Carcinogen Testing: Current Problems and New Approaches

John H. Weisburger and Gary M. Williams

Summary. The classic procedures for testing potential carcinogens in animals have basically not changed in the past 50 years. Considerable knowledge of the mechanisms of carcinogenesis has accrued in the last 20 years, particularly concepts on the metabolic activation of chemicals to reactive electrophilic compounds that can interact with nucleophilic cell components including DNA. These developments, in turn, have yielded a framework for integrating into carcinogen testing the determination of genetic effects of chemicals. A systematic decision point approach to carcinogen testing has been developed which entails a sequential decision-making process as specific tests are performed and evaluated prior to initiation of higher order, more complex tests. Compared to conventional bioassays in rodents, this approach provides knowledge based on mechanisms of carcinogenesis, yields a substantial amount of data at minimal cost, and forms a solid base for eventual health risk assessment.

In recent decades, scientists and the public at large have been much concerned with questions on the environmental causation of cancer. Based on the information that chemicals producing cancer in humans also are carcinogenic in animals, animal models were developed to assess potential cancer risks to humans. Initially, such tests were conducted mostly by academic scientists with an interest in structure-activity correlations, using specialized experimental assays. Later, certain testing approaches were elaborated and standardized before their limitations were apparent and before the mechanisms of carcinogenesis were adequately understood.

Difficulties with Current Approaches to Carcinogen Testing

The standard typical bioassays for the detection of chemical carcinogens as developed by the National Cancer Institute (NCI) requires the use of male and female rats, mice, and occasionally hamsters of strains selected for their sensitivity to carcinogens (*1*). The standard test involves determination of the maximally tolerated dose (MTD) of a product, after which, groups of 50 male and female animals are given the MTD and half-MTD in a 2-year test and, in some instances, a lifetime test. The animals are then examined postmortem with an extensive review of their tissue pathology, and the incidence of neoplasms in the experimental groups is statistically evaluated in comparison with control groups.

Such testing requires not only large resources in time and money but also scarce specialty skills such as veterinary medicine and pathology for reliable execution. Since the time these tests were first developed, largely by the NCI, other organizations such as the Food and Drug Administration, Environmental Protection Agency (EPA), Consumer Protection Safety Commission, Occupational Safety and Health Administration, and National Toxicology Program (NTP) have emerged to require as part of their mission specific additional tests and expanded data. When the NCI began carcinogen screening programs in 1962, a test of a given chemical performed in one species took as little as 8 months and cost about $10,000 to $15,000. Ten years later a more extensive test in two species with larger numbers of animals required about 30 months and cost about $75,000. Now, another 10 years later, tests of a chemical for multiple observational end points require even larger resources, more time (up to 64 months), and as much as $300,000 to $500,000 (*2*).

The results for approximately 245 chemicals tested by standardized procedures have been published as NCI or, more recently, as NTP reports. For these chemicals, 32 tests were judged inconclusive and 23 equivocal (*3*). A number of tests gave borderline results that presented statistical difficulties, and without additional data points and mechanistic understanding they were subject to much subjective and even controversial interpretations.

Animal bioassays by themselves can yield ambiguous results, especially in relation to human risk assessment. In the past it was the practice to take the data at face value. When an experiment yielded a statistically significant excess of cancer in the test series compared to the controls the test substance would be labeled a carcinogen, and regulatory agencies would be inclined, or indeed forced, to take appropriate steps to remove such a product or otherwise protect potentially exposed individuals. This approach is justified with agents that are obviously carcinogenic, such as those yielding a high incidence of cancer at a given site in several species in a short time. In fact, most known human carcinogens do exactly that, and thus, in order to define human risk, relatively little additional information may be needed for such compounds.

A variety of chemicals, however, yield less definitive evidence upon testing but nevertheless have been represented as being human cancer risks (*4*). For example, amaranth (FD&C Red Dye No. 2) seemed to yield a statistically significant incidence of total tumors in female rats (but not in male rats) in the absence of an increase at any specific site. Nitrite was reported to increase slightly the incidence of spontaneously arising splenic sarcomas in rats. With high levels of saccharin, evidence of carcinogenicity was seen in small numbers of rats in a two-generation test. Thus, amaranth was banned in the United States (but not in other countries), and regulatory actions were formulated but not implemented for nitrite and saccharin.

Moreover, a substantial portion of the chemicals tested under the NCI protocols, especially those belonging to the class of halogenated hydrocarbons, produced an increase in the incidence of liver tumors that have a 20 to 40 percent spontaneous occurrence in the mouse

Dr. Weisburger is vice president for research, American Health Foundation and director of the Naylor Dana Institute for Disease Prevention, the Research Institute of the Foundation. Dr. Williams is associate director of the Institute and chief of the Division of Pathology and Toxicology. The authors are also research professors of pathology, New York Medical College. Their address is American Health Foundation, Valhalla, New York 10595.

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strains used, but had no other major carcinogenic effect (*5*). The significance and interpretation of this finding, in particular, should be viewed together with collateral evidence on the possible mode of action of this group of chemicals.

In an effort to provide a comprehensive overview of chronic toxicity and carcinogenicity, more complex and expensive bioassays were developed. Even so, some of these bioassays have yielded false negative results. A recent example is the NCI testing of vinylidene chloride, which was reported to be inactive. In a smaller, earlier test series, this chemical yielded distinct positive results and was also mutagenic (*6*). The negative result in the large-scale bioassy therefore requires an explanation; furthermore, it indicates the need for a more systematic approach to carcinogen assays in order to avoid such problems.

Another consideration of increasing importance is the ethics of routinely using large numbers of animals in testing programs when other approaches are available to delineate hazard. The concern of the public with this issue is reflected by the reintroduction in the 1981 Congress of bill H.R. 556 which is intended to establish a National Center for Alternative Research to develop and coordinate alternative methods of research and testing that do not require the use of live animals.

Chemical Carcinogenesis

For the interpretation of animal studies, an operational definition of a carcinogen is applied to any chemical or product that under the conditions of the test leads to a statistically significant increase in neoplasms in specific target organs (*7*). The general use of this operational definition requires reevaluation in the context of the sizable advances in knowledge of the mechanisms of carcinogenesis that have occurred during the last 20 years.

Research on the correlations between structure and activity and related approaches led to the discovery of a great variety of chemical carcinogens. Although some of these carcinogens had totally different structures, they affected the same target organ. For example, the mold toxin aflatoxin B_1, the plant product safrole, the synthetic chemicals 4-dimethylaminoazobenzene, 2-acetylaminofluorene, and dimethylnitrosamine all caused liver cancer when administered under suitable conditions to laboratory rodents such as mice, rats, or hamsters; in addition, some of these carcinogens were active in larger animals such as dogs or monkeys (*8*). Since the agents mentioned caused cancer at points remote from the point of application, it seemed logical that they required some form of metabolism in the target organ. This, in turn, led to research on the metabolism and mode of action of typical chemical carcinogens.

Thus it was found that pro- or precarcinogens, or indirectly acting carcinogens, were converted to a more active agent, the proximate carcinogen, which in turn was further metabolized to the ultimate carcinogen (*7*). The last agent could interact directly with the critical targets in the cell initiating a sequence of steps leading to cancer. Synthetic, direct-acting carcinogens have such properties inherent in their structure. The ultimate carcinogens are electrophilic reactants, a concept generalized by the Millers (*9*). Such products possess a positive charge that can react covalently with the nucleophilic components in cells, among which the genetic material DNA has emerged as potentially the most significant (*10*). This, in turn, led to an association between carcinogenicity and mutagenicity.

The correlation between mutagenicity and carcinogenicity was greatly expanded when Ames and co-workers (*11*) introduced the use of histidine-requiring mutants of *Salmonella typhimurium* for detecting mutagens and carcinogens. The need for mammalian enzyme activation systems was met by adding a subcellular fraction (S-9) of liver, consisting of microsomes and a soluble fraction obtained by sedimentation of a rodent liver homogenate at 9000*g*. The fraction functions well, but is metabolically different from a whole liver cell in vitro and even more so from liver in vivo (*12*). In particular, S-9 is deficient in enzymes that yield conjugated metabolites and possesses different ratios of specific metabolizing enzymes including the cytochrome systems. This accounts for certain quantitative but usually not qualitative differences between mutagenic activity and carcinogenicity. The failure to detect mutagenicity with known carcinogens in vitro frequently hinges on deficient conversion, by the biochemical activation system used, of the promutagen to the ultimate electrophilic form. Modification of the activation steps often leads to a resolution of the differences observed. At the same time that reliable microbial systems were being developed (*13*), a variety of other short-term tests were introduced that extended knowledge of the mutagenic effects of carcinogens (*14*).

There are several exceptions to the correlation between mutagenicity and carcinogenicity. For example, DDT and other chlorinated hydrocarbons, hormones such as diethylstilbestrol and even naturally occurring estrogens, and drugs such as phenobarbital caused tumors in classic animal bioassays yet were uniformly inactive in mutagenicity tests (*15*). By the operational definition discussed above, all such compounds would be called carcinogens, yet, just as the concept of electrophilic reactants has proved useful in following through the many structural types of organic carcinogens, it seems clear that additional mechanistic concepts will have to be developed for these other carcinogens.

The decision as to whether a chemical has the potential for interaction with genetic material, that is, has genotoxic properties, can be made qualitatively on the basis of several criteria: (i) a reliable, positive demonstration of genotoxicity in appropriate prokaryotic and eukaryotic systems in vitro; (ii) studies on binding to DNA; or (iii) evidence of biochemical or biologic consequences of DNA damage (*16*). Genotoxic chemicals appear to exert their effects by interacting directly, or after conversion to an ultimate carcinogenic form, with DNA. In a quiescent nonduplicating cell this DNA can be repaired. However, duplication of a cell with modified DNA results in mispairing of bases and gives rise to progeny with an abnormal genome corresponding to what is commonly called the dormant or latent tumor cell. Under permissive conditions, such abnormal cells can proliferate and give rise to a neoplasm.

In contrast to genotoxic carcinogens, certain hormones, chlorinated hydrocarbons such as DDT, and phenobarbital exert their carcinogenic effects through incompletely known mechanisms that might best be called epigenetic interactions. Evidence indicates that epigenetic agents require an antecedent change in the mammalian genome. By themselves, epigenetic agents presumably are incapable of causing conversion of a normal cell to a neoplastic one but permit the expression of preexisting latent neoplastic cells. In such instances where the induction of neoplasia by an epigenetic agent might have occurred, it is essential to determine what possible other antecedent reaction might have led to the gene change.

On the basis of these differences in carcinogenic mechanisms, carcinogens can be classified into two broad types, genotoxic and epigenetic, and further divided into eight subclasses of compounds (Table 1) (*16*). Experimental sup-

Table 1. Classes of carcinogenic chemicals. From data in (*6*).

Type	Mode of action	Example
	Genotoxic	
1. Direct-acting	Electrophile, organic compound, genotoxic, interacts with DNA	Ethylene imine
2. Procarcinogen	Requires conversion through metabolic activation by host or in vitro to type 1	Vinyl chloride, benzo[*a*]pyrene, 2-naphthylamine, dimethylnitrosamine
3. Inorganic carcinogen	Not directly genotoxic, leads to changes in DNA by selective alteration in fidelity of DNA replication	Nickel, chromium
	Epigenetic	
4. Solid-state carcinogen	Exact mechanism unknown; usually affects only mesenchymal cells and tissues; physical form vital	Polymer or metal foils; asbestos
5. Hormone	Usually not genotoxic; mainly alters endocrine system balance and differentiation; often acts as promoter	Estradiol, diethylstilbestrol
6. Immunosuppressor	Usually not genotoxic; mainly stimulates "virally induced," transplanted, or metastatic neoplasms	Azathioprine, antilymphocytic serum
7. Cocarcinogen	Not genotoxic or carcinogenic, but enhances effect of type 1 or type 2 agent when given at the same time. May modify conversion of type 2 to type 1	Phorbol esters, pyrene, catechol, ethanol, *n*-dodecane, SO_2
8. Promoter	Not genotoxic or carcinogenic, but enhances effect of type 1 or type 2 agent when given subsequently	Phorbol esters, phenol, anthralin, bile acids, tryptophan metabolites, saccharin

port for this classification is growing, and similar concepts have been adopted by national policy groups (*17*). The recognition of multiple modes of action for carcinogens has major implications for the design of test procedures and for the interpretation of results.

In this article we describe a decision point approach to delineating possible human carcinogenic and mutagenic risks. This approach utilizes the major advances in knowledge of the mechanisms of carcinogenesis to provide reliable and economic methods for the testing of carcinogens.

The Decision Point Approach

An essential feature of the decision point approach to carcinogen testing is that the sequence of tests is such that the results can be evaluated at certain key points in the test series and decisions made regarding the potential carcinogenicity of a given chemical (Table 2).

The concept of diverse mechanisms of action is addressed in the decision point approach in two ways: (i) by using a battery of short-term tests to detect agents operating through genotoxic mechanisms and, in some instances, by epigenetic mechanisms; and (ii) by using a systematic approach that provides a guide to minimal testing but takes into account the possibility that testing for periods other than long-term may not detect chemicals that induce tumors in animals only under specific conditions after prolonged administration.

The use of a carefully chosen battery of short-term tests may either eliminate the need for further testing of the chemical or enable the verification of carcinogenic potential in one of five limited bioassays in vivo. This test battery can also add essential data for risk evaluation when an already completed series of long-term tests has yielded ambiguous results.

The decision point approach, therefore, provides a framework in which to minimize and optimize the necessary testing and at the same time develop an understanding of the mechanism of action of a test chemical (Table 2). At the end of each phase, the significance of the data in relation to the testing objective is critically evaluated and assessed. A decision is made as to whether the data available are sufficient to reach a definitive conclusion or whether a higher level of tests is required. Attention is paid to qualitative—yes or no—answers, and to semiquantitative—high, medium, or low—effects. Since the value and implications of each test have been described (*16*), we discuss here only the essential details of the sequence.

Stage A. Structure of the chemical. The evaluation starts with a consideration of the structure of a given chemical, with particular regard to its potential for activity as an electrophilic reactant either in its present form or after metabolism. For chemicals with structures related to known carcinogens that form electrophiles, structure-activity correlations can be estimated with fair success within several structural classes (*8, 18*).

Stage B. Short-term tests in vitro. This stage of testing is aimed primarily at detecting genotoxins and thus utilizes a battery of short-term tests in vitro, most of which identify genetic effects. Additional tests sensitive to epigenetic carcinogens will have to be developed (*19*).

Multiple tests in vitro are necessary (*20*) because no single test has detected all the known genotoxic carcinogens. The critical issue in structuring such a battery is to define the criteria for selection of appropriate tests. Moreover, since testing is becoming more complex and expensive, it is important to reduce the number of tests to an essential core.

Criteria for a Battery of Short-Term Tests

Data from several key tests are needed before a decision can be made on the potential hazard of a chemical. A battery corresponds to the initial "detection" phase used in most tier approaches to testing. However, the main difference between a battery and the tier approach is that a battery combines "detection" and the next step of a tier, "confirmation," in one stage. Inherent in this approach is the recognition that current short-term tests may yield false positive or false negative results. Thus, parallel simultaneous results are essential for judicious interpretation. The battery approach requires that no conclusion should be drawn or decision made without the data from the entire battery being considered.

Test batteries for carcinogenicity can be validated against data in vivo. The EPA Gene-Tox program, which is currently evaluating short-term tests with reference to carcinogenicity data, should provide important information on this subject. Thus far 23 systems have been evaluated and the assessments of seven are to be published in *Mutation Research* [see (*20*)]. A similar effort is being made by the International Commission for Protection Against Environmental

Mutagens and Carcinogens (ICPEMC) (*20*).

Because oncogenic mechanisms of a nongenetic nature are clearly not detectable in tests measuring a genetic end point, it is important that chemicals operating by indirect epigenetic mechanisms should not be expected to be positive in short-term tests; neither should the results for these chemicals in short-term tests be considered "false negatives." Rather, the short-term tests provide useful information on the mechanisms of action of the chemical, which must be taken into account in risk evaluation.

Some results observed, such as malignant transformation and sister chromatid exchange (SCE), may be caused by events other than a direct attack on DNA; such reactions may be indicative of non-DNA–damaging carcinogens. Efforts are under way to develop tests in vitro for tumor promoters (*19*), but the data available are not sufficient to justify routine inclusion of such tests in a battery. Therefore, in using batteries for the detection of carcinogens it must be recognized that a whole category of chemicals that operate by nongenetic mechanisms, such as saccharin, hormones, bile acids, certain organochlorine compounds and pesticides, and miscellaneous pharmaceuticals, will not be detected.

More than 100 short-term tests are available, but most tiers or batteries center around seven systems: bacterial mutagenesis, eukaryote mutagenesis, *Drosophila* mutagenesis, mammalian cell mutagenesis, DNA damage, chromosome damage, and malignant transformation. The design of a battery should consider certain key principles. First, the end points of the tests should be reliable and have clear biologic significance; that is, they should actually determine what they are supposed to measure, and should have conceptual relevance to mutagenicity or carcinogenicity. Second, a battery should optimize the metabolic reactions underlying all tests. Thus, tests with intact cells would extend the metabolic capacity of the commonly used enzyme preparations, since the latter often cause an artifactual enhancement of activation over detoxification reactions (*12*). Several national and multinational testing programs, particularly a program in Japan (*21*), are now making effective use of test batteries in vitro.

Essential components of a test battery are the microbial mutagenesis tests, developed mainly by Malling, deSerres, Ames, Rosenkranz, Matsushima, and Sugimura, because these are the most sensitive, effective, and readily performed screening tests available thus far (*11, 13*). In deciding what other tests should be included, it is essential to consider metabolic capability, reliability, and biologic significance of the end point.

Table 2. Decision point approach to carcinogen testing. Modified from data in (*16*).

Stage A. Structure of chemical
Stage B. Short-term tests in vitro
1. Bacterial mutagenesis
2. Mammalian mutagenesis
3. DNA repair
4. Chromosome tests
5. Cell transformation
Decision point 1: Evaluation of all tests conducted in stages A and B
Stage C. Limited bioassays in vivo
1. Skin tumor induction in mice
2. Pulmonary tumor induction in mice
3. Breast cancer induction in female Sprague-Dawley rats
4. Altered foci induction in rodent liver
5. Assays for promoters
Decision point 2: Evaluation of results from stage A through all the appropriate tests in stage C
Stage D. Long-term bioassay
Decision Point 3: Final evaluation of all the results. This evaluation must include data from stages A and B to provide basis for mechanistic considerations

Tests for mutagenesis systems in mammalian cells were developed primarily by the groups of Szybalski, Chu, and DeMars [see (*22*)]. Such tests are required in a battery because they provide definitive end points similar to those provided by tests for bacterial mutagenesis but involve the more highly organized eukaryotic genome (*22*).

Damaged DNA or altered chromosomes provide evidence that a chemical can change genetic material. Indicators for DNA damage that have been proposed include DNA binding, DNA fragmentation, inhibition of DNA synthesis, and DNA repair (*23*). Of these, DNA repair is a specific response to DNA damage which is simple to measure and, unlike DNA fragmentation and inhibition of DNA synthesis, cannot be attributed to toxicity. Thus, a DNA repair test provides an end point of high specificity and biologic significance.

A chromosomal test is included to detect effects at the highest level of genetic organization. Such tests, however, may respond to nongenotoxic agents through effects on DNA replication or chromosome separation, for example. Sister chromatid exchange can be readily monitored and is therefore recommended as a chromosome test (*24*). Use of this test will extend the data base and provide a further basis for judging the value and limitations of this test.

A test for cell transformation [see authors cited in (*25*)] is considered for inclusion in the battery because such transformation may be directly relevant to carcinogenesis. The first reliable system for detecting chemical transformation of cultured mammalian cells was introduced by Sachs and associates. Their system utilizing hamster fibroblasts was subsequently developed into a colony assay for quantitative studies by DiPaolo and has been adapted as a screening test by Pienta. In addition, a quantitative focus assay for transformation in mouse cells has been devised in the laboratory of Heidelberger, and a quantitative assay for growth of BHK cells in soft agar has been developed by Styles. The correlation between transformation and malignancy appears to be good in these systems, but the high frequency of transformation is of concern. Moreover, transformation assays are difficult, less widely available than other systems described, and have given positive results with chemicals not likely to have genotoxic properties. Therefore, at present we recommend performance of the first four tests and use of a transformation assay only if the results of this battery require amplification.

Short-Term Tests Selected

Bacterial mutagenesis. Because of the extensive data base and good correlation with carcinogenicity, the Ames test (*11*) in its recent versions, including liquid-phase incubations, is recommended as the first choice for a bacterial mutagenesis test (*13*).

Genotoxic metabolites may be excreted in urine or stool which can be examined in the Ames test as an indication of such products formed in vivo.

Mammalian mutagenesis. The best characterized mutational system in mammalian cells is mutation at the hypoxanthine-guanine phosphoribosyl transferase locus which can readily be measured by conversion of cells to resistance to toxic purine analogs. The target indicator cells used in purine analog resistance assays have almost all been fibroblast-like, such as the V79 and CHO lines that possess little ability to activate carcinogens. This deficiency is met by either cocultivated cells or enzyme preparations. The CHO system has been extensively validated by Hsie and co-workers (*22*). Mutations can be induced in liver epithelial cultures by activation-dependent carcinogens; hepatocyte-me-

diated mutagenesis of several cell types including human cells has been described (*26*). These systems, therefore, may provide useful approaches to monitor the generation of mutagens through intact cell metabolism.

DNA repair. Of the systems available, the use of hepatocyte primary cultures for the DNA repair test developed by Williams [see (*27*)] has proved sensitive and reliable with activation-dependent procarcinogens, including some not readily detected in other systems. This test is considered an essential component of the battery, particularly since cells with intact metabolism are used.

Chromosome tests. As with the mammalian mutagenesis tests, SCE assays generally involve cell types that require addition of an exogenous metabolizing system for biotransformation. The best validated system at present is that in which CHO cells are used, but the recent development of liver cell systems with intrinsic metabolic capability promises to provide an important adjunct (*24*).

Cell transformation. Most transformation assays involve fibroblasts and measure a morphological alteration in the cells. Assays for changes in growth properties related to neoplasia, such as growth in soft agar as used by Styles [see (*25*)], and incorporation of more relevant cell types such as epithelial cells are desirable. The systems of Pienta and of Heidelberger appear to be sufficiently widely used to be considered as potential supplements to the other four tests if deemed necessary (*25*).

Decision Point 1

The six steps (stage A plus steps 1 to 5 in stage B) are the basis for preliminary decision-making (see Table 2).

If definite evidence of genotoxicity in more than one test has been obtained, a chemical is highly suspect. In particular, because of their complementary nature, positive results in the test systems of Ames and of Williams provide strong and possibly certain evidence of carcinogenicity. Since there is some redundancy between bacterial and mammalian mutagenicity, these two systems support rather than extend the significance of positive results. An agent that is mutagenic, DNA damaging, and clastogenic is certain to be carcinogenic and represents an unequivocal toxic hazard.

In contrast, genotoxicity in only one test requires interpretation with caution. For example, several types of chemicals such as intercalating agents are mutagenic to bacteria but not reliably carcinogenic. Positive results have also been obtained in bacteria with synthetic phenolic compounds or natural products with phenolic structures such as flavones. In vivo, such compounds are conjugated and excreted readily. Their carcinogenicity in vivo thus depends on the conjugate being split, which is more likely to occur in coprophagic laboratory rodents than in humans, because of the sizable microflora in the upper gastrointestinal tract of rodents. Therefore, positive evidence of bacterial mutagenesis must be evaluated with regard to chemical structure and metabolism. Similarly, positive results only for mammalian mutagenesis or SCE must be interpreted with caution. However, evidence of DNA damage in the hepatocyte repair test strongly indicates covalent binding to DNA, an established property of carcinogens and mutagens.

A wide variety of organic chemical structures capable of forming a reactive electrophile have been carcinogenic in limited bioassays in vivo (*16*). Other substances, such as solid-state materials, possibly some metal ions, hormones, and promoters, which are negative in tests for genotoxicity, operate by complex and poorly understood mechanisms. Rapid bioassay tests for metal ions could be based on the concept proposed by Loeb and co-workers [see (*28*)] that such ions interfere with the fidelity of enzymes performing DNA synthesis. Chemicals with hormone-like properties, in addition to the natural androgens and estrogens, are potential cancer risks mainly because they affect normal physiological endocrine balances (*16*), but there are no rapid tests for such promoting properties and either specific promotional assays (see Table 2, stage C) or the standard long-term bioassay (stage D) are necessary. Potential promoters could be detected through systems in vitro (*19*) or in vivo by treating animals with a limited amount of a genotoxic carcinogen for a specific target organ (stage C). Most promoters affect one tissue in particular and thus require specialized procedures.

Any positive results of the test battery in vitro can be extended through limited bioassays in vivo (stage C) without the need to conduct a full-scale, costly, and time-consuming long-term bioassay. If all the preceding test systems yield no indication of genotoxicity, however, the priority for further testing depends on two criteria: (i) the structure and known physiological properties (for example, hormone) of the material and (ii) the potential for human exposure to the compound. If substantial human exposure is likely, careful consideration should be given to the necessity for additional testing. The chemical structure and the properties of the material provide obvious guidance on proper course of action.

Stage C. Limited bioassays in vivo. This stage of test development is designed to yield further evidence of the potential carcinogenicity of genotoxic chemicals without the necessity for undertaking a long-term bioassay. The tests recommended are those that will provide definitive evidence of carcinogenicity, including cocarcinogenicity and promotion, in a relatively short period (40 weeks or less). Unlike the tests in vitro, these are not applied as a battery but rather used selectively according to the information available on the specific properties of the chemical. These tests have been discussed in detail (*16*) and are summarized here.

Bioassays Selected

Skin tumor induction in mice. The carcinogenicity of a limited number of chemicals and crude products can be revealed readily upon continuous application to the skin of mice, in which they produce papillomas or carcinomas, or upon subcutaneous injection, when they may yield sarcomas. The activity of such compounds as initiating agents can be rapidly determined by the concurrent or sequential application of a promoter such as phorbol ester.

Pulmonary tumor induction in mice. Induction of lung tumors in specific, sensitive mouse strains was developed as a bioassay by Shimkin (*29*). Results are expressed as percentages of animals with tumors compared to controls, and the multiplicity of tumors is an additional indication of potency. Most chemicals active in this system are also carcinogenic in other longer-term animal tests. A negative result does not signify safety since not all classes of chemical carcinogens induce lung tumors.

Breast cancer induction in female Sprague-Dawley rats. Some chemicals rapidly induce cancer in the mammary gland of young female Sprague-Dawley rats (*30*). In this test also, a positive response has usually been confirmed in long-term tests, but a negative response does not prove lack of carcinogenicity.

Altered foci induction in rodent liver. Several distinct hepatocellular lesions regularly precede the development of hepatocellular carcinomas in rats. The earliest of these, the altered focus, can be visualized in routine histologic tissue

sections by sensitive histochemical techniques, including reactions for the enzymes γ-glutamyl transpeptidase, glucose-6-phospatase, and adenosinetriphosphatase; resistance to iron accumulation; and resistance to the cytotoxic effect of carcinogens (*31*). In mice, hepatomas can be induced rapidly but may result from an epigenetic effect.

Assays for promoters. In addition to providing further evidence of genotoxicity, limited bioassays in vivo can also be used to test for promoting substances. A genotoxic carcinogen that is active at a specific target organ, such as mouse skin, breast, colon, urinary bladder, or liver, is applied in small initiating doses, after which the test compound is administered. The liver of certain commonly used mouse strains reacts in this test as if it already has an abnormal genome, and thus responds positively to promoters for liver carcinogenesis.

Decision Point 2

The presence of positive results in two or more of the rapid tests in vitro together with a definite positive result in the limited bioassays in vivo would make a product highly suspect as a potential carcinogenic risk to humans. This is especially true if the results were obtained with moderate dosages. In addition, convincing evidence would be a finding of a good dose response, particularly with respect to the multiplicity of lung or mammary gland tumors, and positive results for mutagenicity and DNA damage.

The demonstration of promoting activity in any of the modified assays in the absence of genotoxicity indicates that the chemical deserves investigation as an epigenetic agent.

Stage D. Long-term bioassay. The long-term bioassay is used as a last resort for confirming questionable results in the more limited testing or evaluation of compounds that are inactive in the preceding stages, but where extensive human exposure is likely. Long-term bioassays would also develop data on possible carcinogenicity through epigenetic mechanisms. In the latter situation, multispecies and dose response data are most important if the data are to be applied to safety evaluation. The elimination of unnecessary long-term testing for all chemicals by the decision point approach makes more extensive testing of suspected epigenetic agents economically feasible. Methods for conducting long-term bioassays have been reviewed (*1, 16*), and we need only emphasize here that expert judgment is required for design of the test procedures as well as for reliable evaluation and interpretation of the results.

Decision Point 3

Long-term bioassays as an end point in the decision point approach should yield definitive data on carcinogenicity provided the bioassays are properly conducted. Nonetheless, the results of the short-term tests in vitro must be taken into account for an assessment of mechanisms of action and extrapolation of risk to humans. Thus, convincing positive results in the tests in vitro together with documented carcinogenicity in vivo permits classification of the chemical as a genotoxic carcinogen. Such a chemical would have properties typical of other genotoxic carcinogens, namely, the ability under some conditions to be effective as a single dose, cumulative effects, and potential additive effects or synergism with other genotoxic carcinogens. If there is no convincing evidence of genotoxicity, but nonetheless an indication of carcinogenicity in certain animal bioassays, the chemical may be an epigenetic carcinogen. The reliability of this conclusion depends on the relevance of the tests in vitro. For example, the fact that some stable organochlorine pesticides do not show genotoxic properties in liver culture systems which represent the target cell type in vivo is substantial evidence for an epigenetic mechanism of action. Epigenetic mechanisms are poorly understood and are probably distinct for different classes of carcinogens; for example, they may involve long-term tissue injury, immunosuppressive effects, hormonal imbalances, stimulation of cell proliferation, release of existing altered cells from growth control, or other processes not yet known. Most epigenetically acting agents are active only at high, sustained doses and, up to a certain point, the effects they induce are reversible. Thus, these types of agents may represent only quantitative hazards to humans, and it may be possible to formulate safe levels of exposure after appropriate toxicologic dose-response studies are conducted.

Conclusions

We have developed a decision point approach to the testing of potential carcinogens. This approach is based on the mechanistic classification of chemical carcinogens, whether they be synthetic industrial chemicals or naturally occurring products, into two broad classes—genotoxic and epigenetic—and depends on results obtained from a battery of tests conducted in a logical sequence. The sequence of tests is such that at a number of key points decisions can be made regarding the carcinogenic or genetic risk of a given material. It is sometimes possible to obtain definitive information early in the test series and to avoid the necessity for further time-consuming bioassays that can cost several hundred thousand dollars. This approach, which is based on contemporary concepts of the mechanisms of carcinogenesis and is thus buttressed by a strong collateral research base, is well suited for integration into a broader toxicological evaluation of chemicals (*32*). However, the demonstration of carcinogenicity would for most purposes obviate the need for other types of toxicity testing, because carcinogenicity can usually be shown with lower doses of a genotoxin than are required for the demonstration of other toxic effects.

Because the decision point approach is based on a mechanistic understanding of carcinogenesis, and does not depend on the mere performance of routine bioassays that have changed little in the past 50 years, the results obtained are of greater value in expanding our knowledge of carcinogenic processes. A further advantage of the decision point approach is that nongenotoxic chemicals that are selected for bioassay because of concern for human exposure can be tested over a more extensive dose range to delineate dose-response characteristics and possibly identify thresholds. While the methods basically yield qualitative answers in detecting and classifying carcinogens and mutagens, application to health risk analysis necessarily requires consideration of relative potencies and other quantitative aspects (*16, 33*).

An essential adjunct to the adoption of the proposed new approach to carcinogen testing is a more informed process of data analysis. The best effort in data analysis is now provided by the International Agency for Research on Cancer (IARC) through its monograph series *Evaluation of the Carcinogenic Risk of Chemicals to Humans.* Such efforts should be expanded to incorporate all relevant data collected by individual national groups into the evaluation of chemical hazards and to make possible the adoption of uniform standards of safety worldwide. The International

Commission for Protection Against Environmental Mutagens and Carcinogens is currently working toward several of these goals.

Clearly, it is time to use fundamental knowledge in improving the technology and science of mutagen and carcinogen testing.

References and Notes

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2. These cost estimates, which are derived from our past and current involvement in conducting such bioassays, usually do not include hidden costs such as program management and consultants for design and data evaluation. M. Henry [*Ann. N.Y. Acad. Sci.* **329**, 131 (1979)] noted a cost of $300,000 per compound.
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