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Bioassay of Carcinogens: In Vitro and In Vivo Tests

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CANCER IS A GENERIC TERM covering a variety of neoplastic diseases. Approximately 70–90% of all cancers in humans have been attributed to environmental causes (*1–4*). If the agents in the environment that cause such cancers were identified, measures could be designed either to minimize or eliminate their impact. Some success in such efforts has been achieved by controlling the use of chemicals known to induce cancer in occupational settings. However, the pattern of occurrence of the major types of cancer suggests that cancers resulting from occupational exposures represent only a small portion of the total cancer incidence in the world (*5–7*). Therefore, to definitively solve the cancer problem, the etiology of the various key types of cancer must be addressed. Examples of these types of cancer are lung cancer in many areas of the world; gastric cancer in Japan and certain parts of Europe; liver cancer in Africa; and colorectal, breast, and prostate cancer in the western world, particularly the Anglo–Saxon countries (*8, 9*).

The best way of eliminating each type of cancer is to define precisely the nature of the carcinogenic chemical, mixture of chemicals, or enhancing factors that may be operating in the environment to produce the cancer. Clues to the nature of possible causative agents responsible for human cancer are provided by epidemiologic studies. To confirm the carcinogenic potential of suspect agents, studies must show that the chemical can indeed cause, or at least induce or enhance, cancer development in an animal model even though the site affected may not be the same as in humans.

Bioassay systems specifically designed to detect carcinogenic chemicals have a great predictive capability. New synthetic chemicals can be tested for carcinogenic potential prior to extensive human exposure and unnecessary risk. For example, in 1938 before DDT and other pesticides were known, 2-fluorenamine was being considered as a potent and promising insecticide. However, one of its derivatives, N-2-fluorenylacetamide, was discovered to be a powerful carcinogen (*10*), and all plans to use the parent chemical were dropped. If 2-fluorenamine had been used widely as a pesticide, with consequent contamination at the sites of production and

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use, a higher cancer risk in exposed populations might have resulted. Unfortunately, in a number of other instances where chemicals were found to cause cancer in animals, the warning was not applied promptly and avoidable tragedies resulted (11–18).

A key aim of carcinogen bioassay is to assess the carcinogenic risk of chemicals early in the stage of technical development. It is wasteful and, indeed, imprudent to conduct carcinogen bioassays on any chemical late in the development and production stages, when a finding of adverse effects would make futile all of the previous work leading to possible commercial use. Evaluation of the carcinogenic potential of chemicals should include two types of agents: (1) synthetic chemicals that may enter or currently are in the human environment, and (2) naturally occurring chemicals that may be responsible for the diverse types of existing human cancer.

Bioassay systems should be sensitive, reliable, specific tools for the detection of possible carcinogenicity of chemicals. The systems should also be economical and as rapid as possible for agents that are already in the environment. They should, if possible, mimic the human conditions of exposure. Aside from a few recent refinements, the design and conduct of chronic bioassays in laboratory rodents are not much different from the pioneering procedures of decades ago. Often they are slow, expensive, and sometimes even misleading when they are not coupled with a full evaluation of the biological characteristics of the test chemical (19–22). Fortunately, much progress has been made in the last few years in understanding the complex, step-wise mechanisms of carcinogenesis, the structure–activity relationships among chemicals capable of causing or enhancing cancer, the molecular events associated with each part of the carcinogenic process, and the relationship between carcinogenesis and mutagenesis (23–31). In turn, this clarification has given rise to rationally designed rapid bioassay tests that can, together with animal bioassays, yield a reasonably sound approach to health risk estimates—a primary goal of carcinogen bioassays.

Knowledge of the possible carcinogenic properties as revealed by a carefully selected battery of in vitro and in vivo tests is an essential component of the data base for proper health risk assessment of a given product whether naturally occurring or synthetic. The evaluation has two steps: The first step is qualitative yes or no answers, and if the first step is affirmative, then quantitative analysis is conducted. This chapter will provide the scientific and mechanistic background and describe the test systems appropriate for systematic approaches to health risk analysis through in vitro and in vivo bioassays.

History of Carcinogen Bioassay

The appearance of neoplastic diseases in humans has been known from antiquity and probably occurred even earlier in the evolution of the species. An 18th century British surgeon, Percivall Pott, was the first to note that cancer might relate to specific causes. He diagnosed a number of scrotal cancers in relatively young men and associated these occurrences with their occupation as chimney sweeps. Subsequently, other case reports documented an association of specific cancers with specific populations, mostly occupational (32).

The first experimental production of cancer was achieved by Yamagiwa and his assistant. They attempted to reproduce occupational exposure conditions in an animal model by applying coal tar to the ears of rabbits; they thereby demonstrated that coal tar contained carcinogens (*33*). The rabbit ear was also used by Shope when he induced neoplastic disease with an extract that contained a virus; Rous later showed that injury to the treated area enhanced the process (*see* reviews, *34* and *35*).

Subsequently, other researchers successfully used mouse skin as an assay system to demonstrate the carcinogenicity of coal tar and to aid in the isolation of some of the pure components, namely, polycyclic aromatic hydrocarbons (*36, 37*, reviews, *38, 39*). These findings, in turn, led to numerous investigations that used either mouse skin painting (carcinoma induction) or subcutaneous injection (sarcoma induction) to determine structure–activity relationships of many diverse polycyclic aromatic hydrocarbons and heterocyclic hydrocarbons (*40*).

Japanese investigators found azo dyes carcinogenic to rodent liver when administered in the diet or subcutaneously (*see* review *41*). Following the observation that certain aniline dye stuff intermediates could cause urinary bladder cancer in humans, Hueper successfully induced the disease in dogs for the first time in 1937 by the administration of large doses of 2-naphthylamine (*32, 42*). Other investigators adopted the approach using dog bladders and reproduced Huepers' results (*38, 43–46*). Since then, the U.S. Food and Drug Administration (FDA) has required toxicity studies in dogs as part of safety evaluations (*47*).

In the late 1940s, many organic chemists were involved in the synthesis of chemicals related to the known carcinogens, especially polycyclic aromatic amines, azo dyes, and polycyclic aromatic hydrocarbons. This activity resulted in many structure–activity studies, but rather arbitrary dosages and periods of administration and observation were used (*48*).

An intensive effort at quantitative analysis of carcinogenesis was performed by Bryan and Shimkin (*49*). They injected subcutaneously single doses of three hydrocarbons (methylcholanthrene, dibenzanthracene, and benzopyrene) into mice at levels ranging from 0.00024 to 8.0 mg and evaluated the development of sarcoma. After these carcinogenic hydrocarbons had been isolated from tobacco smoke, Wynder et al. (*50*) performed a dose–response study on them. He applied them cutaneously and measured the development of carcinoma quantitatively. About the same time, Druckrey (*51*) published a number of elegant dose–response studies with certain carcinogenic azo dyes and aromatic amines and later with nitrosamines. The results of these studies provided the basis for the concept that the final yield of neoplasms was related to the total dosage of administered carcinogen and that the latent period, which was recognized as an important parameter of carcinogenic potency, was inversely associated with the daily dose rate.

Scientists at the FDA, who were concerned with safety assessment of food- and drug-related chemicals and pesticides, were the first to standardize procedures for bioassay in animal models, mainly rats, dogs, and occasionally mice. Although some of the results were published in the appropriate pharmacological or toxicological literature, many of the specific procedures, as well as their defense and documentation, were published in small specialized monographs of limited

circulation (*47a*). In 1982, the FDA released for comment a draft of a manual embodying current procedures for safety assessment (*47b*). A number of workshops on carcinogen testing were organized by the Union Internationale Contre le Cancer (UICC) and resulted in the publication of pamphlets on the subject (*52, 53*).

In 1961, the National Cancer Institute created a special group, the Carcinogen Screening Section in the Division of Field Studies, to develop procedures for the detection and quantification of carcinogenic risks of various chemicals. This activity led to an evaluation of optimal chronic bioassay methods and the application to the screening of many chemicals (*54–56*). Recommendations for further refinement and standardization were given.

The findings that some carcinogenic nitrosamines were mutagenic in bacteria and yeast (*57, 58*) and that the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was a powerful carcinogen (*59*) prompted a new approach to carcinogenicity screening: the use of prokaryotic organisms to detect mutagens and, by extrapolation, carcinogens in a rapid and efficient manner (*see* review, *60*). In the meantime, numerous mechanistic studies led to the unified concept of Miller and Miller (*29*). They proposed that carcinogens usually are reactive intermediates, as electrophilic reactants or radical cations, that can interact with cellular macromolecules. The convincing demonstration in the newly available systems that many carcinogens were mutagens supported this concept. More specifically, for this type of carcinogen, reaction with DNA was the crucial event in the initiation of the carcinogenic process. However, many agents, such as plastics, asbestos, and hormones, have now been identified as carcinogenic but not mutagenic, and neither their structures, nor the structures of their metabolites, suggests a likely electrophilic character. Alternative mechanisms of carcinogenesis must exist (*61, 62, 82*), especially in regard to the developmental stages of the process. Indirect processes of activation, such as production of active oxygen species (*83–86*), aberrant methylation, (*87, 88*), or chromosomal alterations (*89*) have been described.

Definition of Chemical Carcinogens

Chemical carcinogens can be defined operationally by their ability to increase the occurrence of neoplasms (*62*). Four types of increased response are generally accepted as evidence: (1) the development of types of neoplasms not seen in controls; (2) an increased incidence of the types of neoplasms seen in controls; (3) the occurrence of neoplasms earlier than in controls; and (4) an increased multiplicity of neoplasms in individual animals. Although the term "carcinogen" literally means giving rise to carcinomas, which are epithelial malignancies, in general usage it also includes agents producing sarcomas of mesenchymal origin. Furthermore, under certain conditions, the production of benign neoplasms can be accepted as evidence of carcinogenicity. This practice is justified by two considerations. First, the distinction between benign and malignant neoplasms is often made entirely on the microscopic appearance of the neoplasms, and the interpretation varies with the expertise and biases of the pathologist. Second, agents that induce benign neoplasms usually yield malignancies under severe protocols.

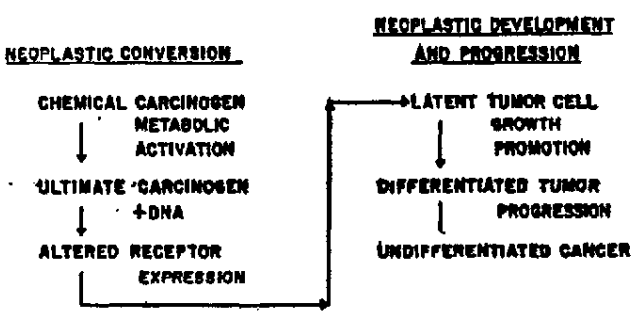
Substances capable of producing an increase in neoplasms, and thereby classified as carcinogens, are a highly diverse collection of chemical substances, including organic and inorganic chemicals, solid-state materials, hormones, and immunosuppressants (*62*). For the chemicals that appear to operate as neoplasm enhancers or promoters, the designation "carcinogen" is unfortunate, because their agents probably cannot initiate the process leading to cancer. Nevertheless, this classification is currently unavoidable because in specific situations these chemicals do increase the yield of cancers, although usually the neoplasms are ones that have a significant spontaneous occurrence, such as mouse liver neoplasms. Knowledge of the specific mechanisms of carcinogenesis should be considered in the testing and evaluation of each potential carcinogen for a proper health risk analysis (*62–66*).

The Carcinogenic Process

The production of cancer in humans and animals by chemicals is thought to be the end result of a complex series of individual reactions that are subject to and controlled by a number of modifying factors. These reactions can be grouped into the two sequences shown in Scheme I. In the first sequence, the normal cell is converted to a neoplastic cell. In the second sequence, the neoplastic cell develops into an overt neoplasm. Chemicals are involved in diverse ways in both sequences.

Neoplastic Conversion. BIOTRANSFORMATION BY HOST ENZYME SYSTEMS. Different enzyme systems can function in the detoxification and elimination of xenobiotics (*19, 67–69*). Many carcinogens, however, undergo enzymatic activation to a reactive ultimate carcinogen. A small number of carcinogens, mostly industrial intermediates and chemotherapeutic drugs, are reactive in their parent form and therefore do not require activation.

INTERACTION OF THE ULTIMATE CARCINOGEN WITH SPECIFIC CELLULAR AND MOLECULAR RECEPTORS. Carcinogens that form reactive species undergo covalent reactions with a variety of cellular macromolecules, including DNA (*70*). Abundant evidence now supports the concept that reaction with DNA is a critical event (*29, 68, 70*),



Scheme 1. Sequence of complex events during chemical carcinogenesis

although other interactions, such as with the mitotic apparatus, may also be important (24). The damaged DNA is subject to removal and restoration by repair enzyme systems (71–73).

FIXATION OF CARCINOGEN DAMAGE. If the cell replicates while DNA damage is persistent, permanent alterations in the genome can be produced in several possible ways: the mispairing of bases leading to point mutations; errors in replication yielding frame-shift mutations; transpositions resulting in codon rearrangement; and combinations of these alterations in sequential steps. Codon rearrangement may involve sequences known as oncogenes, which are emerging as critical gene sequences for transformation (74, 75). In the case of interactions with the mitotic apparatus, chromosomal mutations and aneuploidy could result. All these alterations generate a permanently abnormal cell with an altered genotype and distinct phenotypes.

Neoplastic Development and Progression. MULTIPLICATION OF THE ALTERED CELLS. Abnormal cells may be held in check by tissue homeostatic factors or, if the conditions of exposure or abnormalities generated in the cells permit, they may undergo limited proliferation to form "preneoplastic" lesions. During these processes, further alterations of DNA as a result of transpositions and other error-prone processes are possible.

PROGRESSIVE GROWTH TO NEOPLASM FORMATION. Cells with the requisite abnormalities may have the capacity to proliferate beyond tissue constraints to form neoplasms. This step is facilitated by promoters.

PROGRESSION. Neoplasms can undergo qualitative changes in their phenotypic properties, possibly including transition from benign to malignant behavior (76). This change probably reflects the selection during growth of a population with a genotype coding that produces advantageous phenotypic properties. New genotypes could arise in neoplasms through errors in DNA replication as described by Loeb (77) or alterations in chromosome constitution.

Modifying Factors

Many of these specific steps are controlled and modified by numerous endogenous and exogenous factors. Intrinsic factors, such as species, strain, sex, and age, affect the processes in certain steps, particularly biotransformation and DNA repair. In addition, hormonal, immunologic, and other endogenous factors may enhance or diminish the extent and rate of the carcinogenic process. Among the exogenous elements, nutritional factors are heavily involved. Furthermore, the mode, dose, and frequency of exposure to a carcinogen; the physical environment; and the presence of other agents, both synthetic and naturally occurring chemicals, are all extrinsic contributing factors.

Mechanisms of Carcinogenesis

The neoplastic state is generally considered to be heritable at the cellular level. In other words, the progeny of neoplastic cell division inherit the neoplastic potential. Thus, mechanistic theories of how chemicals convert normal cells to malignant

ones must ultimately explain how the effect becomes permanent. Historically, investigators concerned with the interactions of carcinogens with proteins and RNA have postulated that effects on these macromolecules can eventually be rendered permanent through epigenetic mechanisms on gene expression that create a new stable state of differentiation (*8, 78–80*). Others have proposed indirect mechanisms but these would seem to arise only in exceptional cases (*81*).

Classes of Chemical Carcinogens

Theories on the mechanisms of action of chemical carcinogens have been intended to provide a unifying account for the activity of all agents, but such generalizations do not describe the action of all chemical carcinogens. It would be truly remarkable if such a structurally diverse group of agents all acted on cells in the same manner. Most likely, more than one mode of action is involved in the overall carcinogenic effect by different chemicals. To facilitate consideration of this possibility, we proposed several years ago a mechanistic classification of the agents into two major categories: genotoxic and epigenetic (*62*).

Carcinogens that interact with and alter DNA are classified as genotoxic. This category contains the "classic" organic carcinogens that are electrophilic reactants either in their parent form or after metabolism. Carcinogens in this category can be identified by the biochemical demonstration of DNA damage or by the demonstration of genotoxic effects in short-term tests, as will be described. Most likely, DNA alteration is the key event in the initiation of carcinogenicity by these compounds. These agents operate primarily in the sequence of neoplastic conversion, but may also promote the carcinogenic process through reactive or other metabolites. Because some inorganic chemicals have displayed certain effects including the induction of DNA repair (*90*), they have been tentatively placed in this category. The effects of inorganic chemicals on the fidelity of DNA polymerases suggest that carcinogenic metals might yield abnormal DNA by a distinct mechanism involving alteration of the fidelity of DNA polymerases (*77*). In any event, such inorganic carcinogenic chemicals can be construed to yield cells with altered DNA.

The second broad category, designated as epigenetic agents, comprises those chemicals for which no evidence exists of direct interaction with genetic material, but which produce another biological effect that could be the basis for their individual carcinogenicity. This category contains cytotoxic agents, solid-state carcinogens, hormones, immunosuppressants, and promoters.

Carcinogenic agents for which no evidence of direct genotoxicity has been found by appropriate, reliable studies and which produce another biologic effect that could be the basis for their involvement in the carcinogenic process are classified as epigenetic carcinogenic agents. Possible mechanisms for epigenetic effects include chronic tissue injury, hormonal imbalance, immunologic effects, or promotional activity on cells that are either genetically abnormal or have been independently altered by genotoxic carcinogens. Thus, epigenetic agents operate primarily in the sequence of neoplastic development.

Categorization of carcinogenic chemicals along these lines has been applied by other investigators (*63–65*). However, in some cases, epigenetic agents are

Table I

Classes of Chemical Involved in the Carcinogenic Process

Type	*Mode of Action*	*Example*
Genotoxic		
Direct-acting carcinogen	Organic electrophile that interacts with DNA	Ethyleneimine, ethylnitrosourea
Procarcinogen	Requires conversion through metabolic activation by host or in vitro to electrophile	Vinyl chloride, benzo[*a*]pyrene, 2-naphthylamine, dimethylnitrosamine
Inorganic carcinogen	Some may be electrophiles, but others appear to lead to changes in DNA by selective alteration in fidelity of DNA replication	Nickel, chromium
Epigenetic		
Cytotoxin	May involve chronic cell killing that leads to increased cell proliferation and neoplastic development	Nitrilotriacetic acid, polychlorinated hydrocarbons
Solid-state carcinogen	Physical form is vital; may involve cytotoxicity	Polymer or metal foils, asbestos
Promoter	Augments effect of type 1 or type 2 agent when given subsequently; also may enhance development of "spontaneously" transformed cells into neoplasms	Phorbol esters, phenol, anthralin, bile acids, tryptophan metabolites, saccharin
Hormone	Mainly alters endocrine system balance and differentiation; often acts as promoter	Estradiol, diethylstilbestrol
Immunosuppressor	Appears to stimulate "virally induced" transformed cells	Azathioprine, 6-mercaptopurine

designated simply as "nongenotoxic." This term describes only one aspect of their nature and does not characterize the property that seemingly underlies their role in the carcinogenic process. Nevertheless, until these proposed concepts are fully substantiated, flexibility in terminology is appropriate.

Moreover, the categorization may eventually have to be expanded to include other types of agents. As one possibility, types of chemicals that are not genotoxic per se, but may indirectly produce DNA damage, if these effects can be reasonably established to be mechanisms of carcinogenesis, might be classified as indirect genotoxins. This classification would recognize the fact that a preceding biological effect (i.e., toxicity) must be produced for the genotoxicity to occur. Also, if the production of chromosomal effects by non-DNA damaging agents can be shown to occur in vivo and to be related to carcinogenesis, agents producing this effect could be categorized as karyotoxic to distinguish them from genotoxins.

Genotoxic carcinogens, probably because of their effects on DNA, are occasionally effective after a single exposure and frequently carcinogenic at subtoxic doses. They act in a cumulative manner and act together with other DNA-reactive carcinogens having the same organotropism. They usually produce neoplasms in more than one target organ and have a short latent period.

In contrast, epigenetic agents are a highly diverse group; generalizations are difficult. Nevertheless, epigenetic agents usually affect only select organ systems where they produce physiological perturbations that usually require a long latent period to show their effects. In some classes, such as promoters, carcinogenic effects occur mainly with high and sustained levels of exposure that lead to prolonged physiological abnormalities, hormonal imbalances, or tissue injury.

In the carcinogenesis literature, the recurring statement is found that promoters, especially strong ones, also possess weak intrinsic carcinogenicity. Usually the experimental evidence is derived from one of two test systems: (1) continued high-level administration of promoters such as croton oil to mouse skin or (2) oral administration of DDT or phenobarbital to rats. Both systems have yielded a small but definite yield of benign and malignant neoplasms without any obvious initiation by a genotoxic carcinogen. An explanation or reclassification is needed because promoters by definition do not have intrinsic properties of altering the genetic apparatus. One classic case has supplied an explanation that is possibly applicable to the others. When croton oil was applied to the skin of mice, it appeared to induce by itself a high incidence of papillomas and carcinomas (91). Careful analysis revealed that the mice used were purchased from a supplier who housed the mice in creosoted cages. Thus, the mice had been exposed to genotoxic carcinogens in the creosote prior to the application of croton oil. Similarly, the said weak carcinogenicity of DDT or phenobarbital may stem from prior exposure of the animals to small amounts of carcinogens, possibly mycotoxins or certain nitrosamines in the diet (92–94).

In conclusion, the mechanisms relevant to each test system or human setting in which neoplasia is observed must be rigorously analyzed. For an informative mechanistic analysis, the nature, type, amount, and duration of exposure must be known for any genotoxic carcinogen; any nongenotoxic, epigenetic agent; and any modifying element (promoter or inhibitor).

Carcinogen Testing

The standard bioassays for the detection of chemical carcinogens use male and female rats, mice, and occasionally hamsters. The strains are selected for their sensitivity to carcinogens (*54–56*). The typical test begins with the careful determination of the maximal tolerated dose (MTD) of a product. Usually, two dose groups and one control group of 50 male and female animals are used. The MTD is given to one dose group and one-half or one-third of the MTD is given to the other dose group for at least 2 years. In some cases, exposure continues for the animal's lifetime. Thorough necropsy, microscopic pathology, and statistical evaluation of the incidence of neoplasms in the experimental groups is then compared with the control group. Such testing is still the definitive way to demonstrate whether a chemical is carcinogenic. However, chronic bioassay involves large resources of time and money and requires scarce specialty skills such as veterinary medicine and pathology for reliable execution.

Especially crucial is the escalation in the cost and magnitude of the tests since they were first developed. When the NCI began carcinogen screening programs in 1961, a test of a given chemical performed in one species took as little as 8 months and cost about $10,000–$15,000. A more extensive test given 10 years later in two species with larger numbers of animals required about 30 months and $75,000. Another 10 years later, tests of a chemical for multiple observational endpoints require even larger resources, more time (up to 64 months), and more money (as much as $400,000–$600,000). Even so, for approximately 250 tests using standardized protocols a fair number had results that were judged inconclusive or equivocal (*95–99*). Several tests gave borderline results that presented statistical difficulties and controversial interpretations. In addition, chronic bioassay alone does not provide the mechanistic information needed for proper hazard assessment.

Another important consideration is the ethics of the routine use of large numbers of animals in testing programs. The toxicologist has a clear responsibility to seek alternative approaches that provide equally valid data. As will be described in this chapter, systems are now available that provide superior data for risk evaluation with reduced animal usage (*100*), as well as mechanistic information.

The Decision Point Approach

As a model for a systematic approach to evaluate carcinogenicity, we have developed the decision point approach that involves a sequence of steps of increasing scope (*101, 102*). This approach was formulated to incorporate into carcinogen evaluation the concept that chemicals could lead to an increase in the incidence of neoplasms in exposed animals by several distinct mechanisms, each having different theoretical and practical implications.

The decision point approach takes the two main categories of genotoxic carcinogens and epigenetic agents into account in two ways: (1) the battery of short-term tests is constructed to identify genotoxic carcinogens at an early stage and includes systems that may respond to epigenetic agents; and (2) testing is structured with the recognition that all forms of subchronic testing may fail to

Stage A. Structure determination of chemical
Stage B. Short-term tests in vitro
- Mammalian cell DNA [illegible]
- Bacterial mutagenesis
- Mammalian mutagenesis
- Chromosome tests
- Cell transformation

DECISION POINT 1: Evaluation of all tests conducted in stages A and B

Stage C. Tests for promoters
- In vitro
- In vivo

DECISION POINT 2: Evaluation of results from stages A through C

Stage D. Limited in vivo bioassays
- Altered foci induction in rodent liver
- Skin neoplasm induction in mice
- Pulmonary neoplasm induction in mice
- Breast cancer induction in female Sprague–Dawley rats

DECISION POINT 3: Evaluation of results from stages A through C and the appropriate tests in stage D

Stage E. Long-term bioassay

DECISION POINT 4: Final evaluation of all the results and application to health risk analysis. This evaluation must include data from stages A through C to provide the basis for mechanistic considerations

detect chemicals that can produce neoplasms in animals under chronic administration.

The decision point approach (*see* box) consists of a series of sequential steps. A critical evaluation of the information obtained and its significance in relation to the testing objective is performed at the end of each phase. A decision is made as to whether the data generated are sufficient to reach a definitive conclusion or whether a higher level of testing is required. There are four such decision points. Attention is paid to qualitative (yes or no) answers and to quantitative (high, medium, or low) effects.

Stage A. Structure of Chemical. For a number of reasons, evaluation begins with a consideration of structure. Predictions as to whether a given chemical might be carcinogenic can be made with fair success within certain classes of chemicals (*24, 38, 48, 103–8*). For the series of polycyclic aromatic hydrocarbons, numerous structure–activity tests have demonstrated considerable ranges of activity as a function of structure (*48, 69, 108*). Identical developments hold for the nitrosamines (*104, 105*). Within the large series of azo dyes, many researchers have provided data on carcinogenicity versus structure (*41, 48, 109, 110*). Carcinogens

of this type are usually substituted with amino groups in a *para*-position of a benzene ring. Inclusion of relatively polar substitutents, such as sulfonic acid, abolishes carcinogenicity (*41, 111, 112*). On the other hand, more complex tetraazo dyes that include a potentially carcinogenic benzidine residue which could be released by biochemical reduction, are carcinogenic (*113, 114*). Polynuclear arylamines that are substituted at the *ortho*-position next to the vicinal ring, such as 1-naphthylamine or 1-fluorenylamine, are not carcinogenic, whereas the 2-isomers are powerfully active in rodents—2-naphthylamine particularly is active in humans. This difference in activity results because the 1-isomers are not activated to any significant extent by *N*-hydroxylation but are rapidly detoxified by ring hydroxylation (*38, 46, 67*).

Structure must always be considered in relationship to the metabolic parameters of the test species (*23, 67*). For example the guinea pig, in contrast to other rodents or humans, is endowed with large amounts of the necessary enzymes to carry out C-hydroxylation of the arylamines and conjugation of the resulting phenols. Hence, detoxified metabolites are produced almost exclusively and the proximate carcinogenic products, namely *N*-hydroxy derivatives, are sparingly produced. The capability of an S-9 fraction of guinea pig liver homogenate in converting arylamines to mutagenic *N*-hydroxy derivatives illustrates an important distinction between in vivo and in vitro metabolic capability (*115, 116*). In any case, the arylamines so far tested are not carcinogenic in this species. Other examples of species selectivity based on metabolic capability are well documented (*23, 24, 68, 117–22*).

Thus, information on structure and metabolism permit predictions of carcinogenicity and provide a guide to the selection of appropriate test systems at later stages.

Stage B. In Vitro Short-Term Tests. Among the variety of in vitro assays available (*123–26*), no single system has detected all carcinogens tested; therefore, multiple tests are essential.

The contribution of a test in terms of metabolic capability is critical. Most tests require a mammalian enzyme preparation to metabolize procarcinogens. This factor is often the key limiting part of a test series. A test that employs an enzyme preparation does not expand the scope of a battery in which other detection systems depend on similar subcellular preparations as an essential component.

The two critical elements of a test are the endpoint and the metabolic parameters. The endpoint should be reliable and of definite biological significance.

Most in vitro tests identify genetic effects, and thus, would detect only carcinogens of the type classified as genotoxic. If epigenetic agents are to be detected, additional tests will have to be developed (discussed in the section on assays for promoters).

The rationale for test selection has already been presented in detail (*102*). Briefly, the battery includes tests for the three principal genetic endpoints in mammalian cells—DNA damage, mutagenesis, and chromosome effects—in addition to bacterial mutagenesis. Transformation is included as an optional test to supplement equivocal data. For each of the primary tests, generally available systems have been selected. Cumulative data support the sensitivity and specificity

of this battery, but other equally useful tests, such as mutagenicity in fungi (*127, 128*) or *Drosophila melanogaster* (*129, 130*), could be substituted.

DNA DAMAGE. DNA damage can be measured in numerous ways. The three major types of DNA damage produced by chemicals—base damage, cross-linkage, and strand breakage—all elicit DNA repair synthesis (*71–73, 131*). Therefore, DNA repair has been recommended as a general test for DNA damage (*61, 132*). A variety of systems have been used for this purpose (*133*).

The hepatocyte primary culture/DNA repair assay of Williams (*134, 135*) uses freshly isolated, nondividing liver cells that can metabolize carcinogens and respond with DNA repair synthesis; the repair is measured autoradiographically. This assay has demonstrated substantial sensitivity and reliability with activation-dependent procarcinogens (*135–37*). Within the battery of tests, it offers the specific advantage of providing whole-cell metabolism. So far, evidence indicates that chemicals active both in this system and in bacterial mutagenesis tests are likely to be carcinogenic (*135, 137*).

New approaches under development include the detection of inducible repair responses to damage of bacterial or viral DNA by genotoxic agents (*138–40*). These tests still need validation by a systematic study of known carcinogens. Their advantage would be speed of evaluation.

BACTERIAL MUTAGENESIS. Valuable bacterial screening tests have been developed (*141–45*). The Ames test (*141–45*) measures back mutation to histidine independence of histidine-requiring mutants of *Salmonella typhyimurium*. It can be conducted with strains that are also repair-deficient, that possess abnormalities in the cell wall that make them permeable to carcinogens, or that carry an R factor that enhances mutagenesis. Hence, these organisms are highly susceptible to mutagenesis and make sensitive indicators.

The test developed by Rosenkranz and associates (*144, 145*) uses DNA repair-deficient *Escherichia coli* and measures their enhanced susceptibility to cell killing by carcinogens. In this system, a chemical that interacts with DNA is more toxic to the repair-deficient strain than to the wild-type *E. coli*, because the mutant strain cannot repair the damage. Thus, an indication of DNA interaction is obtained by measurement of relative toxicity. These tests depend on mammalian enzyme preparations for metabolism of carcinogens (*61, 146–49*).

The capability of the Ames test and similar tests that use a mutational event in the bacterial genome to detect carcinogens has been enhanced by two procedures: (1) preincubation of the compound and the biochemical activation system with the test organism (*150–53*) or (2) the fluctuation test (*154*), which is a sensitive variant involving turbidity measurement.

MAMMALIAN MUTAGENICITY TESTS. The three mutational assays in mammalian cells that have been most widely used for carcinogen screening entail mutagenesis at the hypoxanthine–guanine phosphotransferase (HGPRT) locus (*155–59*), the thymidine kinase locus (*160*), or the plasma membrane adenosine triphosphatase (ATPase) locus (*161, 162*). Of these, HGPRT mutagenesis is the most popular. In this assay, mutants lacking the purine salvage pathway enzyme are identified by their resistance to toxic purine analogs, such as 8-azaguanine or 6-thioguanine, that kill cells that can utilize the analogs. This assay has the advantage over ATPase

mutagenesis, which is measured as ouabain resistance, in that it involves a nonessential function; therefore, no lethal mutants result. Its advantage over the measurement of thymidine kinase-deficient mutants by resistance to thymidine analogs is that the gene for hypoxanthine–guanine phosphoribosyl transferase is on the X chromosome rather than a somatic chromosome. Hence, with only one functional copy present in each cell, mutations in wild-type cells can be determined. In constrast, a heterozygous mutant is required for measurable mutation to homozygous thymidine kinase deficiency. A potentially useful aspect of mutagenesis at the thymidine kinase locus is the possibility that both gene and chromosomal mutants can be identified (*163*); but the utility of this identification in carcinogen detection has not yet been established. Other markers to secure evidence for mutant events have been described (*60, 164–66*).

The target cells used in purine analog resistance assays have almost all been fibroblast-like, such as the V79 and CHO lines. Fibroblast lines have displayed little ability to activate carcinogens, other than polycyclic aromatic hydrocarbons. This deficiency has been overcome in several systems by providing exogenous metabolism mediated by either enzyme preparations (*156–58*) or cocultivated cells (*167, 168*). The former provides no extension in metabolic capability over that used for bacterial systems. However, the use of freshly isolated hepatocytes as a feeder system (*167, 169*) offers additional possibilities, because the metabolism of hepatocytes is different from that of liver enzyme preparations (*170–72*). Organ specificity can sometimes be reproduced in whole cell systems (*173*). HGPRT mutagenesis can also be measured in human cells that can be combined with hepatocytes for biotransformation (*174*). Such systems provide clear evidence of effects on the human genome, although thus far mutability of human cells has not differed qualitatively from that in other animal cells. Also, human cell systems have been used to study the metabolism of diverse carcinogens and to serve as a source of metabolic activation enzyme in cell culture mutagenesis (*175*).

CHROMOSOME TESTS. Chromosome tests are of conceptual importance because they reveal damage at a higher level of genetic organization than do mutagenesis assays. Two problems with chromosome tests are that recognition of many chromosomal alterations can be made only be an expert cytogeneticist and karyotype analysis can be very time-consuming. Measurement of sister chromatid exchanges (SCE) overcomes these problems and has shown sensitivity to carcinogens that are not readily detected in other in vitro assays (*176–78*). Moreover, a valuable correlation with potential human effects can be made, because SCEs can be monitored in peripheral lymphocytes of exposed populations.

The assessment of chemically induced SCEs is usually made in CHO (*176–80*) or V79 (*181, 182*) cells, because of the simplicity of their karyotype. These cells require supplementation with an exogenous metabolizing system for biotransformation of most carcinogens. Human liver preparations have been used for this purpose (*183*). Whole cell systems can also be used to mediate SCE induction in cocultured target cells, including human cells (*184, 185*). In addition, liver cell systems with intrinsic activation capability are available (*186–88*).

CELL TRANSFORMATION. The first reliable system for transformation of cultured mammalian cells was introduced by Sachs et al. (*189*). This system, which uses

hamster fibroblasts, was developed into a colony assay for quantitative studies (*190*) and has been adapted as a screening test (*191*). Other transformation assays were subsequently developed (*192, 193*), including the C3H/10T1/2 system of Heidelberger (*194*). The correlation between transformation and malignancy appears to be good in several systems. Transformation assays are not as reproducible or as widely available as other tests and, therefore, we recommend them only to supplement results from the preceding four systems. Nevertheless, transformation provides a useful indication of the potential carcinogenicity of chemicals either through genotoxic or epigenetic mechanisms. For example, certain hormones and sterols that are not likely to be genotoxic (*195*) have led to cell transformation (*196, 197*). Apparently cell transformation does not discriminate between the action of genotoxic and nongenotoxic agents, but in spite of this lack of specificity, it does indicate whether a given product is or can be involved in carcinogenesis. Transformation may involve more than a mutational event; therefore, further clarification is required (*198*). Another approach is the use of cell systems carrying oncogenic viruses as a more sensitive means of detecting transforming chemicals (*199–201*). Because human cancers usually involve epithelial tissues, transformation in epithelial systems is actively being pursued (*202–5*).

Decision Point 1. The five steps of phases A and B recommended thus far provide a basis on which decisions can be made. If clear-cut evidence of genotoxicity in three or more tests has been obtained, the chemical is virtually certain to be a carcinogen; regardless, it represents a clear genetic hazard. Positive results in two tests prove the agent to be highly suspect. In particular, positive results in the Ames test and in the hepatocyte primary culture/DNA repair assay are a strong indication of carcinogenicity.

Evidence of genotoxicity in only one test must be evaluated with caution. Several types of chemicals, such as intercalating agents, are mutagenic to bacteria but are not reliably carcinogenic (*206*). Positive results in bacterial tests have also been obtained with synthetic phenolic compounds or natural products with phenolic structures such as flavones (*25, 26, 207*). In vivo, such compounds are likely to be conjugated and readily excreted. Their carcinogenicity would thus depend on in vivo splitting of such conjugates, that may occur more readily in laboratory rodents than in humans. Therefore, positive evidence of bacterial mutagenesis must be evaluated further in the light of chemical structure and metabolism. For equivocal results, further testing in DNA repair tests, a transformation assay, and the limited in vivo bioassays are indicated.

If all the preceding test systems yield no indication of genotoxicity, the priority for further testing depends on two criteria: (1) the structure and known physiological properties (e.g., for hormones) of the material; and (2) the potential human exposure. 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 guidance on the appropriate course of action (*38, 48, 104–6*). Organic chemicals with structures that suggest possible sites of activation may reveal their carcinogenicity in limited in vivo bioassays. On the other hand, substances such as solid-state materials, hormones, possibly some

metal ions, and promoters that are negative in tests for genotoxicity operate by complex and as yet poorly understood mechanisms. The limited in vivo bioassays would probably not yield any results when these substances are tested alone. The next step recommended for such chemicals is either a specific mechanistic study or an assay for promoting activity; the recommendation depends on the structure.

For example, metal ions may be tested in a rapid bioassay that takes advantage of the concept proposed by Loeb (*77*) that such ions affect the fidelity of enzymes concerned with DNA synthesis. The nature of the metal ion should provide the necessary insight for choosing the type of assay most likely to reveal any adverse effects.

Compounds other than those of the strict androgen and estrogen type can nevertheless exert effects on endocrine glands. Such chemicals are potential cancer risks mainly because they alter normal hormone levels and balances (*62, 208*). For example, certain drugs lead to release of prolactin or other hormones from the pituitary gland. Chronic intake of drugs that cause permanently higher serum and tissue peptide hormone levels might, in turn, alter the relative ratio of other hormones. Any substance with such properties should undergo a chronic bioassay with carefully and appropriately selected doses to evaluate whether endocrine-sensitive tissues would be at higher risk. The interpretation of data must take into account the normal diurnal, monthly, and even seasonal cycles of the endocrine system and whether the balanced, rhythmic system has been altered by the test. More research on testing methods for such properties is required.

Several types of nongenotoxic agents have produced primarily or exclusively liver neoplasms in rodents (*209, 210*). The potential of chlorinated aliphatic hydrocarbons, chlorinated polycyclic hydrocarbons, and hormones to act as promoters of liver neoplasms has been described (*211–15*). As yet, the structural requirements for promoting activity are poorly understood outside the class of phorbol esters. Currently these agents can be identified only in initiation–promotion protocols in neoplasm promotion assays or in chronic bioassay. Thus, when possible promoting potential is suspected on the basis of several criteria—chemical relationship to known neoplasm promoters, differential effect on organ growth, and hormone-like properties—the chemical should be tested in the assays described for Stage C.

Stage C. Assays for Neoplasm-Promoting Agents. Certain epigenetic agents enhance the effects of genotoxic carcinogens or facilitate the development of abnormal cells into neoplasms. In addition to a variety of synthetic chemicals, certain drugs, immunosuppressants, and hormones appear to act in this manner. Therefore, assessment of promoting activity is critical for the safety testing of chemicals. The tests recommended at this stage, however, are only for nongenotoxic agents, because agents with DNA-damaging capability can enhance carcinogenicity in a sequential protocol as a result of summation of DNA damage (*216*).

In Vitro Tests. Numerous in vitro systems have been used to study the classic tumor promoters 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and related phorbol esters that are the active ingredients in croton oil (*217, 218*). Responses related

to interactions with specific membrane receptors (*218, 219*) were noted. Such effects, however, were not found with other types of promoters.

The transformation systems described earlier can be modified to test for promoting substances if a limited amount of a carcinogen is used followed by the agent being tested (*220–23*). To test for cocarcinogenicity, the two compounds can be administered simultaneously. A renal cell system has been adapted to study initiation–promotion in a cell aggregation assay (*224*). Other approaches include the production of sister chromatid exchanges (*225*) and aneuploidy (*226*).

From the concept that important informational molecules are exchanged between cells in contact through gap junctions, researchers have developed systems to detect promoting potential by using chemicals to block this intercellular communication (*227–30*). A similar approach using liver cells has been devised (*231, 232*). This effect in vivo causes cells with an abnormal genome to be isolated from the growth-controlling elements provided by neighboring normal cells and thus released for progressive growth (*211, 227, 230, 233*). This technique shows considerable promise, but still needs further validation.

IN VIVO ASSAYS. Promoters of neoplastic development often display a high degree of organ specificity. Consequently, in vivo assays involve the administration of a genotoxic initiating agent that affects the organ in which the promoting activity of the test substance is to be examined.

To test for promoting activity by a chemical in liver carcinogenesis, investigators give a few doses of a limited amount of an appropriate genotoxic hepatocarcinogen, such as diethylnitrosamine or *N*-2-fluorenylacetamide, followed by the test chemical (*234–39*). Enhancement of carcinogen-induced altered foci, which is evidence of promotion, is then identified by using histochemical markers, such as γ-glutamyl transpeptidase (*235, 236*) or iron exclusion (*237*).

Materials that appear to be enzyme inducers—in particular chemicals that induced some liver tumors in mice but not rats and that were not genotoxic in appropriate in vitro test batteries—have shown promoting activity in such systems (*233, 238, 240*). Mouse liver appears to respond as if it had an initiated DNA and gene structure. With rats this is different, although chronic tests with nongenotoxic promoters have often yielded a low incidence of liver tumors; this low incidence is perhaps related to the presence of mycotoxins or nitrosamines in the diet. Many of the more complex polychlorinated aliphatic and cyclic hydrocarbons also fall into the class of enhancing substances (*64, 241*). Unlike genotoxic carcinogens, they exhibit a nonlinearity in the shape of their dose–time response (*242*).

In the absence of genotoxicity, promoting activity can be tested on mouse skin initiated with small doses of benzo[*a*]pyrene or 7,12-dimethylbenz[*a*]anthracene (*50, 68, 243–45*). A material exhibiting endocrine properties or one affecting endocrine balances in general may modify breast or endometrial cancer induction in animals given limited amounts of methylnitrosourea as an initiating dose or in mice with the mammary tumor virus (*246, 247*). Similarly, promoters for urinary bladder cancer or colon cancer may be tested by pretreatment with limited amounts of a bladder or colon carcinogen (*248–51*). Initiation–promotion schemes, when designed with a number of dose levels that include the possible prevailing environmental level, would provide the necessary background information to establish a threshold level for health risk analysis.

The mechanism of promotion is a subject of considerable current research. It may involve a sequence of steps eventually leading to proliferation and possibly to differentiation comparable with membrane and intracellular effector systems like prostaglandins and cyclic nucleotides. One early biochemical indicator of promotion is the induction of ornithine decarboxylase (ODC) (*30, 252–55*). The induction of this enzyme has been used to discover new promoters (*256*). Increased levels of ODC appear to be associated with increased liver cell proliferation (*257*) and might be related to the mechanisms of the promotion.

Decision Point 2. A positive response in the in vitro test for inhibition of intercellular communication strongly suggests promoting activity. However, this test has not been sufficiently validated yet, largely because of the paucity of proven neoplasm promoters outside of the class of phorbol compounds. Therefore, if a positive response is obtained in this system, an in vivo test for neoplasm promotion should be conducted.

A nongenotoxic chemical that is active in an in vivo test for neoplasm promotion must be regarded as a potential human health hazard. Moreover, such an agent would probably produce an increase in neoplasms with chronic administration of high levels. Therefore, the finding of a positive effect indicates the need for further safety testing. This testing should include multidose administration following a short course of the appropriate genotoxic carcinogen; the multidose requirement is to delineate any threshold level.

Neoplasm promoters are almost always negative in the limited in vivo bioassays; therefore, such tests should not be applied to promoters. A more useful indicator is a chronic bioassay. Because promoting activity indicates potential carcinogenicity, the chronic bioassay should be designed to establish the possible no-effect level; in other words, a broad dose range should be tested. The finding of a no-effect level in chronic bioassay coupled with other negative carcinogenicity tests but evidence for promoting action would lead to a health risk assessment distinctly different than that for genotoxic carcinogens.

Stage D. Limited In Vivo Bioassays. This stage of evaluation employs tests that provide further evidence of the potential hazard of chemicals that have limited evidence for genotoxicity without the necessity of undertaking a full-scale chronic bioassay. Also, some of these bioassays provide relative potency ratings when the design includes positive controls.

A number of tests for in vivo genotoxicity have been developed; these tests include the dominant lethal test, specific locus test, heritable translocation test, host-mediated mutagenicity, chromosomal damage, testicular DNA synthesis inhibition, sperm abnormality, sebaceous gland suppression, granuloma pouch assay, and DNA fragmentation or repair in various organs (*61, 258*). Chemicals that were negative in all the in vitro genotoxicity tests will probably be negative in these in vivo tests, except for those chemicals that are activated to genotoxic metabolites by host bacteria; cycasin and certain nitroaromatic compounds are in this category. In vitro systems can be modified to detect these compounds (*259*), for instance, in vivo–in vitro systems (*260, 261*); therefore, at present, the more complex and costly in vivo genotoxicity tests are not recommended as a primary screen. Even a positive result in one in vivo genotoxicity test would not be conclusive evidence of

carcinogenicity. It would serve only as a further indication of the need for additional testing, which already is the recourse for suspect chemicals that are negative or equivocal in the in vitro tests.

Thus, at this stage, the in vivo tests recommended are those that will provide definitive evidence of carcinogenicity within a relatively short period (i.e., 30 weeks or less). Unlike the in vitro tests, these assays are not applied as a battery but rather used selectively according to the information available on the chemical. As in all in vivo carcinogen bioassays, the tests are designed to use an adequate number of animals in each group, including suitable positive, untreated, and, where indicated, vehicle controls. Another critical design element is the duration of the study. The testing must be terminated before the control group exhibits a large increase in spontaneous tumors that would decrease the effective detection of any induced neoplasms. At the end of the study, appropriate statistical procedures are used to establish whether a given treatment group had evidence of a positive response. If so, consideration is given to percent incidence, multiplicity of lesions of a given type, and latent period of the treated groups versus appropriate controls.

ALTERED FOCI INDUCTION IN RODENT LIVER. Because of its metabolic capability, the liver is the target for many carcinogens. During liver carcinogenesis, several distinct hepatocellular lesions precede the development of carcinomas (*262*). The first lesion to appear is the altered focus, and it can be demonstrated in routine histologic tissue sections when sufficiently developed. However, more sensitive techniques permit reliable and objective identification at early stages by detecting abnormalities characteristic of altered foci. For instance, in rat liver, abnormalities in the enzymes γ-glutamyl transpeptidase, glucose-6-phosphatase, and adenosine triphosphatase can be used for histochemical detection (*263–66*). Another important histochemical marker for foci is their exclusion of cellular iron (*237, 266*). Detection of this property is more sensitive than detection of the enzyme abnormalities under some circumstances, and unlike the enzyme abnormalities, this property can be used to characterize mouse and hamster liver lesions in addition to rat.

Induction of altered foci (or nodules) in rodent liver has been used by several groups as a rapid means for detecting carcinogens (*238, 240, 267, 268*). With known liver carcinogens, foci have been detected within 3 weeks of carcinogen exposure, and they appear in high numbers by 12–16 weeks of exposure. One approach is to subject the animals to 12–16 weeks of exposure to the test chemical in the diet or by gavage with subcutaneous injection of iron (Imferon) during the last 2 weeks to produce the iron load that delineates iron-excluding foci. Sections can be stained for both γ-glutamyl transpeptidase and iron, and the number of foci in standard sections from all lobes can be determined.

Another approach to detecting precancerous liver lesions employs the resistance of altered cells to the cytotoxic effect of chemicals (*265, 267*). In this approach, administration of the test chemical is followed by exposure to a cytostatic agent and partial hepatectomy. The cytostatic agent is preferentially metabolized by normal liver cells, and this process prohibits these cells from proliferating in response to the partial hepatectomy. In contrast, the cells in liver altered by the test chemical proliferate and become extremely conspicuous.

Another finding that may have application in carcinogen screening is that induction of GGT-positive foci is accompanied by an elevation of serum GGT (*269*). This change could be monitored to determine animals for the most appropriate time to terminate the study.

So far, various carcinogens have tested positively for altered foci induction. In the rat, polycyclic aromatic hydrocarbons, arylamines, certain aminoazo dyes, heterocyclic amines, nitrosamines, urethane, ethionine, aflatoxins, safrole, and vinyl chloride were all active. In the mouse, safrole was active, and in the hamster nitrosamines were active.

SKIN NEOPLASM INDUCTION IN MICE. The induction of skin neoplasms in mice has been extensively used as a bioassay (*30, 38, 39, 270*). The carcinogenicity of a limited number of chemicals and crude products is readily revealed when continuous application of the material to the skin of mice produces papillomas or carcinomas or when subcutaneous injection yields sarcomas. Activity of initiating agents is rapidly determined by the concurrent or sequential application of a promoter, such as a phorbol ester. Tars from coal, petroleum, or tobacco are active in such systems, as well as the pure polycyclic aromatic hydrocarbons and congeners contained in such products. Mouse skin responds positively because it appears to have the necessary enzymes to yield the active intermediates that result in initiation, especially if cocarcinogens or promoters are present in the crude products. On the other hand, these mixtures rarely yield visceral neoplasms such as those found in the liver, principally because the liver can detoxify these chemicals quickly. However, lung and lymphoid tissues in sensitive mouse strains can be secondary sites of neoplasms. By selective breeding, strains of mice (e.g., SENCAR) were developed to show extreme sensitivity to carcinogens and promoters for mouse skin (*270–72*).

A positive response in this system, as in others, is indicated by an increase in the number and multiplicity of papillomas and carcinomas in a statistically significant manner over controls. The latent period of appearance and permanence of neoplasms can also be compared. An advantage of this system and of the induction of breast cancer in female rats is that the lesions can be seen or palpated without killing the test animals.

At least seveal hundred chemicals or materials have been tested on mouse skin. This system is useful primarily for polycyclic aromatic and heterocyclic hydrocarbons and direct-acting chemical carcinogens, such as sulfur or nitrogen mustard, bis(chloromethyl)ether, propiolactone, and alkylnitrosoureas.

Arylamines and related carcinogens by themselves usually do not provide a positive response on mouse skin, although two exceptions are 2-anthramine and 3-methyl-2-naphthylamine. These two compounds are active in this system, possibly because they are converted to active epoxy intermediates in the same manner as the polycyclic aromatic hydrocarbons (*273*). On the other hand, mouse skin does not appear to yield a positive result with a basic fraction of tobacco tar, even though this fraction is mutagenic and leads to cell transformation. Some arylamines and urethane give a positive indication even if administered by routes other than cutaneous application, but promotion with phorbol ester is required (*29, 270, 271, 274*).

PULMONARY NEOPLASM INDUCTION IN MICE. Andervont and Shimkin (275) pioneered the system of lung neoplasm induction in specific sensitive strains of mice, especially the A/Heston and related strains. In this system, agents are administered orally or by injection, and the incidence and multiplicity of pulmonary neoplasms is determined grossly, usually within 20–40 weeks. A singular advantage of this assay system is that, in addition to an end point measuring the percent of animals with tumors, the multiplicity of neoplasms is a further parameter expressing the strength of carcinogenic action. Also, significant results can be obtained in 30–35 weeks and sometimes faster. Extension of the test for a longer period is not desirable, because the incidence of pulmonary neoplasms in control animals increases rapidly after 35 weeks, and the test loses sensitivity. Most chemicals that are active in this system are also carcinogenic in longer, chronic animal tests, but not the converse. Not all types of chemical carcinogens yield a positive response. Thus, a negative response in this system does not necessarily mean a compound can be considered safe. This uncertainty of negative results is also present with other relatively rapid in vivo tests.

Compounds that have induced pulmonary neoplasms in the mouse include polycyclic aromatic hydrocarbons, urethane, alkylnitrosamides, alkynitrosamines, alkylating agents, aziridines, and hydrazines. Although arylamines induced neoplasms, they did so poorly.

BREAST CANCER INDUCTION IN FEMALE SPRAGUE–DAWLEY RATS. The rapid induction of neoplasms in the mammary gland of young female random-bred Wistar and, to a greater degree, Sprague–Dawley rats was discovered by Shay and elegantly extended by Huggins (276). For many carcinogens the optimal age for effective breast cancer induction is about puberty or at 45–60 days old when cells appear to proliferate at a maximal rate (277, 278). With some agents, the age factor is not so crucial, and the induction of neoplasms is effective when the treatment begins at an age of 5–6 weeks, as is generally true for chronic bioassays (279). A positive response is indicated by an increase in the incidence and multiplicity of mammary gland neoplasms in the rats. With powerful carcinogens, especially select polycyclic hydrocarbons, arylamines, or nitrosoureas, mammary gland neoplasms are induced in less than 9 months. As with lung neoplasm induction in mice, the multiplicity of mammary neoplasms provides an additional quantitative criterion to denote relative strength of the carcinogenic stimulus.

Decision Point 3. Proven activity in more than one of the limited in vivo bioassays may be considered unequivocal qualitative evidence of carcinogenicity.

A definite positive result in one of the limited in vivo bioassays together with two positive results in a battery of rapid in vitro bioassay tests for genotoxicity also indicates potential carcinogenicity. Even greater concern is warranted if the results were obtained with moderate dosages and if multiplicity indicated a good dose response.

Positive results in one in vivo bioassay and in only one in vitro test makes the agent highly suspect, but further testing is indicated.

A negative result in any of these assays does not constitute proof of noncarcinogenicity, because all of these tests have some limitations.

Stage E. Chronic Bioassay Test Systems. Chronic bioassay is used in the decision point approach as a last resort for confirming questionable results from the more limited testing. For instance, these tests should be used to check compounds that have extensive human exposure even though they tested negatively in the preceding stages or to acquire mechanistic data on possible indirect epigenetic agents. Multispecies and dose–response data are very important for risk assessment. An initiation–promotion test using an initiating agent that has broad tissue responses, such as some nitrosamines or nitrosamides, would also be useful for risk assessment.

The ultimate goal of carcinogen test systems is to provide data that permit evaluation of the carcinogenic risk for humans. Therefore, an important issue regarding chronic bioassays is the degree to which data obtained in such systems actually reflect a human carcinogenic risk.

The specific cellular and molecular systems in humans are essentially the same as equivalent cellular and molecular systems in animals. Because these systems determine how a given exogenous chemical interacts, humans would probably react much the same as the animal models; although quantitative aspects and the tissues affected would vary because of different metabolic pathways.

About 29 chemicals or mixtures have been demonstrated unambiguously to have induced cancer in humans (*4, 5, 8, 29, 280*). Almost every chemical that has led to cancer in humans is highly carcinogenic in an animal model. The converse may very likely be true; a chemical that is reliably carcinogenic in animal models probably would affect humans, especially chemicals that induce a high yield of neoplasms over several dose ranges in a number of species with a reasonably short latent period (15 months or less). Such chemicals, especially if collateral data show them to be genotoxic, are unquestionably human risks. As noted, the known human carcinogens are typically endowed with such properties. Key exceptions are arsenic in the form of inorganic arsenite or inhaled benzene; animal models have failed to provide convincing evidence of carcinogenicity. However, virtually all other human carcinogens, whether synthetic or natural products, can probably be detected quickly and reliably in laboratory animal models and in short-term tests.

Interpretation of the results of chronic bioassays will be discussed under data evaluation. Nevertheless, a qualification should be introduced at this time. Humans are highly heterogeneous; they live in different environments under varied dietary and other exposures and thus have widely varying response patterns to any given carcinogenic challenge. For example, much evidence has established that heavy cigarette smoking leads to lung cancer. However, even with equal numbers of cigarettes smoked per day, the responses of different individuals vary a great deal. The smoking method, the inhalation depth, the nutritional status of the individual, the biochemical activation and detoxification systems, the ciliary and mucous clearance systems, and other defense mechanisms all play a role in determining the overall outcome (*281, 282*).

The detailed methodology of chronic bioassay is described in several monographs (*54–56, 133*). Some critical factors will be reviewed in this chapter. The design of chronic bioassay is crucial—there is no such thing as a routine long-term test.

PURITY OF CHEMICALS. Impurities in a chemical may enhance or diminish toxicity and carcinogenicity. Investigators must have detailed information on the composition of the test substance. If the substance is a technical product, both the technical product with its contaminants, as well as the pure main component, should be tested. Data must be available on the stability of the chemical under the conditions of administration. Data on its behavior in other bioassay systems would be useful.

SELECTION OF ANIMALS. A comprehensive bioassay in animals is best performed on more than one species. Indeed, even powerful carcinogens that affect humans have been inactive in all strains or species tested. For example, 2-naphthylamine, for reasons that are not yet clear, causes cancer poorly in most strains of rats (*283*) but is active in mice and hamsters (*38, 46, 107*). On the other hand, the mold product aflatoxin B_1, suspected as a cause of liver cancer in humans, does not elicit a neoplastic response in the liver of mice but is highly potent in rats under the customary bioassay conditions. Administration to newborn mice, however, produced a carcinogenic effect to the liver and in older mice to the lung (*120*).

Early researchers used larger animals such as rabbits and dogs for carcinogenicity studies. Because the duration of the overall carcinogenic process is to some extent proportional to the lifespan of a given species, model bioassay systems have concentrated on small rodents such as mice, rats, or hamsters. These animals have an average lifespan under good conditions of 2–3 years and thus develop neoplasms within a shorter time frame.

Numerous strains of inbred and noninbred rats, mice, and hamsters (*284–86*) can be used. Considerations that guide the selection of a specific strain include the ease of maintenance of the animals, their relative sensitivity to various test chemicals, freedom from nonneoplastic disease, and low and late occurrence of spontaneous cancer.

The rats most often used for carcinogen bioassay are the Fischer and Sprague–Dawley strains in the United States, the Donryu strain in Japan, and various types of Wistar-derived rats in Europe. These regional differences have tended to disappear with the increasing availability of standard specific pathogen-free rats, such as the Fischer strain. Some of these breeds exhibit an appreciable incidence of spontaneous cancer, especially in endocrine-responsive organs, when kept for their full lifespan. Many testing programs have standardized their systems by using the highly inbred rat of the Fischer strain (*54*) and the noninbred Osborne–Mendel strain. The latter strain was selected for its sensitivity to certain chlorinated hydrocarbons (*287*). Other strains have important roles for specialized assay systems. For example, the Sprague–Dawley strain female rats are excellent for the Huggins breast cancer assay (*276*).

A greater variety of mouse strains is available for carcinogen testing. The noninbred Swiss breeds are widely used for a variety of tests and are quite suitable, provided adequate control groups are used. Inbred strains such as AKR, which exhibit spontaneous leukemia and lymphoma early in life, or C3H, which develop high yields of liver neoplasms in males or mammary neoplasms in carriers of the mammary tumor virus, are not very useful. Strain A mice are excellent because of their tendency to give lung neoplasms in a short time with many carcinogens, but

they cannot be used for long tests because of a high incidence of spontaneous lung neoplasms (*275*). Strains such as Balb/C and hybrid strains, such as the widely used model B6C3F1 (C57BL X C3H F_1) (*288*) now adapted for the NCI bioassay screening program (*54*), exhibit adequate sensitivity, a relatively low spontaneous disease rate, and good longevity.

In addition to sensitivity to pulmonary neoplasm formation, several strains of mice readily develop liver neoplasms. This response has been criticized as not always reflecting a true carcinogenic risk to humans, even though a number of human carcinogens do yield mainly liver neoplasms in mice (*289*). As discussed, a positive response in such mouse strains may be seen also with a nongenotoxic promoter or estrogenic hormones (*210, 211, 213–15*).

Noninbred hamsters have found favor largely because of extensive tests by Shubik and Saffiotti (*see* 286). They are particularly well suited for lung carcinogenesis studies, because they are fairly resistant to the complications of infectious lung diseases, which are more frequent in rats and mice. Hamsters develop urinary bladder neoplasms when high levels of aromatic amines and azo dyes are given. Thus, the hamster can serve as a model for the mechanism of the induction of bladder cancer by such chemicals in humans. Recently a number of strains of inbred hamsters were developed, mainly by Homburger (*286*). When 3-methylcholanthrene is given orally, some of these strains have responded with several types of cancer in the gastrointestinal tract and mammary gland.

The quality of the animals is an important factor for useful carcinogen bioassays. Procurement of animals from unreliable sources can be a costly mistake if such animals prove unsatisfactory for the collection of valid data either through premature loss or through disease processes that may affect their response. The cost of acquiring animals is only a minor item compared with the overall expense of conducting a carcinogen bioassay. Only the best quality will do (*290*).

In some instances, animals at 6–7 weeks old have significantly greater tolerance for toxic chemicals than at 3–4 weeks old, when they are weaned. Hence, 6-week-old rodents may be at an optimal age for initiating exposure to the test chemical with respect to maximizing the sensitivity of the system and allowing the expression of carcinogenic potential, if present.

After the first successful induction of neoplasms in newborn animals by viruses (*291*), more workers adopted this type of experimentation (*69, 292–94*). Mice of various strains and hamsters appeared to be quite responsive to many types of chemicals when treated from birth; with rats, this result was not as common. A few accurate comparative studies have been conducted (*293*). The field of transplacental carcinogenesis has seen renewed interest (*295, 296*), especially in the light of human findings with diethylstilbestrol (*8, 14, 280*). The mechanisms may need elucidation for application to human risk extrapolation. An illustrative example is the problem with the saccharin tests: Two-generation tests with high levels of saccharin (*297–99*) led to a modest yield of bladder cancer in the offspring that were continued on the saccharin-containing diet, whereas similarly treated rats given saccharin only from weaning on had little evidence of effect. Saccharin is now known to be nongenotoxic (*300*); it acts by a promoting mechanism (*248–50*). The results of the two-generation test may stem from an artifactual metabolic imbalance (*301*) and tissue damage that seems to have no counterpart in humans

who consume foods containing relatively low levels of saccharin. Evidence suggests that saccharin modifies protein metabolism and leads to urinary excretion of indoles; this pathway ties the effect of saccharin to that of tryptophan (*46, 250, 302*). Thus, high-level exposure tests over two generations need special justification with respect to potential human exposure modalities. On the other hand, two-generation modes of administration could be useful for the study of macro- or microcomponents of diet, such as fat or minerals, because these have been present in traditional diets over many generations (*251*).

ANIMAL MAINTENANCE. Animals must be housed and maintained under optimal conditions to avoid complications, such as intercurrent infection, that can affect the animal response (*133, 303*). Recommendations on animal husbandry have been made (*133*). Briefly, animals should not be crowded in the cages, nor should racks of cages be crowded into animal rooms. Temperature and humidity need to be closely controlled. Light is usually on for 12 h and off for 12 h. Air flow should be uniform and complete 6–10 changes/h. Recently, in view of the high energy costs, the standards in this respect were modified with fewer changes per hour recommended, provided that animal rooms were not overcrowded. Soiled bedding and cages should be changed at least twice weekly. Bedding quality needs careful selection; certain soft wood chips or cedar shavings can release volatile enzyme inducers that can affect host biochemical parameters, and hence, response to toxic or carcinogenic agents (*304*). The tap-water supply should be clean and the bottles, if used, should be thoroughly sterilized at each refilling. Water can be chlorinated to maintain sterility, or the acidity can be adjusted carefully to about pH 2. Automatic water supplies need to be monitored to ensure a continuing supply of clean fresh water. Adventitious carcinogens can enter the environment of test animals from a variety of sources (*305*), especially the food (*92–94, 120*); therefore, *careful monitoring is essential.*

Numerous commercial diets are available for animal maintenance. Consideration has been given to formulating a standard diet for carcinogen bioassay that is uniform not only in composition, as are the commercial chows, but also in ingredients. A few laboratories use a semipurified diet formulation. However, certain carcinogens have been found to be more toxic when mixed with a semipurified diet than with a commercial good-quality chow. The reason for this difference is not known, but it might stem from multiple effects, such as the presence of natural fibers and certain enzyme modifiers in laboratory chows.

ADMINISTRATION OF TEST CHEMICALS. Test chemicals can be administered in several ways. The mode selected depends in part on the ultimate application and dosages of the chemical found in the human environment. Food additives or drugs given orally are best tested by feeding; volatile air pollutants might be examined by inhalation. On the other hand, if correlations of the relative potency of a given product to its chemical structure are being sought, especially a structure that short-term tests have showed to be genotoxic, the route of administration is somewhat less important. However, sound principles must be applied in the interpretation of data. With certain routes of administration, such as subcutaneous injection or bladder implantation, neoplasms are not necessarily produced because the chemical itself is truly carcinogenic; sometimes the physicochemical nature of the

chemical or solid produces a tissue reaction as a result of persistence at the point of application or insertion. The tissue reaction then results in neoplasms (*8, 306, 307*). Consequently, tests such as bladder implantation are now rarely used. The conclusions drawn must necessarily be based on a complete understanding of the mechanism involved, especially properties such as presence or absence of genotoxicity derived from appropriate tests.

Oral Intake. Test chemicals can be mixed in food, administered in drinking water, or given by gavage or stomach tube. Oral intake appears to be quite reliable in detecting carcinogenic potential. The presence of the chemicals should not prevent consumption of essential food components. If the test agent affects the palatability of the diet, nutritional deficiencies may develop. This problem can be avoided by proper design involving dietary adjustment to ensure the proper balance of macro- and micronutrients.

Also, because of the intake of excessive amounts, deposits of the chemical or its metabolites in certain tissues may lead to adverse effects in organs such as the kidneys and urinary bladder. Such deposits can be avoided with lower yet still meaningful dosages. Unless crystalluria or similar effects are predicted to result in humans exposed to the chemical, such animal data would not accurately forecast human carcinogenic hazard.

If the chemical being tested is not a food itself or an important component thereof, it should not account for more than 5% of the diet. Probably a maximum level of 1% would suffice to evaluate properly the potency of even a weak carcinogen for which preliminary studies indicate genotoxicity. Careful mixing of the test chemical into the diet is essential. Verification of the desired composition by chemical analyses of aliquots of the diet is useful. When the prepared diet is in pellet form, such analyses are essential because the pelleting process often raises the temperature to a point where certain materials decompose, volatilize, or react with other diet ingredients.

Similar considerations hold when oral intake is administered in the drinking water or by intubation. The appropriate dose level would be selected on the basis of (1) prospective use, (2) the actual toxicity of the chemical obtained in preliminary tests to define the maximally tolerated dose, and most importantly, (3) any information derived from previous in vitro tests for genotoxicity and/or enhancing potential.

The use of gavage or intragastric intubation offers the advantage of quantitative administration of a given dose, because rodents do not regurgitate. Also, the handling of test chemicals with this method is less hazardous; contamination that results from spilling is less likely. Gavage can be performed repeatedly, and if the animals are started on a regimen at weaning, they adapt and tolerate daily handling readily. However, administration by tube delivers the entire dose all at once in contrast to administration in the drinking water or the diet where the chemical is delivered and absorbed over a period of time. Animals might show somewhat differently tolerated dosages or effects as a result of different peak levels in the tissues and blood. Another point to be considered is that the taste of the substance being administered might favor or necessitate intragastric intubation.

Many of the carcinogens being tested are often lipophilic compounds that require a suitable vehicle. One approach is to use a suspension of finely powdered

product in a jelling agent, such as agar, methylcellulose, or other such polysaccharide, instead of a solution. Suspensions are reasonably stable, and aliquots can be drawn and inoculated into the stomach. A gel-based diet has been formulated and can incorporate diverse substances satisfactorily and can be administered reasonably quantitatively by pouring the gel in the cages without the use of food containers (*308*).

When a solution is preferable, edible oils and fats can be used. However, oils or fats will increase the percentage of fat intake. Nutritional changes may have undesirable consequences; various carcinogens, specifically with target sites such as the mammary gland, large bowel, prostate, endometrium, and probably pancreas, are potentiated by dietary fat (*251, 309, 310*). Therefore vehicle control animals should be given a suitable positive control compound to assess the impact of such potentiating dietary elements.

Cutaneous Application. The skin of mice or rabbits generally responds better than that of other species to carcinogenic challenges. Additionally, a sensitive strain of mouse, the SENCAR (*272, 273*), has been developed. Mouse skin has been a standard tool to detect and quantitate rapidly the carcinogenic potential of coal, petroleum, tobacco tars, and waxes. Except for tests of liquid chemicals, a solvent such as acetone, toluene, dimethyl sulfoxide, some purified mineral oils, or edible oils is usually necessary. Benzene is less desirable, because it is metabolized to toxic phenol and has adverse effects of its own. Some mineral oils are not useful, because the test chemical is often poorly transferred to the skin. Because of solubility problems, lesser amounts of chemical can be administered by the cutaneous route than by the oral route. Usually papillomas and carcinomas at the point of application to the skin are seen only with ultimate or primary carcinogens unless the chemical is a procarcinogen that can be metabolized by the skin to an active ultimate carcinogen. For example, the polycyclic aromatic hydrocarbons fall into this class. Mouse skin is an ideal test system for such chemicals, because it appears to possess activating but only limited detoxifying capacity. Under some conditions, oral administration of a carcinogen that is not considered active on mouse skin could lead to initiation at that site, perhaps in the sebaceous glands; once the site has been primed, then topical application of a promoter could produce neoplasms (*311*). Cutaneous application of many products also may lead to their resorption. Thus, they can cause adverse effects and cancer in tissues remote from the point of application. Therefore all tissues must be examined for lesions during complete autopsies even when the chemical is applied to the skin, unless previous work has shown that a remote effect does not exist.

Subcutaneous Injection. The carcinogenic components in coal tar, namely the polycyclic aromatic hydrocarbons, were first found to be carcinogenic by this technique. Primary and ultimate carcinogens are detected because injection site sarcomas are produced. Because many chemicals are resorbed from the injection area, information is also obtained on possible effects in remote organs. However, the in vivo circulation after subcutaneous injection may differ substantially from that seen after other modes, such as intraperitoneal and intravenous injection and oral intake. The initial distribution can affect the metabolic activation and detoxification sites and the relative tissue concentrations; therefore, in many

instances, the mode of administration determines the effective key target organ. For example, *N*-dibutylnitrosamine given to rats subcutaneously yields only bladder carcinomas, whereas orally it induces also liver and esophageal cancer (*104*).

Subcutaneous injection of certain products that are difficult to resorb, such as complex dyes, oils, and waxes, can produce sarcomas, even though the chemicals are not really carcinogenic by other criteria relevant to human risk evaluation. Data obtained in the in vitro tests concerning genotoxicity provide the mechanistic basis for interpreting injection site sarcomas.

Intraperitoneal Injection. This method is a convenient way of introducing test chemicals, even repeatedly over long periods of time. Two requirements for sustained exposure are that the chemicals must be somewhat soluble by themselves and their vehicle must not accumulate. Oils tend to be absorbed poorly from the peritoneal cavity. However, even fairly water-insoluble chemicals are resorbed well after intraperitoneal injection into rodents. The amount absorbed should be ascertained in each individual case.

With direct-acting primary carcinogens, local neoplasm production in the form of sarcomas is often seen. With agents requiring chemical or biochemical activation, the spectrum of neoplasms obtained usually is similar to that noted with oral intake.

Intravenous Injection. This technique is the most delicate to execute, especially for multiple dosing. It permits the rapid widespread distribution of a test chemical to many organs, particularly if the agent is relatively water soluble. Even so, the specific organ affinity of carcinogens is often maintained. Injected suspensions, on the other hand, tend to be filtered out by the vascular system in select organs such as the lung, and cancer may develop primarily in those organs.

PRELIMINARY TOXICOLOGY. *Rationale For Dose Selection: Maximum Tolerated Dose (MTD).* Several considerations dictate that carcinogen bioassays must include at least one carefully selected high dose level: Carcinogens, like other pharmacologic agents, have dose-dependent effects (*49–51, 312–23*) such that the higher the dose level, the more elevated the response—in this instance, greater induction of neoplasms. Also the higher the dose, the shorter the latent period of neoplasms. If two different chemicals exhibit identical yields of neoplasms, the one that induces neoplasms faster can be said to be the more powerful agent.

In recent years the question of dose selection and, especially, the use of the MTD has become a controversial area. This controversy stems in part from the interpretation of results obtained in certain bioassays using the MTD and one-half the MTD; the data generated were interpreted on the basis of statistics only without application of other scientific concepts. As a result certain agents were labeled carcinogenic.

Carcinogens administered at high dosages may have specific toxic effects, particularly when the compound obeys nonlinear pharmacokinetics; this behavior complicates extrapolation and health risk analysis, sometimes rendering it impossible (*242, 324, 325*). This kind of problem will arise primarily with nongenotoxic chemicals; for these chemicals four to five screening dose levels are

recommended to acquire a reasonable view of pharmacological and toxicological dose–response behavior. Thus, a nonlinearity will be readily pinpointed. High dose levels are [illegible] with genotoxic carcinogens, because they generally have greater acute and subacute toxicity that is reflected in a lower MTD. Often a linear trend is seen with dose, measured by binding to protein or DNA or by circulating serum levels (*326–31*). This linear trend is interesting, because toxicity depends on metabolic production of the more toxic proximate and ultimate carcinogen from an administered procarcinogen. Any nonlinearity seen with genotoxic carcinogens may stem from saturation, either of absorption due to solubility of a solid or of transfer to pulmonary volume of inhaled gas (*332*).

Erroneous conclusions are drawn too easily from poorly planned or monitored bioassays. To plan a bioassay well, one should first acquire information with a battery of efficient and rapid in vitro tests and determine genotoxicity as well as any promoting or enhancing potential. This data base, the potential use, and the anticipated extent of human contact with a product form the essential components for eventual health risk analysis. Knowledge of potential human contact would involve data on possible routes of entry, frequency, and continuity of the exposure, and above all, on the expected levels of exposure.

If the decision point approach becomes the norm for such tests, then expensive and complex chronic bioassays will be conducted much less often. They should be used for chemicals that have widespread and extensive human contact. Under these conditions, at least four dose levels, including one mimicking the highest potential human exposure level, should be tested. The results of such a study will yield a delineation of the shape of the dose–response curve and data for realistic levels of human exposure. However, one dose level should be at the MTD to provide information on the most severe conditions. A negative result under such conditions would then preclude suspicions that a product might have been active had higher levels been tested.

For example, heavy chronic abusers of the drug phenacetin have eventually developed urological cancer (*333*). A number of tests of phenacetin, even at high doses, failed to disclose a carcinogenic effect, although this drug has a structure typical of carcinogenic aromatic amines and is mutagenic. The carcinogenic effect was not detected until a test was conducted at the carefully selected MTD that reproduced the human condition in an animal model. A corollary comment is that the dose–response curve for this drug has an evident threshold in animals and in humans. In the series of carcinogenic arylamines, the monocyclic arylamines are much less carcinogenic than the higher homologues (*273*), a point to be considered when claims are made that such chemicals are human cancer risks, on the basis, for example, of mutagenicity data only.

Similar considerations hold for chemicals that do not give any indications of genotoxicity in the in vitro prescreens, and particularly, ones that show promoting effects. Human contact with these compounds might provide conditions where possible carcinogenesis-enhancing effects could be expressed. Human epidemiological studies have shown that nutritional elements such as dietary fat level have a decisive effect on risk of breast or colon cancer (*251, 309, 310*): reduction of fat intake from 40 to 10% of calories, or only a four-fold decrease, converts a high-risk situation for those diseases, as seen in Western countries, to a low-risk situation,

typical of that of traditional Japan. Thus, dose selection and the use of multiple doses is very important with such agents.

The problem of dose levels is sometimes confused with statistical considerations. A common misconception is that high dose levels are used in carcinogen testing because the data obtained in relatively small groups of animals (e.g., 100 rodents) need to be extrapolated to large numbers of humans. Actually, the bioassay was standardized with high-level dosing based on two major considerations. The first, and most important, consideration was the realization that high doses are essential to obtain evidence of carcinogenicity with known human carcinogens such as 2-naphthylamine (*39, 107, 283, 334*). Lower dosages of some chemicals with demonstrated human carcinogenicity had appeared negative in chronic rodent tests. The second reason was that in a series of bioassays involving five or six dose levels, evidence of carcinogenicity was obtained over a range of dosages with powerful carcinogens (*319*). However, with somewhat weaker carcinogens, the effect was clearly apparent only at the highest dose levels. Together, these considerations led to the 1967 formulation (*54*) of procedures in which preliminary dose ranging was used to establish the so-called maximally tolerated dose (MTD). The bioassay was conducted at that dose level, and also at a lower dose level, such as one-half or one-third of the MTD. The lower dose was designed to ensure survival of at least one group over a chronic 2-year test. (If the MTD was too high, premature mortality of animals might result.) The approach was designed as a qualitative screening test, although where animal survived at both dose levels, some semiquantitative indication of a dose response might be obtained.

The use of high dose level testing to detect carcinogens might also have a counterpart in the field of human exposure in occupational carcinogenesis. For example, production of aromatic amines began in about 1870, and until the mid 1950s, when adverse effects of these chemicals were recognized, few if any precautions were taken to avoid human exposure to the amines. Workers were subject to high-level exposure, which could account for the observed cases of cancer (*107, 388*). Although there is little quantitative data, the exposure of workmen under these early, unhygienic conditions might have led to the absorption of grams of product per day, and thus, large total amounts over a period of years. Likewise, in worker exposure to vinyl chloride, it seems that up to now angiosarcomas were seen mainly in reactor cleaners (*15, 16, 389, 390*), who were exposed to sizable amounts of this agent.

When cancer develops in humans as a result of occupational exposure to a chemical carcinogen, determining the dosages involved is difficult, if not impossible. Indeed, cancer often develops many years after a variable exposure index. However, in some cases, dosages have been reasonably accurately evaluated. When urinary bladder cancer has developed subsequent to known exposure to a drug as with chlornaphazin or phenacetin, information can be gathered from hospital records (*313, 333*). Lung cancer incidence in cigarette smokers increases and arises earlier the higher the number of cigarettes smoked per day (*281, 282*). Pubertal girls were found to have a rare form of vaginal cancer after their mothers

were treated with high doses of a hormone, diethylstilbestrol, but not after treatment with low doses (*14, 335*).

On the basis of these considerations, the administration of as high a dosage as can be tolerated that will allow survival of the test animals seems most logical.

The next question to be asked is whether administration of such high dose levels could cause cancer as an artifact resulting from the dose levels used. The likelihood of this result is minimal in most instances, however. Many noncarcinogenic chemicals have been administered at high dose levels without eliciting evidence of a neoplastic response. Thus, even at the highest tolerated dose, carcinogenic effects are attributable to the test agent. Nevertheless, a positive response to the MTD does not automatically imply a risk for humans.

Careful analysis must be given where animals treated with high dose levels of a chemical developed not only a carcinogenic response, but also other abnormalities that most likely were responsible for the neoplastic response. For example, the presence of foreign bodies in the kidney, renal pelvis, or urinary bladder may by itself elicit a neoplastic response (*38*). When administration of the test chemical leads to crystalluria and the deposition of stones in the excretory organs as a primary response or leads to necrosis and hence regeneration, the possibility must be considered that the induction of neoplasms is secondary and not necessarily indicative of a direct carcinogenic effect from the chemical. Knowledge of possible genotoxicity facilitates, and in fact is essential for, proper rational interpretation of such findings.

Another problem requires thorough analysis: the induction of neoplasms in the endocrine system consequent to high-dose application of chemicals that affect hormonally responsive tissues over an extended period. Chemicals or drugs of this type may very likely induce cancer in the endocrine system if their administration leads to hormonal imbalance in the animal. Cancer in endocrine-responsive organs in humans may stem from such imbalances (*208, 336–38*). Current views are that such chemicals cannot be evaluated properly for carcinogenic potential in a rodent system that does not have an estrous cycle comparable to that of the human female. However, drugs of this type can be tested for possible carcinogenic effect in nonendocrine target organs. For example, diethylstilbestrol leads to renal carcinoma in male hamsters by an unknown mechanism that seems to involve pituitary hormones (*339*).

Determination of Maximum Tolerated Dose (MTD). Once the mode of administration, species, and strain of animal have been selected, the MTD under the precise experimental conditions must be determined. Typically, groups of five males and five females are given a range of dose levels by the route selected. These tests must mimic the definitive experiment. Thus, if the protocols call for a single dose, such as a classical LD_{10} or LD_{05}, acute toxicity can be determined. Single doses of some chemicals can be carcinogenic, but most chemicals are only active after repeated exposure. Also, more often than not, humans are exposed chronically. Usually, the MTD is determined over a 6– to 8–week period, which gives a fair indication of tolerance for lifespan tests. The dose levels are selected on the basis of mortality

and weight gain in relation to vehicle or untreated controls. The MTD is a dose that leads to no mortality in the 8-week test and that may depress the weight gain of animals 5–20% with an optimal lowering of less than 10%. Except for special cases, which involve newborn animals or extend over two generations, these tests usually begin when rodents are 6 weeks old.

The importance of correctly selecting the MTD cannot be overemphasized. If the first set of tests gives inadequate indication, a second series should examine doses centered at the level indicated in the first series.

Large numbers of animals should not be wasted only to discover at the end of 2 years that the dosages used were too low and that the results cannot be interpreted. Doses on the high side will be noted readily by inadequate weight gain or mortality.

Chronic Bioassays. *Experimental Design.* The steps prior to the initiation of long-term tests are (1) acquiring data on the possible genotoxic effect of an agent, (2) thoughtfully selecting the species and strain of animals, (3) performing the preliminary toxicology to delineate MTD under the exact conditions of the chronic bioassay, (4) determining an experimental design that involves adequate numbers of animals in each group, and (5) including a vehicle or diet control as well as a suitable positive control; the positive control compound should have a structure similar to that of the compound tested.

Participation of statisticians in the tests will optimize the experimental design, enable the experiment to be conducted most economically, and ensure that data yield valid, significant, and meaningful results (*340–48*).

A 10% excess of the required number of animals should be procured so that animals judged inappropriate during the quarantine period can be eliminated. Animals deviating from the average weight by ±10% should not be used. The animals should be randomly assigned to experimental and control groups.

The ultimate refinement of evaluation desired determines the number of animals; experiments range from 25 males and 25 females per dose level to as many as 100 per group. Tests on widely used environmental chemicals occasionally require more animals. Studies involving more elaborate protocols with more than two dose levels might well involve a pyrimidal structure using a smaller number of animals at the highest dose levels where a more severe effect is likely. The groups given lower dose levels, as well as the control groups, may include larger numbers of animals to obtain adequate mathematical significance with the lower effect. Pyramidal structure is most desirable for studying the effects of promoting substances after a standard dose of a genotoxic initiating carcinogen is given. The effect of promoters may be highly dose-dependent, and relatively low effects can be expected with lower dose levels. For a compound present in the environment at low levels, relatively large numbers of animals will be necessary to determine an accurate health risk analysis and to delineate the existence of a threshold.

In chronic tests simultaneous control groups are very important, because the animals used exhibit spontaneous neoplastic and nonneoplastic lesions. If a vehicle is used, there must be vehicle controls involving the same number of animals as in the experimental group. However, if a number of chemicals are studied simultaneously, only one control group is needed. The number of animals in the control

group, C, should be determined by the following equation: $C = E\sqrt{n}$, where E is the number of animals in each experimental group and n is the number of experimental groups. For example, a test series with five chemicals with 50 males and 50 females per chemical run simultaneously requires the use of 50 times the square root of 5 or 112 control males and 112 control females. Of course, all animals required for a given test series should be from the same shipment or should be close in age.

A positive control group exposed to a known carcinogen under established conditions is useful. For this purpose, a smaller number of animals may suffice, although at least 15 females and 15 males should be used. If males and females are known to respond identically, studies can use only one sex, but should use an increased number per group; 25–35 animals is acceptable.

Conduct of Study. If despite careful preliminary toxicology, the weight gain of the animals in the experimental group appears inappropriate, for example, as high as the controls (dosage too low) or below the target 10% weight depression (dosage too high) at the selected MTD, the dose levels can be adjusted within the first 8 weeks of the test. If inadequate dose selection is noted after 10 weeks, the test should be terminated and experimental conditions adjusted properly. Belated alteration in dosages after the weights reflect inadequate dosing often does not yield an adequate change in the weight curves.

Animals are maintained on test (1) until adverse effects are noted, (2) for 104 weeks, or (3) for lifetime. Lifetime studies are worthwhile only under highly controlled conditions in which the aged animals are carefully and frequently examined. In most instances, especially in large-scale test series involving thousands of animals, it seems more efficient to terminate the experiment after a predetermined time, such as 104 weeks. The quality of the tissues collected and freshly fixed is bound to be much better; thus, the interpretation of the experiment is more reliable. Further, a 2-year exposure in rodents under adequately designed and controlled conditions should detect virtually all agents that might represent a carcinogenic risk to humans. Almost all known genotoxic carcinogens quickly lead to cancer induction in high incidence in several species. Thus, a 21–24 month test should be adequately sensitive. Longer maintenance may actually be detrimental, because of the rapidly increasing incidence of varied spontaneous neoplasms with age. Furthermore, the administration of the test compounds should be stopped 3 months before the planned termination of the bioassay for two reasons: (1) a reversible lesion due to toxicity that could confound histopathological evaluation might regress; and (2) the toxicity of the test compound that might be inhibiting the growth and development of certain neoplasms would be eliminated. A really carcinogenic chemical should demonstrate its effect even with a 20-month administration, and there seems no benefit at all in maintaining the treatment.

In the course of the experiment, the animals should be inspected at least once if not twice a day to isolate animals that appear ill. Nothing is gained by maintaining animals that show adverse effects. If cancer is present, it will not grow much more; if cancer is absent, it is not likely to develop in a short time. The groups of control animals must survive at least as long as any of the experimental groups to be valid for comparison. If any group has lost 75–80% of its animals after 21 months, all the animals should be autopsied and the test concluded.

Necropsies should be performed by competent, highly trained personnel experienced in experimental pathology or under the immediate supervision of a qualified pathologist. Tissues should be immersed in adequate amounts of the appropriate fixative solution, and certain hollow organs, such as the urinary bladder or stomach, should be tied off and filled with fixative to avoid fixation artifacts and possible misleading interpretations. After fixation, the tissues are processed by conventional techniques for the preparation of hematoxylin and eosin-stained sections. Lesions are diagnosed according to accepted criteria for the interpretation of neoplastic and nonneoplastic diseases (*349–52*).

Data Acquisition. Throughout the experiment, careful records must be kept of weight gain and of any grossly visible neoplastic lesions or other abnormalities including their location and multiplicity. Regression of benign lesions, such as papillomas or adenomas, is of interest. The U.S. Food and Drug Administration regulations entitled "Good Laboratory Practices" (353) specify accurately the kind of records expected.

The results are tabulated by listing lesions according to types for every tissue. In some organs, such as the mammary gland, lung, liver, and intestinal tract, the multiplicity of the lesions is a more sensitive indicator of relative carcinogenicity than the percent of animals with cancer at a given site. More can be learned by examining the data for each tissue or target site than a record of the overall cancer incidence. Combining the results for all tissues and organs is not very instructive, except when there is a clear-cut increase over controls and several different tissues show cancer. In fact, cases are known where the incidence of neoplasms increased in one tissue and decreased in another so that the overall incidence was almost identical with controls (357).

Evaluation of Results. In the evaluation of a chronic bioassay, the main point is whether the test chemical produces a carcinogenic effect. As discussed earlier, a positive effect is defined as (1) the development of types of neoplasms not seen in controls, (2) an increased incidence of the types of neoplasms occurring in controls, (3) the occurrence of neoplasms earlier than in controls, or (4) an increased multiplicity of neoplasms in individual animals.

In this evaluation, the participation of statisticians is essential for analysis of tumor incidence data (*333, 355–57*), and adjustment for intermittent, treatment-unrelated mortality (*358–60*). In a given experiment, when statistical significance is not achieved by comparing any one dose group versus controls, the data for a number of doses can be examined for trends (361). However, virtually all human carcinogens yield a striking, high incidence of cancer in a relatively short time in several animal models. Thus, it is difficult to visualize a risk associated with a chemical that gave a borderline, albeit statistically significant, effect in a 2-year study. Borderline results can often be attributed to the variability of the incidence of spontaneous neoplasms (362).

Final Evaluation

The results obtained in chronic bioassays should be interpreted in view of the results from preceding stages of testing. Convincing positive results in the in vitro tests coupled with documented in vivo carcinogenicity permit classification of the

chemical as a genotoxic carcinogen. In chronic bioassays, genotoxic carcinogens often produce neoplasms in more than one specific organ and in high yield with a relatively short latent period. Such results in the absence of data on genotoxicity, therefore, should direct attention to interaction with DNA as the mode of action. Squire (*21*) has provided a useful means of quantitative evaluation of long-term bioassays. Consideration is given to a number of species and tissues affected, pathologic nature and severity of the neoplasms, latent periods, and dose–response relationships, and important weight is assigned to genotoxicity.

If chronic bioassays have been performed in the absence of any data on genotoxicity, the appropriate short-term assays should be conducted before analyzing the long-term data. Such information appears to be crucial to practical interpretation and health risk analyses. For example, bioassays of chemicals that have not exhibited any evidence of genotoxic properties may yield artifacts at the MTD; positive results might reflect a promoting action or might result from metabolic aberrations that would not occur under lower level, realistic exposures, as is the case with nitrilotriacetic acid (NTA) and probably saccharin. Analysis of the positive results with NTA is instructive. Fed at high levels (1–2%) in the diet, metal ion complexes of NTA led to physiologic and pathologic abnormalities in kidneys and bladder of mice and rats, which, in turn, led to cancer in these organs (*363*, *364*). At low levels, no carcinogenicity occurred. By the operational definition, NTA would be labeled a carcinogen. By using contemporary mechanistic considerations, NTA, a chemical without any evidence of genotoxicity, is not a hazard under conditions of low-level exposure in which chelate formation does not affect the host.

A simultaneous study of NTA and another chelating agent, ethylenediaminetetraacetic acid (EDTA) led to neoplasia in kidney and bladder with NTA but not with EDTA. The MTD for EDTA (7,500 ppm) was lower than that for NTA (15,000–20,000 ppm). The proper conclusion in this instance is not that NTA is more carcinogenic than EDTA but that it is less toxic. Identical considerations hold for the effect by butylated hydroxyanisole (BHA) (MTD of 12,000–20,000 ppm) (*365*) versus that of butylated hydroxytoluene (BHT) (MTD of 4,000–5,000 ppm) (*366*).

Health Risk Assessment

Carcinogens and promoters involved in human cancer induction were first discovered through carefully conducted epidemiologic studies of specialized population groups. The effect of suspect agents was then tested in animals models or in in vitro systems. Newer techniques currently in development use exfoliated cells to ascertain exposure to certain genotoxic materials (*367–70*). Highly sensitive and specific monoclonal antibody techniques may be able to trace carcinogen–macromolecular adducts in human tissues, and hence verify exposure to certain carcinogens (*371–372*). The ideal, of course, in carcinogen testing is to identify human hazards before exposure and thereby prevent it (*373–81*).

The possible results in the decision point approach and the accompanying hazard assessments are summarized in Table II.

If data show that the product is genotoxic and positive bioassay results have been obtained in the relatively rapid animal models, then the relative potency of

Table II

Results in the Decision Point Approach

Stage B: Genotoxicity	*Stage C: Promoting Activity*	*Stage D: Activity in Limited In Vivo Bioassay*	*Stage E: Carcinogenicity*	*Evaluation and Recommendation*	*Risk Assessment*
+				Likely carcinogen; can confirm at Stage D	Caution for human exposure
+		+		Genotoxic carcinogen	Control human exposure
−				Possible epigenetic agent; perform tests for promotion if exposure warrants	Not a qualitative carcinogenic hazard
−	+			Epigenic agent; delineate dose–response and perform chronic bioassay over dose range	May have safe level of exposure
−	+		+	Epigenetic carcinogenic agent	If a no-effect level found, it probably represents a safe level of exposure

the product should be determined in relation to a suitably selected positive control compound and from the dose–response curve.

If in vitro tests failed to disclose evidence of genotoxicity, a preliminary conclusion would be that the substance, at worst, acts indirectly, perhaps as a promoter. In vitro tests for promoters are now being developed, and the chemical ought to be subjected to such tests. In addition, the product should be assessed in rapid in vivo tests with suitable initiation using a genotoxic carcinogen and appropriate positive controls for the suspected target organ so that the relative strength of the agent can be evaluated. An example is the effect of butylated hydroxytoluene in enhancing liver carcinogenesis in relation to phenobarbital or estrogen (*213, 382*).

If the structure and the results in in vitro systems suggest genotoxicity, then limited in vivo bioassays should be used to obtain clear-cut evidence. Positive findings indicate the need to control human exposure.

If the potential human exposure or other criteria have led to a full-scale, chronic bioassay, then a number of points must be considered before drawing conclusions. For instance the range of dosages exhibiting activity in each group of animals should be compared with positive and negative controls. If only two dose levels were used then sound health risk analyses are difficult unless a high incidence of cancer was induced in several species. The various mathematical treatments relative to data evaluation and health risk assessments in the literature (*340–48, 358–64*) have been derived from data on carcinogens now recognized as being genotoxic, and therefore, are applicable only to genotoxic carcinogens and not to epigenetic agents. The shape of the dose–response curve might reveal the possibility of a promoting effect, for example, if activity is seen only at the MTD and much less or no activity at lower dose levels. Under these conditions, a suitable experiment should be designed with an appropriate genotoxic carcinogen as initiator and a broad range of dose levels of the epigenetic promoting agent. Such a study should permit the assessment of the existence of a threshold from which a suitable no-effect level for humans can be derived (*383*). However, the classical statistical calculations designed for risk assessment do not apply to agents acting by nongenotoxic, epigenetic mechanisms. For these compounds, conventional toxicologic dose–response relationships should be used in risk assessment.

For genotoxic carcinogens, evidence shows that they can act by somewhat different mechanisms and are quantifiably quite distinct. For example, four proven carcinogens—(1) acetamide (12,500 ppm; 15 months); (2) safrole (5000 ppm; 12 months); (3) diethylnitrosamine (40 ppm; 6 months); and (4) aflatoxin B_1 (0.015–0.1 ppm; 6–12 months)—all cause liver cancer, but at very different dosages. The last two, especially aflatoxin B_1, are considerably higher risks for humans than the first two chemicals. Different models can produce widely different quantitative estimates of risk. If the available data show that humans are not more sensitive than experimental animals to the chemical carcinogen (*384*), then the simple linear extrapolation can be used to obtain a crude upper limit to the true risk. However, current understanding of the complexities of chemical carcinogenesis and their nonlinear kinetics suggests that departure from linearity and a threshold should exist at low doses (*385*). Indeed, for the more simple process of chemical

mutagenesis in vivo, a drop below linearity at low doses has been demonstrated (*386*). Such facts have recently been taken into account in formulating a more conservative model (*387*) that is appropriate for low-dose extrapolation.

Concluding Remarks

Since the first edition of this monograph, sizable progress has been made in determining the mechanisms underlying carcinogenesis. A key advance was the discovery that the great diversity of chemical structures capable of causing cancer eventually act via an electrophilic reactant; sometimes metabolic activation is needed. Also, further insight has been obtained into the molecular target of genotoxic electrophilic carcinogens, namely DNA, the genetic material in the cell. Researchers have also discovered that DNA with covalently bound carcinogens can be repaired and that some of the observed biological effects, including organotropism, depend as much on such repair processes as on the metabolic activation and interaction with DNA.

The interaction with DNA has yielded the necessary connection to relate mutagenicity to carcinogenicity. This finding has provided a sound basis for utilizing the property of mutagenicity as an assessment of potential carcinogenicity for some chemicals. We have classified chemical carcinogens into two groups: (1) genotoxic agents, and (2) agents capable of increasing cancer risk through mechanisms other than genotoxicity—through epigenetic mechanisms. Most classes of agents operating through an epigenetic mode of action do so in a reversible, highly dose-dependent fashion. Theoretical and also regulatory implications have indicated that epigenetic agents should be treated differently from genotoxic agents. Further research on methods to detect epigenetic agents will provide better classification and control of all types of potentially harmful substances, instead of the blind, blanket indictment of all agents regardless of mechanism. Excellent evidence indicates that currently prevailing human cancers are caused as much by the presence of agents operating via epigenetic mechanisms as by genotoxic carcinogens. Examples are cancer of the lung due to smoking of cigarettes; and cancer of the colon, breast, prostate, and perhaps pancreas due to certain dietary habits, especially the level of fat consumed. Thus, there is hope that these major types of cancer can be controlled by modifying the environment not only with respect to genotoxic carcinogens but also with respect to epigenetic carcinogens, thereby lowering the complex risk factors for these diverse human cancers.

The public is much more aware of environmental cancer risks. There is legislation and regulation at the state and federal level and in international agreements to control possible causes of environmental cancer. Nevertheless, the public, while concerned with the issues, is not well informed. Problems that come to public attention through the press and media are not necessarily those that would be most relevant to the protection of the public. We hope that the application of the methods and principles presented in this chapter will be of value to professionals in the field of carcinogenesis and will result in activities enabling the public to have sound and useful facts for avoiding cancer hazards.

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Environmental Cancer and Heart and Lung Disease

Seventh Annual Report to Congress

1984

Task Force On
Environmental Cancer and Heart and Lung Disease

U.S. Environmental Protection Agency
Public Health Service
- National Cancer Institute
- National Heart, Lung, and Blood Institute
- National Institute for Occupational Safety and Health
- National Institute of Environmental Health Sciences
- National Center for Health Statistics
- Centers for Disease Control
- Food and Drug Administration

Department of Energy
Consumer Product Safety Commission
Occupational Safety and Health Administration
Department of Agriculture

Prepared by the Task Force Working Group
Scott Baker, Chairperson

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TASK FORCE ON
ENVIRONMENTAL CANCER AND HEART AND LUNG DISEASE

Please address your reply to:

JAN 4 1985

William D. Ruckelshaus
Administrator
U.S. Environmental Protection
Agency
Washington, D.C. 20460

Honorable Thomas P. O'Neill, Jr.
Speaker of the House of Representatives
Washington, D.C. 20515

Dear Mr. Speaker:

As Chairman of the Task Force on Environmental Cancer and Heart and Lung Disease, I am pleased to submit the Seventh Annual Report to Congress in accordance with Public Law 95-95, Section 402(b)(5).

During the past year the scope of the Task Force was broadened by the addition of new members: the Consumer Product Safety Commission, the Department of Agriculture, and the Occupational Safety and Health Administration as an observer. The addition of new members increases the opportunities for interaction and ensures that the environmental disease problem is addressed from a variety of perspectives.

This report highlights the Task Force activities during the past year and identifies five specific recommendations for research derived from those activities. Three of the recommendations focus on environmentally related nononcogenic lung diseases, the subject of a Task Force-sponsored workshop. The other recommendations relate to the widely recognized need for better exposure data and the general need for a coordinated, multi-disciplinary approach to medical research.

Although much remains to be learned about the relationship between environmental pollution and human disease, I believe progress is being made. The Task Force on Environmental Cancer and Heart and Lung Disease is an effective mechanism for facilitating interagency cooperation and coordination and is actively contributing to our improved understanding of the environmental disease problem.

Sincerely,

William D. Ruckelshaus
Chairman

Environmental Protection Agency • National Cancer Institute
National Heart, Lung, and Blood Institute • National Institute for Occupational Safety and Health
National Institute of Environmental Health Sciences • National Center for Health Statistics
Centers for Disease Control • Food and Drug Administration
Department of Energy • Consumer Product Safety Commission
Occupational Safety and Health Administration • Department of Agriculture

TASK FORCE ON
ENVIRONMENTAL CANCER AND HEART AND LUNG DISEASE

Please address your reply to:

JAN 4 1985

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Washington, D.C. 20460

Honorable George Bush
President of the Senate
Washington, D.C. 20510

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PREFACE

The Task Force on Environmental Cancer and Heart and Lung Disease is an interagency group established by Congress to promote cooperation and coordination and recommend research through its deliberations to determine and quantify the relationship between environmental factors and human disease as a means of reducing or preventing environmentally related cancer and heart and lung disease. As a result of Task Force consideration and the member Agencies' approval, recommendations have evolved from the needs identified by scientists at Task Force-sponsored workshops.

Section 402 of Public Law 95-95, the Clean Air Act Amendments of 1977, designated the initial members as the U.S. Environmental Protection Agency (EPA); and from the Public Health Service (PHS) of the Department of Health and Human Services, the National Cancer Institute (NCI), the National Heart, Lung, and Blood Institute (NHLBI), the National Institute of Environmental Health Sciences (NIEHS), and the National Institute for Occupational Safety and Health (NIOSH-Centers for Disease Control). Seven other Government organizations have joined the Task Force by invitation: three PHS Agencies, the National Center for Health Statistics (NCHS), other components from the Centers for Disease Control (CDC), and the Food and Drug Administration (FDA); the Department of Energy (DOE) in 1983; and the Consumer Product Safety Commission (CPSC), the Occupational Safety and Health Administration (OSHA) of the Department of Labor, and the Department of Agriculture (USDA) in 1984.

To meet its reporting requirements to Congress, this report describes the accomplishments of the Task Force during its seventh year, September 1983 to August 1984. Chapter 1 introduces the Task Force and its various subgroups. Chapter 2 describes the substantive activities of the Task Force during its seventh year, and Chapter 3 outlines planned activities for the eighth year. Recommendations are contained in Chapter 4.

CONTENTS

CONTENTS (Concluded)

EXECUTIVE SUMMARY

The Task Force reports on its activities yearly to Congress. During its seventh year, the Task Force activities were focused in the following areas:

- Exposure assessment and mechanisms of toxicity
- Environmental health professional education
- Quality assurance in analytical methodology
- Legal impediments to epidemiologic research.

Based on the findings and conclusions developed by scientists participating in meetings sponsored by the Task Force, the following recommendations for research are offered to the Congress for its consideration:

1. Research should be supported to develop approaches and methods for assessing human exposure to environmental pollutants and the dose of pollutants to tissues.
2. Methods are needed to identify cellular, molecular, or immunologic markers that might aid in detection, prognosis, and treatment of environment-related respiratory diseases.

3. Research is needed on the relationship between acute respiratory responses and the development of chronic, irreversible, environmentally related nononcogenic lung disease.

4. Basic studies of the cellular, molecular, and genetic events that may contribute to the pathogenesis of respiratory diseases should be undertaken.

5. A coordinated, multidisciplinary approach, involving all the tools of medical research, is necessary to elucidate the relationship between environmental factors and human disease.

The Subcommittee on Exposure, together with the Scientific Liaison Staff of the Food and Drug Admintration, published several papers that were presented at the Task Force-sponsored symposium, Exposure Assessment: Problems and Prospects.

The Subcommittee on Metabolism completed its draft report, "Strategies for Determining the Mechanisms of Toxicity." The report describes strategies for using mechanistic approaches to study problems of toxicity.

The Interagency Education Program Liaison Group produced an annotated bibliography of materials on occupational and environmental health available from Federal organizations. The information was compiled to assist health professionals in identifying and selecting appropriate materials for the clinical management of environmentally related disease.

The Project Group on Quality Assurance in Analytical Methodology prepared an inventory of standard reference materials that are used to ensure the reliability of chemical analytical results. The Group

also sponsored a seminar on Laboratory Quality Assurance--A Management Approach. The seminar addressed the role of the laboratory manager in quality assurance, complex managerial problems, and management concepts in statistics, measurements, and sample analysis.

The Working Group's assessment of the legal impediments to conducting epidemiologic research was completed. The study findings indicate that

- Legislative changes have not occurred to significantly modify existing privacy and confidentiality laws or restrictions on use of Agency data
- Several Federal Agencies are exploring ways to increase access to Federal data and allow record linkages of specifically defined data elements for approved epidemiologic research projects
- Resource-use data are not available to quantify the added costs of current research impediments
- Even if legal and administrative impediments are relaxed, information processing barriers and limitations to the most efficient use and linkage of existing Federal data may continue to hamper the conduct of epidemiologic research studies.

The Task Force is planning a Workshop on the Contribution of Airborne Pollutants to Respiratory Cancer as a means of assessing research to determine and quantify the relationship between environmental factors and human disease and to develop strategies, research, and other measures to reduce the risk.