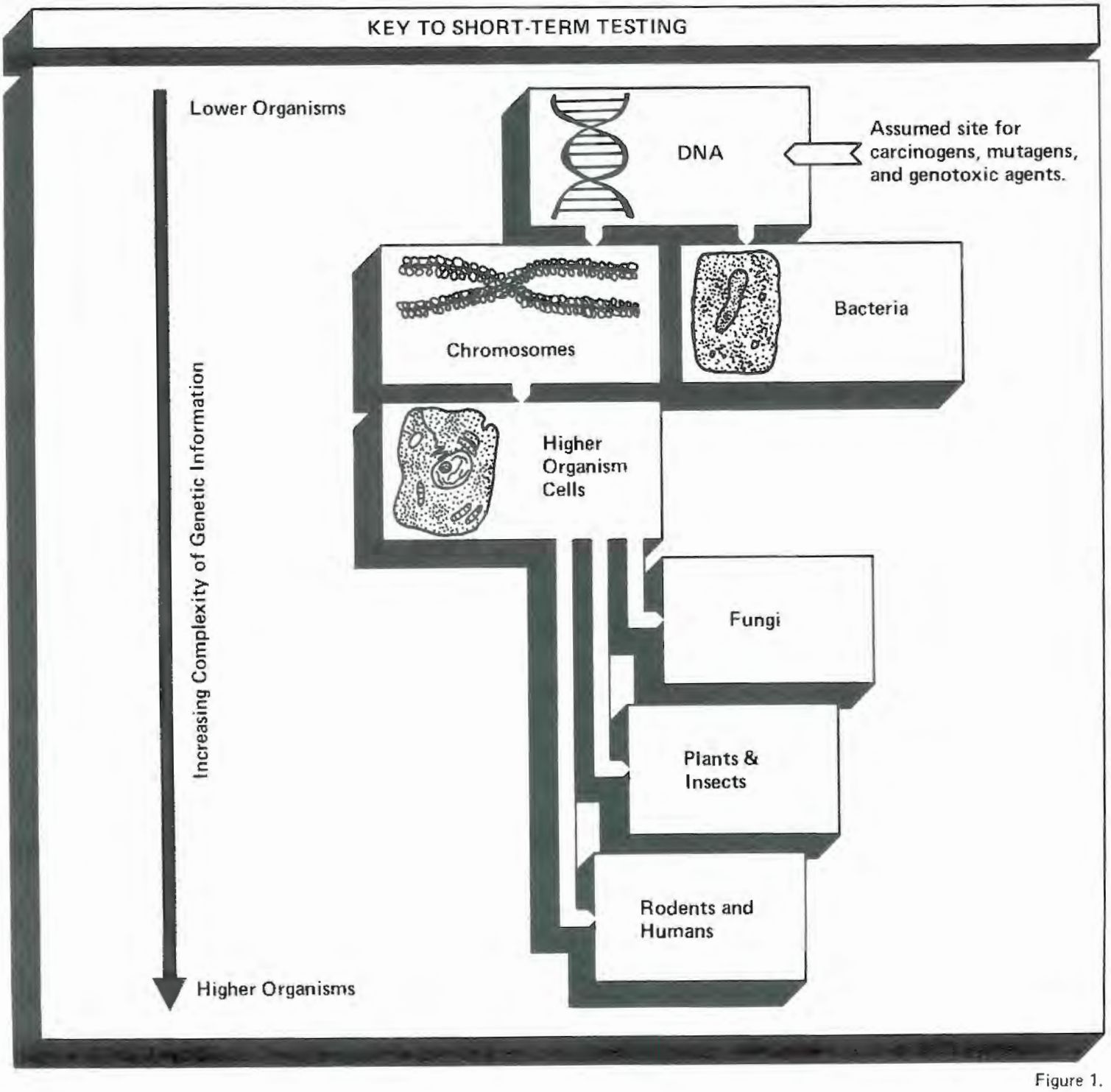


Figure 1.

Scientific Basis

Short-term tests for genotoxicity look for the ability of a chemical to damage the genetic material (DNA) of a cell. Since DNA controls all the functions of the individual cells that make up an organism, even a small change in the DNA can have severe consequences for an organism or its offspring. As was stated in the introduction, an increasing amount of evidence exists to indicate that latent diseases such as cancer, birth defects, and genetic disease may be caused by alterations in DNA.

The key to short-term testing is the fact that the fundamental structure of DNA is the same in all organisms (see Figure 1). Thus, a chemical that affects the DNA of a single cell or organism in a short-term test can theoretically have a similar effect on the DNA of an exposed human.

Although the fundamental structure of DNA is the same in all organisms, the amount and complexity of DNA varies according to the complexity of the organism. Generally, the more complex a test organism and its form of DNA organization, the more likely it is to approximate the human response. Thus, mammalian cells are better models than bacteria for human cells.

How They Work

All short-term tests follow the same basic format. The substance of interest is applied in some predetermined concentration to the test system or organism. The substance is then metabolically activated either within the organism or by the addition of a special enzyme treatment. After a suitable period of time to allow the effect to take place, the test system is examined for signs of genotoxicity. This may be done in any of several ways, as indicated in Table 1 at the end of this section.

Metabolic activation is an essential element of short-term testing. As Figure 2 indicates, many chemicals are not toxic themselves, but can be converted into a toxic chemical by an organism's normal chemical conversion processes (metabolism).

Compared to animals and humans, the microorganisms and isolated animal cells used in short-term tests have only a limited capacity for metabolizing chemicals. For this reason, compounds that are not directly active (these compounds are sometimes called procarcinogens or promutagens) cannot be detected in short-term tests unless some form of metabolism is supplied to "ectivate" the chemical. Fortunately several types of metabolic activation are available. Three methods are illustrated in Figure 3. Of these three, *in vitro* activation is the most commonly used because of its simplicity and effectiveness.

Lethal Toxicity Determination

Before any test is made of a chemical's biological effects, it is important to determine the dose or concentration that will allow the test to be performed effectively. Too large a dose ca kill the test organisms, so that the effect being studied never has tha chance to appear. Too small a dose can give a misleac ing impression of safety, since the effective dose may not be achieved.

Fortunately, relatively straightforward techniques exist for determining the optimal range of concentrations for testing. Several different concentrations of a chemical are applied to the test system and the survival at each dose is determined. Doses that exhibit some toxicity, but that do not drastically reduce the cell populations, are generally the ones used for short-term testing.

Types of Biological Activity

Five different types of biological activity related to genotoxicity can be studied in short-term tests:

- DNA damage and repair.
- Gene mutation.
- Chromosome alterations.
- Cancer-like (oncogenic) cell transformation.
- Tumor formation

Each of these classes of activity is discussed below.

— **DNA Damage and Repair**
Whenever a cell's DNA is disturbed or damaged, cell mechanisms come into action to repair the damaged parts. DNA damage and repair tests take advantage of this fact in searching for evidence of genotoxic effects. These tests look either for direct evidence of alterations in the DNA, or for evidence that DNA repair mechanisms are in action. If the repair mechanisms can be demonstrated to be operating above the normal level, then damage to the DNA is indicated. DNA damage and repair tests are available using bacteria, yeast, mammalian cells, and whole animals.

— **Gene Mutations**
Gene (or point) mutations are submicroscopic DNA alterations occurring in a single gene[1] and leading to an altered gene product. Most gene products are proteins. Since proteins are involved in all the chemical reactions taking place in a cell or organism, the biological consequences of even a small change can be severe. For instance, a minute change in an enzyme (a type of protein) can interfere with a cell's normal functioning by making a key chemical reaction impossible.

Gene mutations are most easily detected by looking for altered gene products, such as enzymes. An enzyme deficiency can manifest itself in any number of ways that can be conveniently measured. In test systems using bacteria, yeast, or mammalian cells in culture, a cell's requirement for a certain nutrient may change, or its tolerance of a chemical poison may be altered. In short-term tests involving whole organisms such as fruit flies or plants, specific changes in the test organism's features (i.e., color or shape) can be observed as evidence of mutation.

[1]A portion of the DNA that directs the formation of a single product.

— **Chromosome Aberrations**
Chromosome alterations or aberrations are microscopically visible disturbances in chromosomes.[2] They can include the loss or gain of entire chromosomes, chromosome breaks, and faulty assembly processes such as nondisjunctions and translocations. Chromosomal aberrations are a major cause of heritable human disease, and their occurrence is often associated with cancer. They are detected either by searching for microscopically evident alterations or by examining tissues or organisms for traits known to result from such alterations. Cells from insects or mammals are frequently used.

— **Oncogenic Transformation**
Oncogenic transformation is the chemically induced conversion of normal cultured mammalian cells into malignant-like cells. Whether or not transformed cells are actually malignant (or cancerous) can be ascertained by injecting them into whole animals to see if they give rise to tumors. Most frequently, transformed cells are distinguished in culture by abnormal growth patterns that are visible under the light microscope.

— **Tumor Formation**
Tumor formation in rodents is a definitive indicator of a chemical's carcinogenicity. Short-term tests measuring tumor formation use special strains of mice and rats that develop tumors especially rapidly — within 10 to 26 weeks of chemical treatment. (In traditional long-term or lifetime tests for carcinogenicity, 2 or more years may elapse before tumors appear.)

In short-term tests for tumor formation, the number of tumors appearing in treated animals is compared to the number that have appeared spontaneously in an untreated control group of animals. A higher number of tumors in the treated animals indicates potential carcinogenicity. Nonmalignant tumors may be counted when their presence correlates with the later appearance of malignant tumors.

[2]A form of DNA organization found in higher organisms.

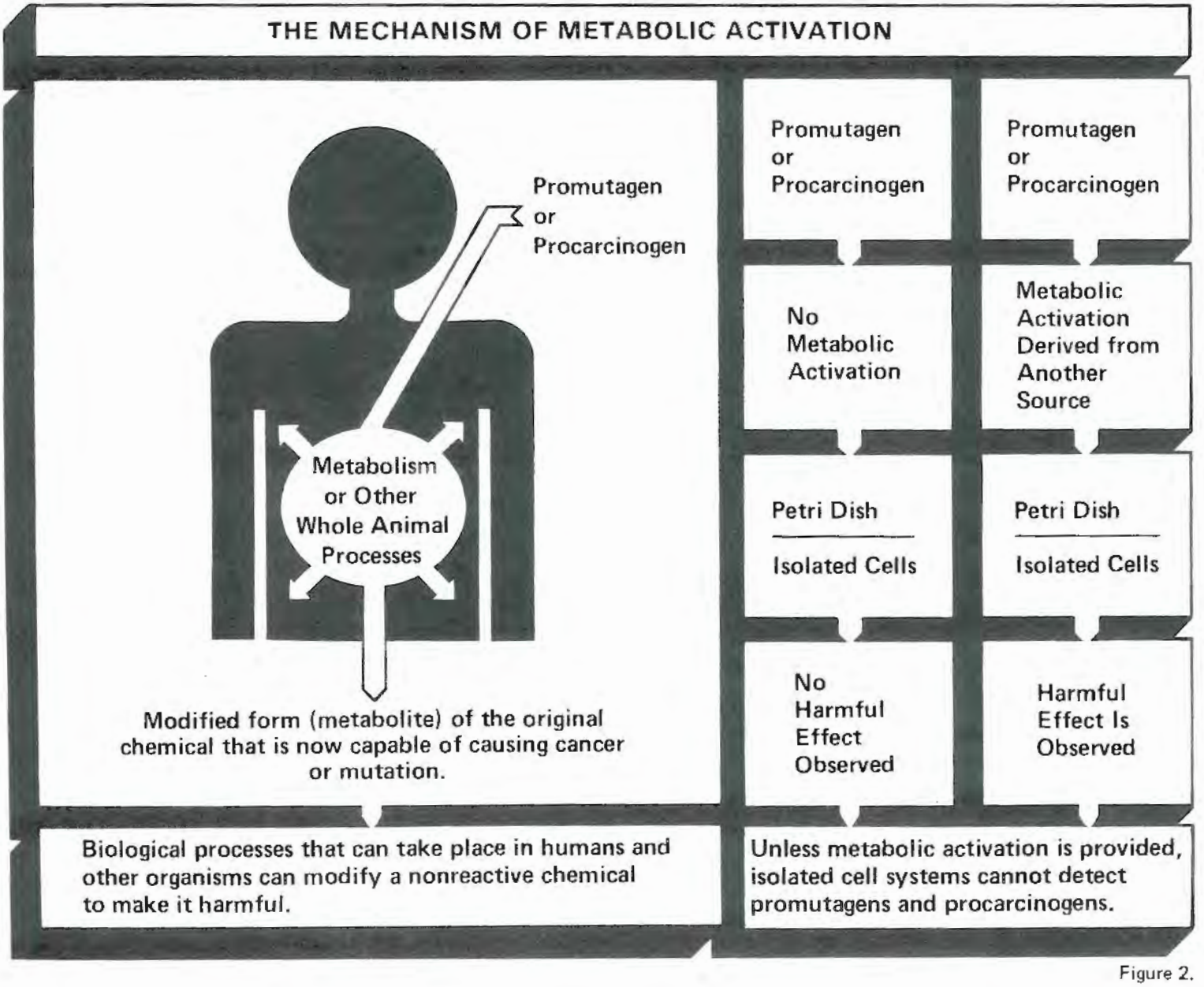

THE MECHANISM OF METABOLIC ACTIVATION
Promutagen or Procarcinogen
Metabolism or Other Whole Animal Processes
Modified form (metabolite) of the original chemical that is now capable of causing cancer or mutation.
Promutagen or Procarcinogen
Promutagen or Procarcinogen
No Metabolic Activation
Metabolic Activation Derived from Another Source
Petri Dish
Isolated Cells
Petri Dish
Isolated Cells
No Harmful Effect Observed
Harmful Effect Is Observed
Biological processes that can take place in humans and other organisms can modify a nonreactive chemical to make it harmful.
Unless metabolic activation is provided, isolated cell systems cannot detect promutagens and procarcinogens.

Figure 2.

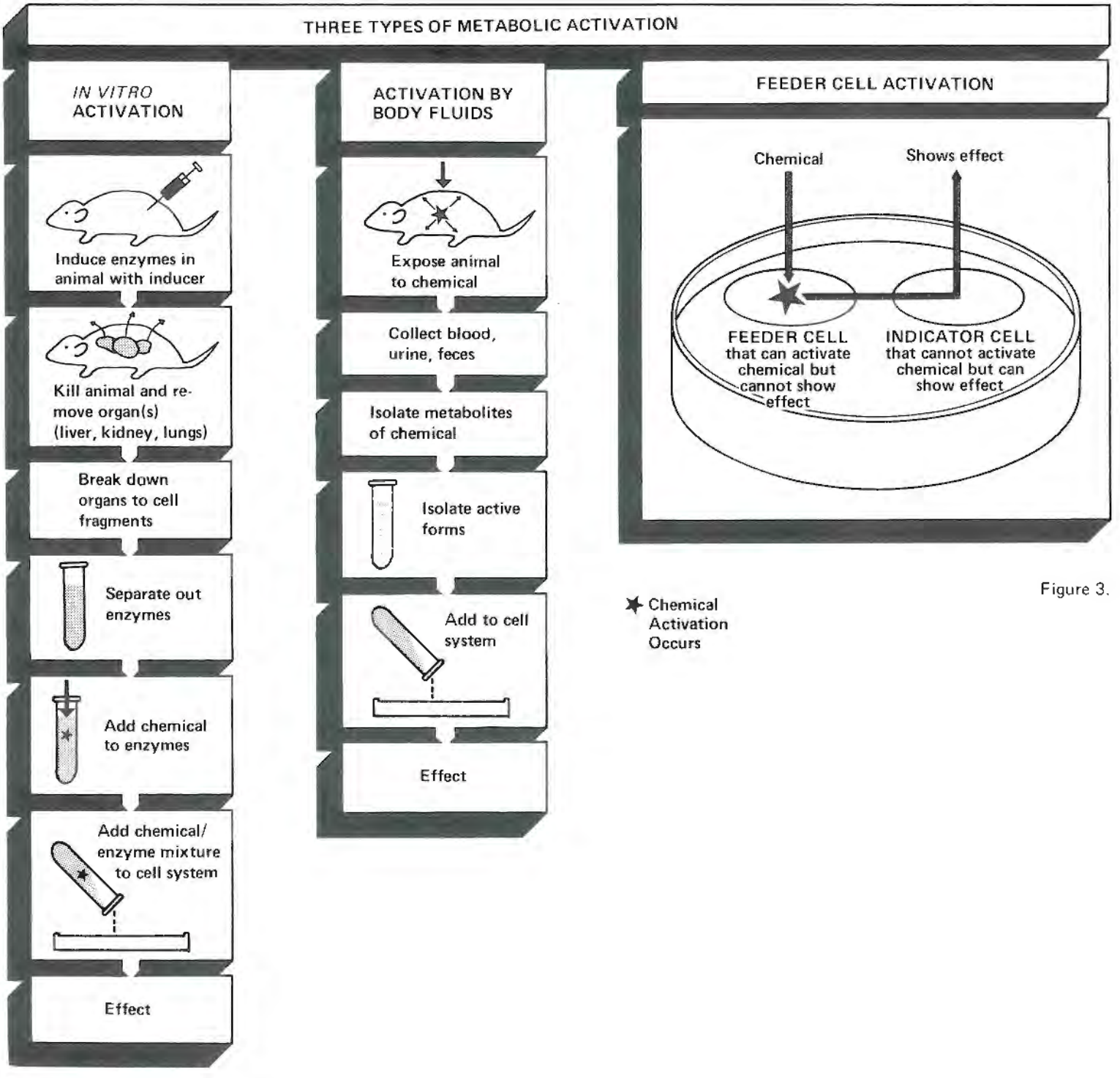

THREE TYPES OF METABOLIC ACTIVATION
IN VITRO ACTIVATION
Induce enzymes in animal with inducer
Kill animal and remove organ(s) (liver, kidney, lungs)
Break down organs to cell fragments
Separate out enzymes
Add chemical to enzymes
Add chemical/ enzyme mixture to cell system
Effect
ACTIVATION BY BODY FLUIDS
Expose animal to chemical
Collect blood, urine, feces
Isolate metabolites of chemical
Isolate active forms
Add to cell system
Effect
FEEDER CELL ACTIVATION
Chemical
Shows effect
FEEDER CELL that can activate chemical but cannot show effect
INDICATOR CELL that cannot activate chemical but can show effect
Chemical Activation Occurs

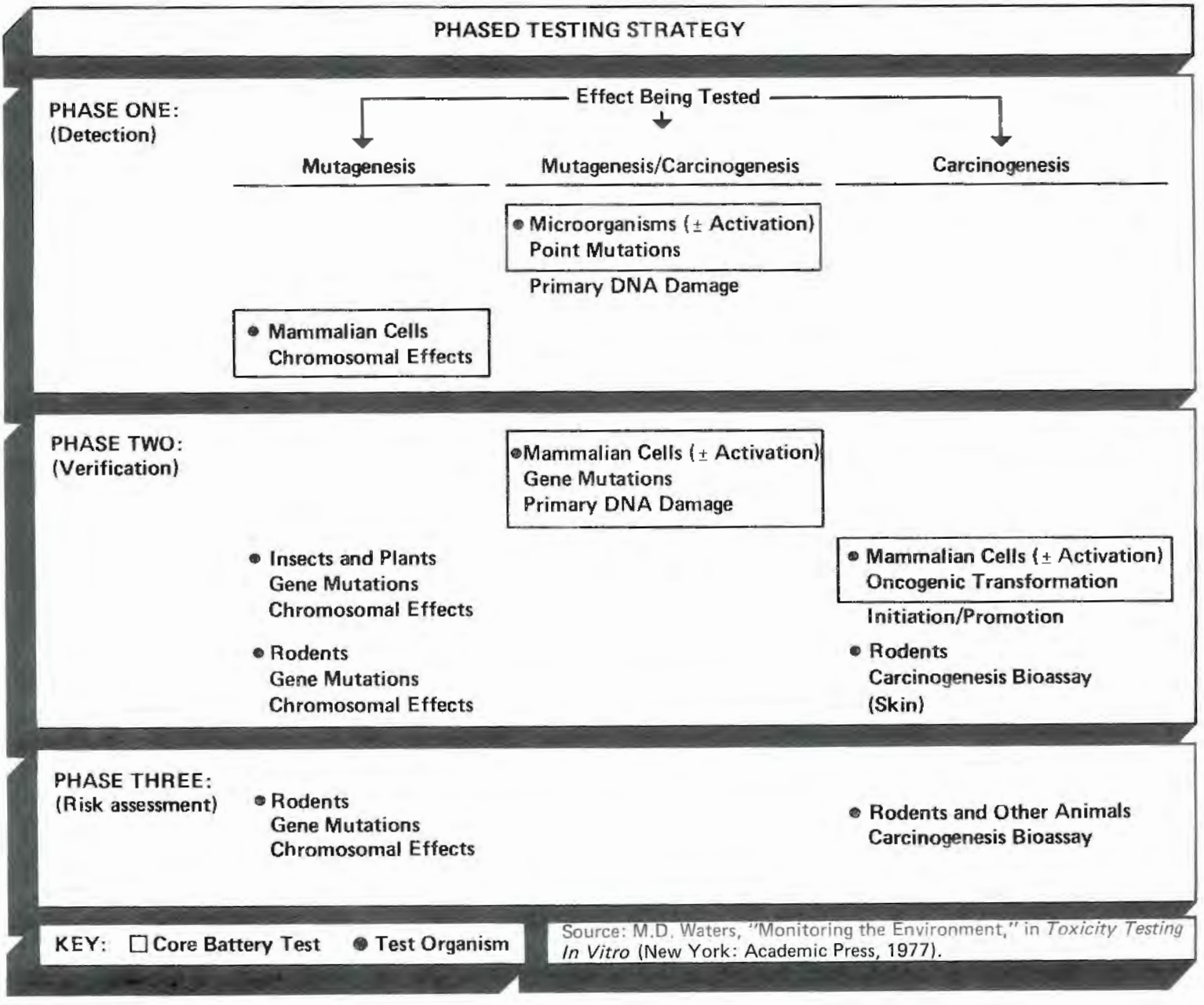
Figure 3.

Table 1
Basic Ways Short-Term Tests Are Done

How to tell a positive response	Systems[a]
Visual observation of whether growth has occurred under special conditions	• DNA damage in microbes • Gene mutation in microbes and isolated mammalian cells
Visual observation of abnormal growth patterns	• Oncogenic transformation of isolated mammalian cells
Microscopic examination for gross changes in the genetic material	• Cytogenetic assays for chromosome alterations • Unscheduled DNA synthesis using mammalian cells • Micronucleus test with mammalian cells • Sister chromatid exchange in mammalian cells • Sperm morphology test
Visual observation for unusual color or shape	• *Tradescantia* — plant gene mutation test • DNA damage in yeast
Observation in whole animals of birth losses and unusual offspring	• Gene mutation or chromosomal alterations in fruit flies (*Drosophila*) • Dominant lethal test in rodents or fruit flies • Heritable translocation in rodents • Specific locus test in mice

[a]See appendix for more complete information.

Phased Testing Strategies



Source: M.D. Waters, "Monitoring the Environment," in *Toxicity Testing In Vitro* (New York: Academic Press, 1977).

Short-term tests are rarely used individually for risk assessment, since no single short-term test is capable of detecting all the types of effects that may be caused by a genotoxic chemical. More often, a group of tests is used, with the particular testing strategy depending on time considerations, cost, and the type, scope, and accuracy of information desired.

Recently, EPA scientists have been exploring the advantages of a phased testing strategy for chemical hazard assessment in which testing is conducted in one or more distinct stages or phases. The extent of testing at each phase is determined by the test results from the previous phase and by the degree of potential hazard suggested by factors such as production volume, projected human exposure, and the known toxicity of related chemicals. By considering this information, limited testing resources are utilized in a manner that provides for the protection of human health in proportion to the anticipated risk involved.

In the phased approach, tests are organized into three phases. Phase One tests principally involve work with microbes and cost on the order of $2,000 or less. They are less definitive than Phase Two short-term tests which commonly use mammalian cells, insects, and plants and cost about 10 times as much as Phase One tests. Phase Two tests are used to confirm the effects detected in Phase One and to characterize more specifically the nature of the effects (i.e., whether the chemical is carcinogenic or mutagenic). Phase Three tests are generally whole-animal studies with rats or mice. They may cost $250,000 or more and take several years to complete. Phase Three tests provide the most authoritative evidence concerning the degree of risk posed by a chemical and may be used to establish acceptable levels for environmental exposure to a chemical. Because of the vast time and expense involved, Phase Three tests are best reserved for a limited number of high priority chemicals.

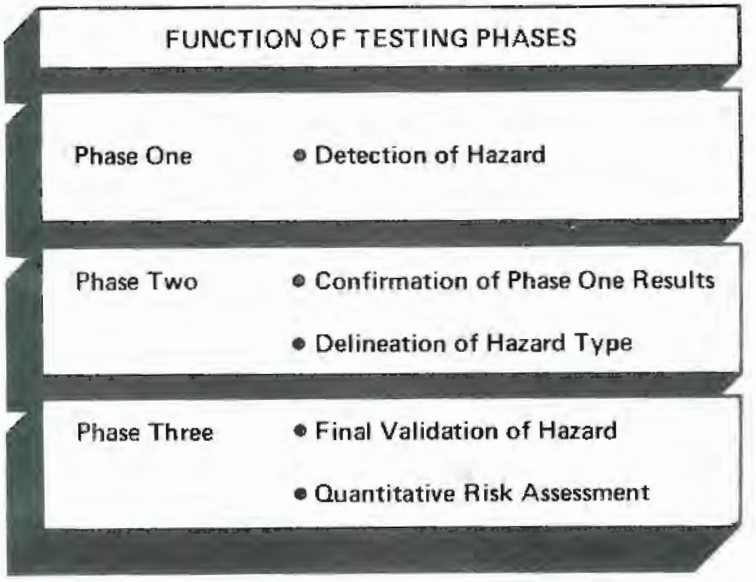
The specific tests used in each phase vary from chemical to chemical, but the basic concept is the same for all phased strategies. Simpler, less expensive tests are first conducted to gain a preliminary indication of a chemical's toxic potential. More thorough and expensive testing is performed in successive phases only if it appears to be merited on the basis of preliminary test results or the degree of anticipated risk.

To ensure thorough testing, a "core battery" of short-term tests may be performed. The "core battery" is a group of essential tests that scientists agree must be conducted in order to determine with reasonable confidence whether or not a chemical may be mutagenic or carcinogenic.

The core battery includes four types of Phase One and Phase Two tests:

- Gene mutations in microorganisms and isolated mammalian cells.
- Chromosome aberrations, preferably in cells from treated animals.
- DNA damage in mammalian cells.
- Malignant-like changes (oncogenic transformation) in mammalian cells.

Phased testing strategies can take advantage of the fact that some tests tend to give false positive results while others tend to give false negatives. False positive-prone tests may be deliberately chosen for Phase One so as to maximize the chance that a hazardous chemical will be detected and sent on for further testing. In designing Phase Two, scientists can use tests that are less likely to give false positives so as to minimize the chances that a relatively innocuous chemical will be sent on for expensive Phase Three testing.



The EPA has begun to use phased testing strategies on a limited scale in its research activities. In this context, phased strategies have proven particularly helpful in analyzing complex mixtures for their hazardous components. An example of the EPA's use of phased testing is described under "Diesel Exhaust" on page 18.

Program Applications

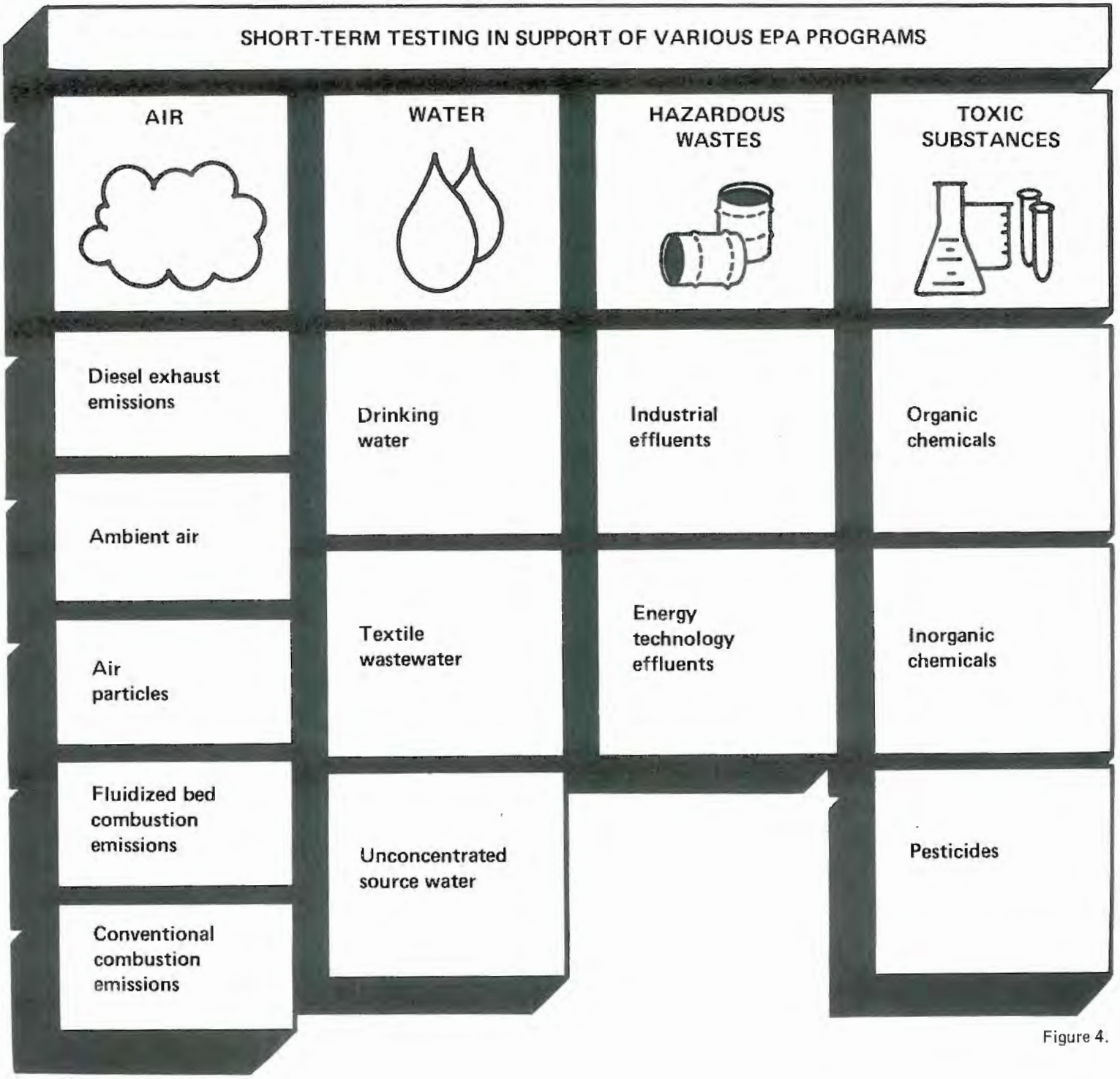


Figure 4.

Although the primary purpose of the EPA's Research and Development programs for short-term tests is to develop, refine, and apply test systems, chemicals that are of genuine public health concern are often selected for use in the research program. This practice enables preliminary toxicity data to be obtained at the same time that test development is taking place. A number of EPA regulatory programs have been assisted in this way (see Figure 4). Two prime examples of research applied to regulatory program uses are discussed below.

Pesticides

Several years ago, the EPA initiated a short-term testing program on 39 pesticides representing several different chemical classes (i.e., organophosphates, chlorinated hydrocarbons, and carbamates). As many as eight different short-term tests were performed for each compound to determine how these biocidal (life-killing) materials would behave in short-term tests.

Specific issues addressed were:

- Would the highly acute toxic properties of pesticides interfere with the ability of the short-term tests to detect genotoxic effects?
- Would every compound show up positive in at least one test, and would this be a realistic indication that all the compounds were genotoxic?
- Which short-term tests were most suitable for use with pesticide chemicals?
- What was the potential of the pesticides to cause long-term health effects such as mutations and cancer?

Since a phased testing strategy was not being used, Phase One, Two, and Three tests were performed concurrently. Phase One tests consisted of assays for bacterial DNA damage and gene mutations, while Phase Two tests looked for unscheduled DNA synthesis in mammalian cells and gene mutations in the fruit fly *Drosophila*. The Phase Three tests involved assays for dominant lethal mutations and heritable chromosomal translocations in mice. Due to resource limitations, only 20 compounds could be tested in *Drosophila,* only 10 in the dominant lethal assay, and only one in the heritable translocation assay.

As it turned out, the short-term tests were adaptable to these highly biocidal materials. Test results for each chemical were considered as a group, so that a single positive response was taken to mean that a chemical was a potential threat. Contrary to what had been feared, pooling the test results in this way did not make every pesticide a potential threat. Seventeen of the 39 chemicals were uniformly negative. The pooling of test results was thus shown to be a practicable method for discriminating between genotoxic and nongenotoxic compounds.

For the 22 compounds that registered at least one positive response, follow-up testing has been started on a case-by-case basis. Compounds that gave a positive response in only one of the tests are being retested to verify the positive results, and certain chemicals that were not originally tested in *Drosophila* and mice are now being tested in those systems. Other Phase Two tests are also being used to look for the potential of pesticides to cause oncogenic transformation and chromosomal effects.

Early results from the pesticide testing program have contributed to an increased understanding of the sensitivity and adaptability of short-term tests. The program has also provided valuable preliminary genotoxicity data which have been used by the EPA's Office of Pesticide Programs to help assess the chronic hazards associated with the various pesticides.

Once Phase Two and Phase Three test results for the 39 pesticides are available for verification and validation purposes, scientists will be able to determine the concordance (or degree of agreement) between the various short-term tests, as well as their accuracy in detecting potentially mutagenic or carcinogenic pesticides. This information should enable scientists to specify which of the tests studied are most suitable for use with pesticides and other biocidal materials. In addition, Phase Three test results will provide the authoritative information needed for pesticide hazard assessment.

Diesel Exhaust

The increasing use of diesel engines in automobiles has prompted EPA concern about the concomitant rise in atmospheric levels of diesel exhaust and the potential health hazards this may create. Diesel exhaust is a complex mixture of thousands of different chemicals, most of which have never been identified chemically.

To isolate all the substances in diesel exhaust and examine each one individually for health effects would be prohibitively difficult, expensive, and time-consuming. The EPA is therefore using a phased testing strategy in combination with chemical analysis to identify which portions (or fractions) of diesel exhaust are hazardous and should be subjected to further chemical analysis and testing. Fractions that appear to be relatively nontoxic are being assigned a low priority for further analysis, so that limited resources will not be devoted to substances less likely to threaten human health.

The testing strategy for the diesel exhaust program has proceeded in several steps. Diesel exhaust was first examined using Phase One core battery tests. One group of tests — the microbial mutagenicity tests — registered positive, indicating that diesel exhaust may contain mutagenic (and possibly carcinogenic) chemicals and therefore should receive further testing.

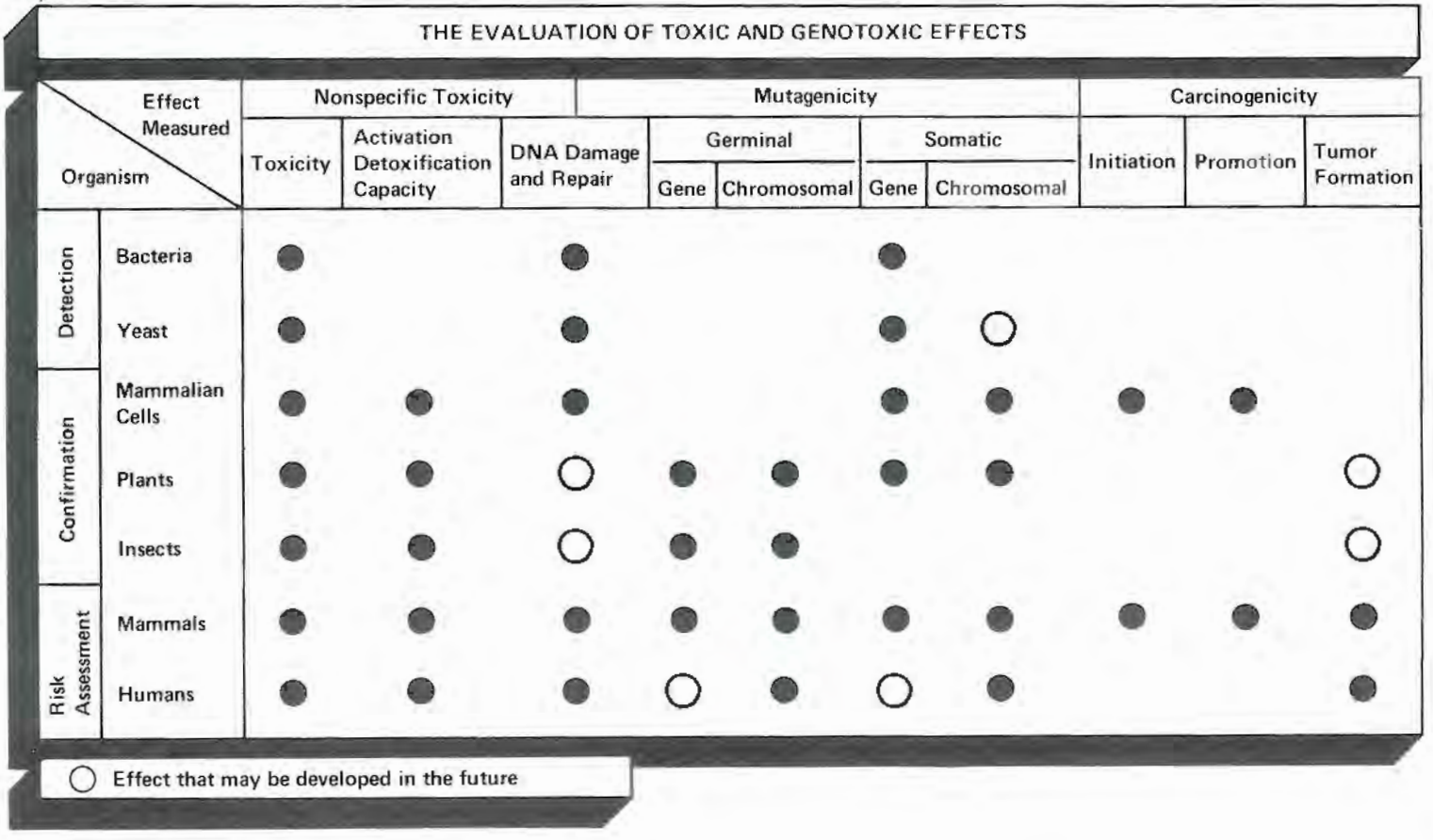
To get a better idea of which portions of the exhaust were potentially hazardous, chemical procedures were used to divide (or fractionate) the exhaust into several distinct fractions. Each fraction was subjected to Phase One microbial mutagenicity tests. The most mutagenic of these fractions were fractionated further, and the resulting subfractions were then tested in the microbial mutagenicity tests. Several of these proved to be mutagenic.

To confirm the activity indicated by the Phase One tests, Phase Two tests were performed on the positive fractions and subfractions. When these tests registered positive as well, it was decided to perform Phase Three whole animal tests on the diesel exhaust to further confirm earlier results and to determine the magnitude of the health threat.

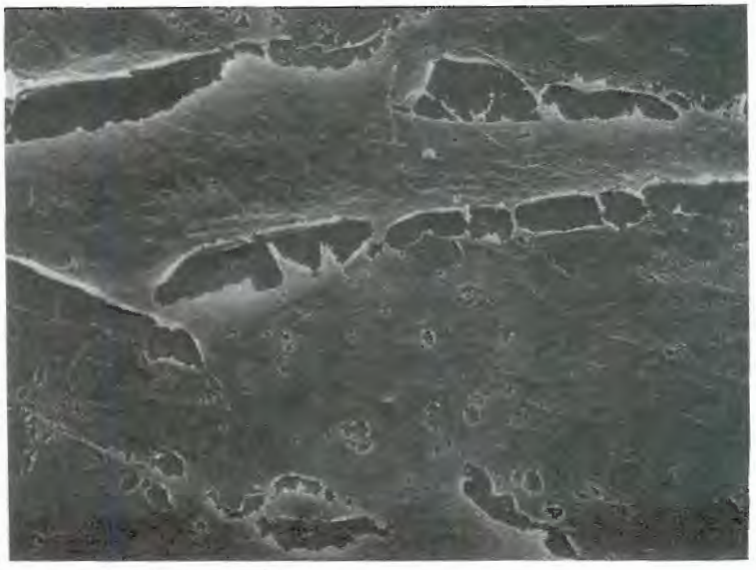
Mutagenic subfractions are currently being analyzed to determine their chemical composition. The pure compounds that are identified by these procedures will be tested individually in mutagenicity tests in an effort to pinpoint precisely which chemicals in diesel exhaust are potentially hazardous.

Although the diesel exhaust program is still in progress, it has already produced some very important and useful results. It has demonstrated two important applications of short-term tests: they can be used with complex mixtures to indicate whether or not a hazard may exist, and they can also be used to pinpoint which fractions of the complex mixtures are responsible for the observed mutagenicity. (These two features may facilitate hazard assessment of common environmental pollutants that are complex mixtures.) In addition, the results of the diesel exhaust program have contributed to the EPA's preliminary assessment of the potential health impacts that may be associated with the increased use of diesel engines.

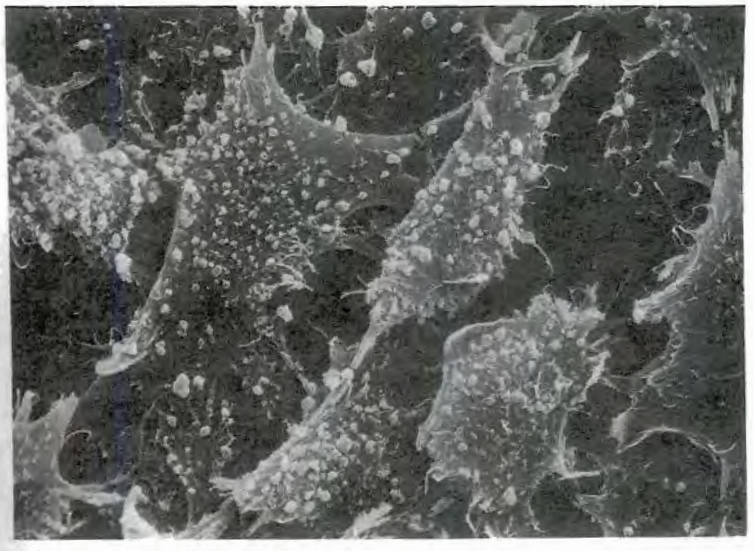
Research Trends



Research on short-term tests is currently proceeding in several areas. Existing test data are being compiled and evaluated in order to document the accuracy and utility of short-term tests. At the same time, laboratory research is being conducted to refine and improve the tests, and several important applications of short-term tests are being explored. Some current areas of research are described below.



A. Normal Cells (3000x)

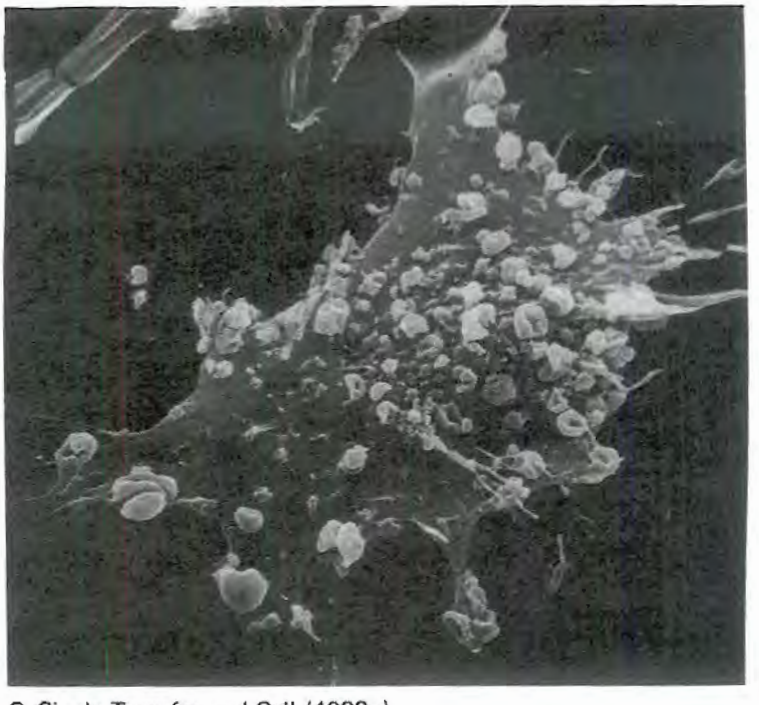


B. Transformed Cells (2000x)

Streamlining a Short-Term Test

Currently, there is no Phase One test for the ability of a chemical to cause oncogenic transformation, because the short-term tests that are presently used to detect this effect take too long to serve as rapid screening tests. Generally, 6 weeks must elapse after chemical exposure before the cell system exhibits the usual signs of transformation (abnormal growth patterns) that are visible under a light microscope.

The possibility of shortening the time for detection of transformation is currently being explored. Investigators have noticed that transformed cells exhibit a strikingly different surface from normal cells when viewed under a scanning electron microscope (SEM) (Figure 5). Research is currently under way to investigate how soon these changes occur after chemical exposure, and whether they are as accurate an indicator of the transformed state as abnormal growth patterns. If the SEM can detect transformation quickly and accurately, oncogenic transformation may become usable as a rapid prescreening test in the first phase of testing. This would help make Phase One testing a more comprehensive indicator of potential carcinogenicity.



C. Single Transformed Cell (4000x)

Figure 5.

Tackling the Sample Size Problem

Each short-term test requires a certain amount of the chemical in order to adequately test for genotoxic potential. If too small a concentration is used, the chemical's toxic properties may go undetected. This sample size requirement can make short-term testing impossible if the substance to be analyzed is in short supply. Gas samples are particularly troublesome since the materials in them are highly diluted, and it is often difficult to obtain a concentrated sample for analysis. The volume of material available for testing purposes may be reduced even more if the sample must be broken down chemically into smaller and smaller fractions in order to pinpoint the hazardous components (see "Diesel Exhaust" on page 18).

Research is currently being conducted to modify short-term tests so that they will require smaller sample amounts. Already, the standard Ames test for carcinogens and mutagens (see Figure 6) has been modified so that effectively one-fifth as much sample is required. By using a "well test" procedure, a one-milligram sample can be used to make four or five different measurements that would each normally require milligram quantities. Scientists are also attempting to develop innovative chemical fractionation techniques that will allow larger samples to be derived from complex mixtures.

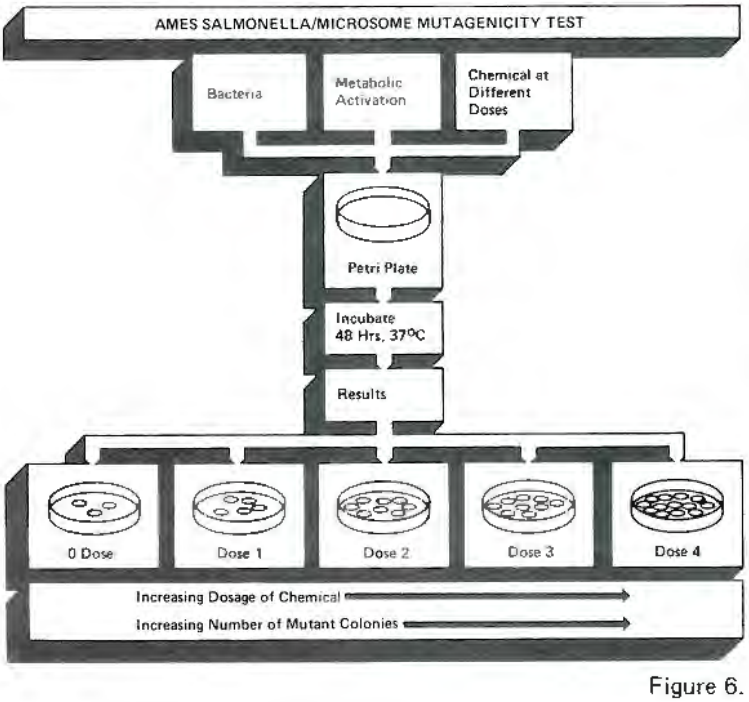


Figure 6.

Developing Human Cell Systems

The most obvious system to use for detecting potential human carcinogens and mutagens is one that employs human cells. Many attempts have been made to develop such a system, but only recently has the work proven successful. In 1978, a group of scientists showed that cell lines derived from human foreskin could be cultured and used to detect oncogenic transformation. At about the same time, other human cells were shown to be amenable to studying mutagenic effects. These advantages prompted the EPA to initiate research into human cell systems for mutagenesis and oncogenic transformation.

Research is proceeding in several areas. Techniques are being developed to allow the continued propagation of human cells under culture conditions, and different types of human cells are being tested for their ability to survive in culture. Along these lines, scientists are exploring the possibility of developing model systems using cells from organs that are often the sites of cancer (i.e., lung, intestine, and prostate). If this effort is successful, such model systems could provide a more accurate means of predicting the effects of chemicals on specific sites in the human body.

Multi-Effect Tests

Most short-term tests that use mammalian cells have been designed to measure only one type of biological effect. EPA scientists are currently working to develop mammalian cell systems that will be able to detect two or more different kinds of effects. Various mouse and human cells that may be capable of detecting both mutation and oncogenic transformation are being explored, and special attention is being focused on a Chinese hamster ovary (CHO) cell system that may be able to simultaneously test for four different types of effects (general toxicity, gene mutation, chromosomal effects, and DNA damage).

Development of multi-effect test systems has important ramifications. Such systems would enable scientists to gain greater insight into the relationships between the various effects being tested. By eliminating the differences that may result from the use of different cell systems, it should be possible to see if there is some underlying connection between the various effects that a chemical may have. Enhanced understanding of the mechanisms underlying the genotoxic effects measured by multi-test systems could eventually lead to major refinement of testing strategies and better understanding of the significance of test results.

Improving Metabolic Activation

Metabolic activation is such an important step in short-term tests that deficiencies in activation systems are often suspected as the cause of inaccurate test results. EPA scientists are currently exploring means of improving existing systems and developing more potent methods of activation. Research is proceeding in several areas. Existing activation systems are being biochemically analyzed in order to gain a better understanding of the important enzymes involved. At the same time, scientists are working to develop test systems using organisms and cells known to have a high metabolic capacity. Such systems could hopefully provide their own metabolic activation and thus not require the addition of enzymes from another source (exogenous activation).

Researchers are also exploring the possibility of using enzyme inducers to activate mammalian cells in culture. Currently, inducers are injected into whole animals to raise their enzyme levels, and various organs (usually the liver) are then ground up and applied to test systems. It is hoped that direct application of inducers to mammalian cell cultures will raise the cells' enzyme levels and eliminate the need for exogenous activation. Such activated mammalian cells could also be used to activate another cell system by the feeder layer technique (see Figure 3).

The Gene-Tox Program

Since short-term tests were first developed more than 10 years ago, a significant amount of information has been generated concerning their accuracy and reliability. The purpose of the Gene-Tox[1] program, which is being directed by the EPA's Office of Toxic Substances, is to compile all the available information on short-term tests and to provide an up-to-date evaluation of their status.

Twenty-seven different short-term tests have been scheduled for the initial evaluation. For each test, expert scientists drawn from government, industry, and academia will review and evaluate the available information. Answers will be sought to such questions as: How accurately can the test system detect carcinogenic and/or mutagenic chemicals? Is the accuracy greater with certain classes of chemicals? Can the actual magnitude of the hazard to human health associated with a chemical be predicted? In addition to addressing these questions, investigators will attempt to determine which groupings of tests are most suitable for specific purposes; for example, which tests would together most effectively test for a specific type of genetic damage. This information will be extremely valuable in designing testing strategies that are both accurate and cost-effective.

An important part of the Gene-Tox program will be the development of a computerized data management system for the storage and analysis of the significant data. This will facilitate the review process by enabling the information to be readily accessed according to such parameters as chemical, chemical class, type of test system, or organisms used. The computerized data file will also permit future test data to be added as they become available, thereby providing an accessible and up-to-date record of all relevant information on the tests.

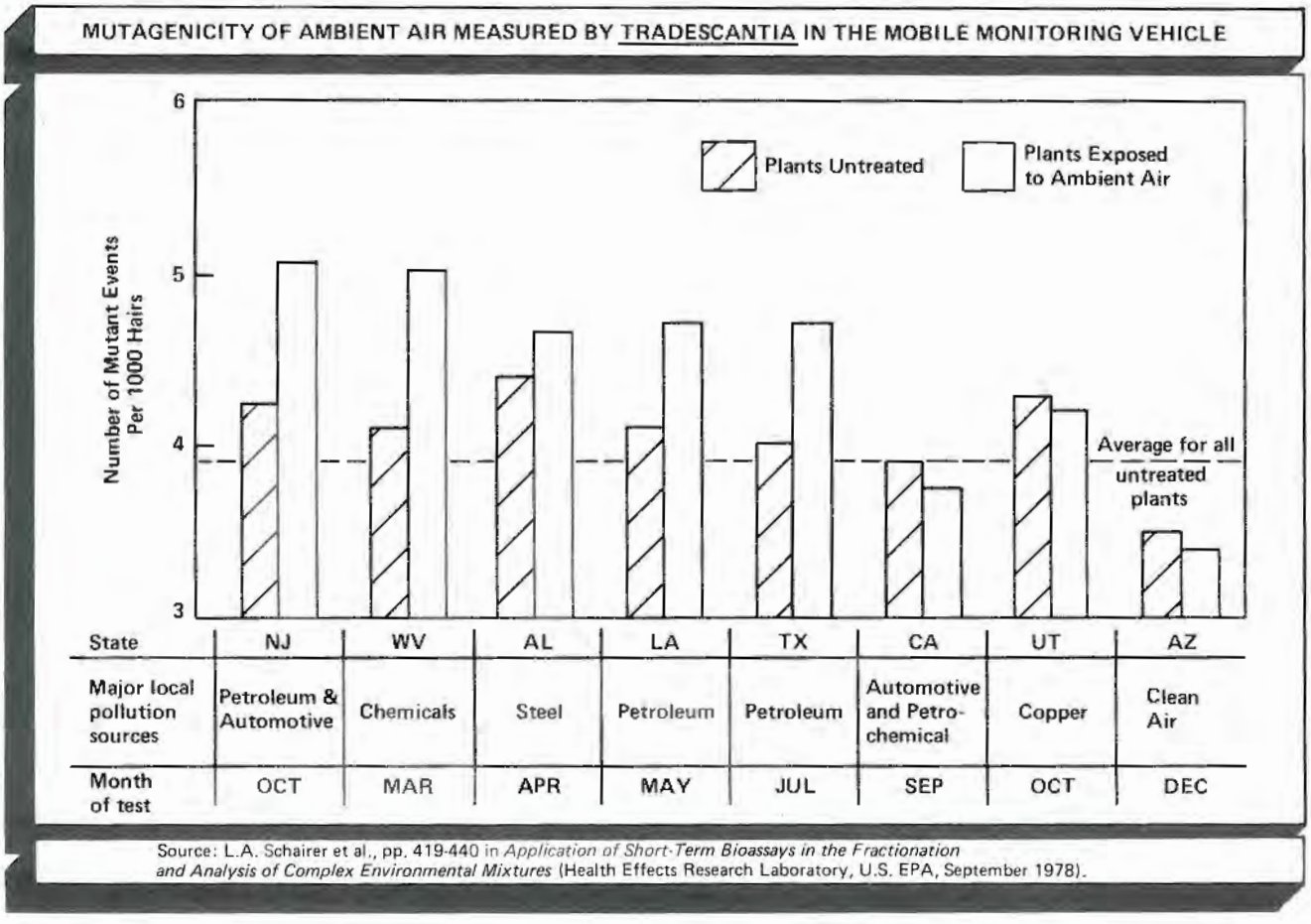
In addition to providing a state-of-the-art evaluation of short-term tests, the Gene-Tox program will also accomplish another important task. It will help to identify aspects of short-term tests that require further development and validation. Such information will be of great value in designing and guiding future research programs.

Plants and Humans

Scientists have recently discovered that plants have enzymes similar to those found in animals. This intriguing finding has inspired two related avenues of research. One seeks to exploit plants as a source of metabolic activation for short-term tests. The other is concerned with the possibility that chemicals applied to plants may be converted by plant enzymes to new chemicals that may be toxic to humans ingesting the plants.

So far, this research has produced some exciting, but possibly disturbing findings. Tissues taken from plants treated with pesticides were ground up and applied directly to the Ames assay with no additional metabolic activation. While the pesticides themselves had shown no mutagenic activity in the Ames assay (either with or without animal-derived metabolic activation), the pesticide/plant mixture showed weak positive activity. Such a finding suggests that plants may have a previously unrecognized capacity to transform substances into potentially mutagenic and carcinogenic compounds.

[1]Derived from the program's official title: *An Evaluation of the Current Status of Bioassays in Genetic Toxicology*.



Source: L.A. Schairer et al., pp. 419-440 in *Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures* (Health Effects Research Laboratory, U.S. EPA, September 1978).

Figure 7

Field Monitoring

One exciting short-term test application currently being explored by the EPA is the use of the *Tradescantia* plant system for monitoring on-site air quality. Traditionally, air has been monitored using chemical methods to determine the concentrations of specific known hazardous chemicals. The *Tradescantia* plant test may provide an improved means of measuring overall air quality by allowing ambient air to be screened for the presence of mutagenic chemicals.

The concept behind the *Tradescantia* test is a simple one. When exposed to gaseous mutagenic chemicals, the stamen hair cells of the *Tradescantia* flower mutate from blue to pink. By counting the stamen hair cells that change color, a measure of potential mutagenicity can be obtained. *Tradescantia* is particularly suited to field testing because it can tolerate a broad range of conditions and, unlike some other short-term tests, it does not require special sterile conditions.

In a collaborative research program,[1] a mobile laboratory for exposing *Tradescantia* to ambient air has been tested successfully in eight different natural and industrial environments, including sites in New Jersey, California, Texas, Utah, and the Grand Canyon in Arizona. The mutagenicity findings are presented in Figure 7. Further research will have to be performed to gain a better idea of the potential and limitations of the *Tradescantia* test, but the results obtained so far suggest that the test can be used as a sensitive and rapid indicator of ambient air quality.

[1]Involving Brookhaven National Laboratory, the National Institute of Environmental Health Sciences, and the Environmental Protection Agency.

Perspective

One of the highest priorities of the EPA Research and Development Program is the protection of human health through the identification and control of toxic substances. Short-term tests have been singled out for intensive research and development because they are potentially valuable tools for achieving this goal.

Many types of short-term tests are being developed, but this document has focused on short-term tests that attempt to detect a chemical's potential to cause cancer and genetic disease. These disorders are among the most devastating that a chemical may cause, and short-term tests in this area have consequently received great attention. This attention has been rewarded by highly encouraging results.

Our advanced industrial economy uses and produces thousands of chemicals, most of which did not even exist until the past several decades. To reduce the threat of these substances to human health, scientists must be able to discriminate toxic materials from those that are nontoxic. This is an awesome task, but not an impossible one if short-term tests fulfill their early promise for rapid and effective detection of potentially hazardous chemicals.

Appendix: How Effects are Measured in Various Short-Term Tests

DNA Damage in Bacteria (Pol A test, rec test)
Two strains of bacteria are used that are identical except in their ability to repair DNA damage; one strain can repair damage while the other cannot. Both strains are exposed to the test substance, and the extent to which cells are killed is measured for each. If the repair-deficient strain has a greater degree of cell killing, DNA damage is assumed to have occurred.

DNA Damage in Yeast (Mitotic recombination, mitotic gene conversion, or mitotic crossing over)
Special strains of yeast cells are used to test for these effects. When the cells change color from white to either pink or red, DNA damaging potential is indicated.

Gene Mutation in Bacteria or Fungi (Ames test, WP2 assay, yeast assays, and others)
Special strains of bacteria are used which cannot grow without a nutritional supplement. Certain types of mutations will permit these bacteria to grow in unsupplemented media. By treating the cells and then seeing if they can grow in unsupplemented media, mutagenicity can be measured. Distinguishing mutated bacteria from nonmutated bacteria is not necessary using this procedure, since only mutant cells can grow and form visible colonies.

DNA Damage in Mammalian Cells (Unscheduled DNA Synthesis and Sister Chromatid Exchange)
Abnormal distribution of a DNA marker indicates whether DNA damage has occurred. Ways of detecting this abnormal distribution include microscopic examination and photographic and machine measurements.

Gene Mutation in Mammalian Cells (HGPRT, TK, and Na/K-ATPase assays)
In these systems, mutations that confer resistance to a poison are measured. Cells are first treated with a test chemical and then exposed to the poison. Since only mutant cells can survive and grow, mutagenicity can be measured simply by observing the extent of growth in the poisonous environment.

Gene Mutation in Plants (*Tradescantia* and maize waxy locus)
Mutations in these plants are detected by looking for color changes in the stamen hairs or pollen grains. In *Tradescantia,* mutation causes the stamen hairs to change from blue to pink. In maize, mutated pollen grains can be detected by the purple color they acquire when they are treated with iodine.

Chromosomal Effects in Isolated Cells or Whole Organisms (Cytogenetics assays)
Treated cells (or cells from treated organisms) are stained and then examined under the microscope for various chromosomal abnormalities. Lost, broken, or disarranged chromosomes indicate genotoxicity.

Oncogenic Transformation (Transformation assays)
When certain types of mammalian cells are treated *in vitro* with carcinogens, they undergo cancer-like transformation. If these cells are injected into appropriate experimental animals, tumors will appear. Most frequently, transformed cells are distinguished by their unusual growth patterns in culture, such as abnormal piling-up and disorientation of cells.

Micronucleus Test
Animals are treated with a chemical, and their red blood cells are removed, stained, and examined under the microscope. If small fragments of the genetic material (micronuclei) are observed, chromosomal damage is indicated. Normal red blood cells will not contain any genetic material or fragments of genetic material.

Drosophila melanogaster (Sex-linked recessive lethal test for gene mutations; nondisjunction and heritable translocation assays for chromosomal effects)
Drosophila have a variety of "marker" traits that can be used to signal whether gene mutations or chromosome disturbances have occurred. In general, *Drosophila* tests involve treating specially "marked" male or female flies with a substance, mating them, and then observing whether their offspring have certain specific features, such as unusual eye color or shape. Depending on the test, genotoxic events can be indicated either by the presence or the absence of a specific feature in the offspring.

Appendix: Representative Short-Term Tests for Genotoxity

Type of Test	Specific Test	Organisms Used
DNA Damage in Microbes	Pol A test	*Escherichia coli*
	rec test	*Bacillus subtilis*
	Mitotic recombination, mitotic crossing over, or mitotic gene conversion in yeast (D3, D4, D5, or D7 Assays)	*Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*
DNA Damage in Mammalian Cells	Unscheduled DNA Synthesis (UDS)	WI-38 strain human cells or various rodent cells
	Sister-chromatid exchange (SCE)	Various cell lines or animal sources
Gene Mutation in Bacteria and Fungi	Ames test	*Salmonella typhimurium*
	WP2 Assay	*Escherichia coli*
	Yeast "forward" and "reverse" assays	*Saccharomyces cerevisiae; Schizosaccharomyces pombe*
	Miscellaneous	*Aspergillus nidulans; Neurospora crassa*
Gene Mutation in Higher Systems	HGPRT, TK, and Na/K-ATPase Assays	L5178Y mouse lymphoma cells; Chinese hamster ovary cells (CHO); Chinese hamster lung cells (V-79)
	Sex-linked recessive lethal assay	*Drosophila melanogaster*
	Plant tests	*Tradescantia;* maize waxy locus
Chromosomal Effects in Isolated Cell Systems	*In vitro* cytogenetics assays	WI-38 strain human cells; Chinese hamster ovary cells (CHO)
Chromosomal Effects in Whole Organisms	*In vivo* cytogenetics	Various rodent species
	Micronucleus test	Various rodent species
	Nondisjunction assay	*Drosophila melanogaster*
	Heritable translocation assay	*Drosophila melanogaster*
Oncogenic Transformation	Transformation assays (clonal or focus)	Syrian hamster embryo cells (SHE); BALB/c3T3 mouse cell line; C3H10T1/2 mouse cell line
Tumor Formation	Mouse skin tumorigenesis	Sencar mice
	Mouse pulmonary adenoma	Strain A mice
	Rat tracheal transplant	Various rat strains

acute effect	a health effect of short duration that is usually reversible
Ames assay	a well-known short-term test that measures a chemical's ability to cause mutations in a specially engineered strain of the bacteria *Salmonella typhimurium*
bioassay	a test to determine the effect of a chemical on a living organism
carcinogenic	able to cause cancer
chromosome	a form of DNA organization found in higher cells and organisms
chromosome aberrations	changes in the number, shape, or structure of chromosomes
chronic effect	a prolonged health effect that may involve irreversible change or damage
complex mixtures	a grouping of several different chemicals
DNA (deoxyribonucleic acid)	a large molecule that contains the genetic information responsible for cell growth, function, and reproduction
enzyme	a protein that acts as a catalyst to allow a specific chemical reaction to take place in a cell
epidemiology	the science of correlating exposure to a substance with the appearance of a specific disease or other effect in a human population group
false negative	a test result which indicates that a chemical is harmless when it is actually hazardous
false positive	a test result which indicates that a chemical is hazardous when it is actually harmless
fractionation	the process of chemically separating a complex mixture into a series of simpler mixtures (fractions)
gene	a portion of DNA that directs the formation of a single product
gene mutation	a mutation in a single gene
genetic material	see DNA
genotoxic	able to damage genetic material
germinal cell	a reproductive cell (i.e., sperm, egg)
hazard assessment	the evaluation process for determining if a substance is hazardous to humans

heritable	capable of being passed from one generation to another
in vitro	pertains to a procedure that takes place in an artificial medium (lab equipment) without the use of live animals. Literally means "in glassware"
in vivo	pertains to a biological reaction or test which occurs within the body of a live animal
indicator system	a cell or organism that shows (or indicates) a specific effect
malignant	refers to the cancerous cells or tumors that may grow, proliferate, and eventually kill the organism
metabolic activation	the process whereby an inactive material is changed into an active one (in the context of short-term testing, this involves the conversion of a procarcinogen to a carcinogen or a promutagen to a mutagen)
metabolism	the physical and chemical processes in an organism which transform chemicals into simpler or more complex forms
metabolite	a product of metabolism
microbes	microorganisms such as bacteria or yeast
mutagenic	able to cause mutations
mutation	a stable change in the genetic material
oncogenic	able to cause tumors
oncogenic transformation	a cancer-like change that can be brought about in isolated mammalian cells by chemical treatment
procarcinogen	a substance which is converted into a carcinogen by an organism's metabolic processes
promutagen	a substance which is converted into a mutagen by an organism's metabolic processes
protein	a large biological molecule essential for many cell structures and functions
somatic cell	any nonreproductive cell in a multicellular organism
stamen hairs	the part of the flower that produces pollen
target cells	isolated cells or cells within an organism which react in a specific manner to a toxic chemical or other stimulus
toxic	able to produce an adverse effect
transformed	see oncogenic transformation

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