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The Distinct Health Risk Analyses Required for Genotoxic Carcinogens and Promoting Agents

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Health risk analysis needs to apply newer developments in the understanding of the underlying mechanisms of the carcinogenic process which has allowed for the classification of chemical carcinogens into those that damage genetic material directly (genotoxic carcinogens) and those that operate by indirect or epigenetic mechanisms. We propose a systematic decision point approach for detecting and evaluating substances for carcinogenic risk. This approach recognizes that genotoxic and epigenetic agents operate by different mechanisms and distinguishes between these two categories of carcinogens primarily on the basis of results in a battery of short-term tests that includes systems which reliably detect genotoxic carcinogens and others which may respond to epigenetic agents. Genotoxic carcinogens at very low dosages may have practical, effective threshold no-effect levels, but, nevertheless, because of their mechanism of action they are regarded as a qualitative hazard. The action of epigenetic agents of the promoter class is highly dose-dependent and reversible, and thus, a distinctively different health risk analysis is required for these agents to take account of their quantitatively lesser hazard.

Introduction

Substantial progress towards an understanding of the risk factors for specific kinds of cancer and the underlying mechanisms of carcinogenesis has taken place in the last twenty years (*1-3*). This knowledge of the nature of the carcinogenic process has led to the recognition that chemicals which produce tumors in animals may do so by a variety of modes of action.

Chemical carcinogens have been divided by Weisburger and Williams (*4*) into two broad categories based upon their ability to damage genetic material, or the lack thereof, and then further divided into a total of eight subgroups (Table 1). A corollary of this classification is that one type of health risk analysis is appropriate for genotoxic carcinogens that damage DNA, because of their specific mechanisms of action, whereas agents that operate by indirect nongenotoxic means require a different type of evaluation (*5, 6*).

Genotoxic agents undergo a series of competing reactions, ultimately reacting with DNA which appears to be the critical event in carcinogenesis (Fig. 1). Once cell duplication with the so-generated abnormal DNA has occurred, the effect is basically irreversible. In contrast, the action of agents operating by epigenetic mechanisms, which are as yet unclear and require much more research, usually necessitates their presence at high levels for a long time and, indeed, is reversible up to a certain point. Moreover, in many cases, their action is also tissue-specific. For example, bile acids are powerful promoters of colon cancer but act as inhibitors when tested in the classic mouse skin system (*7, 8*). As another example, there is sound evidence that saccharin belongs to the category of epigenetic agents and acts as a promoter for cancer of the urinary bladder (*9-11*). The many attempts to use standard techniques of health risk analysis for saccharin have yielded controversial results simply because such techniques do not apply to this type of agent. In fact, new procedures to define the mode of action of nongenotoxic or epigenetic agents need to be developed for better risk evaluation.

This new understanding of the mechanisms of carcinogenesis and the classification of chemical carcinogens has been made the basis of a rational, sequential system for evaluating the carcinogenic po-

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Table 1. Classes of carcinogenic chemicals.

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

tential of chemicals (*12*). The key aim is to detect substances posing potential health risks, as well as to acquire knowledge as to which health risks are associated with which kinds of cancer, via the simplified, accelerated, more economical, and more reliable means afforded by the systematic decision point approach. This system will be delineated as a foundation for a discussion of elements required for health risk analysis, itself an essential step in the prevention of cancer.

The Decision Point Approach

The decision point approach involves five sequential steps, A-E, in evaluating the potential carcinogenicity of a chemical (Table 2).

This approach was formulated to incorporate several newer developments in carcinogenesis into the evaluation of the toxic effects of a chemical. Of prime importance was the concept that some chemicals could produce an increase in the tumor incidence in exposed animals, i.e., be carcinogenic, by several distinct mechanisms, each having different theoretical and practical implications. One of the mechanisms proposed (*3*) involves the formation of an electrophilic reactant which would react covalently with cellular macromolecules. Work in several laboratories (*2-4, 13, 14*) has strongly indicated that DNA is in fact the critical cellular target. Other chemicals, however, do not react covalently with DNA but are nevertheless carcinogenic or oncogenic in some animal bioassays. We, therefore, suggested that chemical carcinogens could be divided into two main categories based upon their capacity to damage DNA.

Table 2. Key steps in decision point approach to delineate possible carcinogens or promoters.

	Step
A	Structure of chemical
B	Battery of *in vitro* short-term tests
	1. Bacterial mutagenesis
	2. Mammalian cell mutagenesis
	3. Mammalian cell DNA repair
	4. Mammalian cell chromosome effects
	5. Mammalian cell transformation
C	Limited *in vivo* bioassays
	1. Skin tumor induction in mice
	2. Lung tumor induction in mice
	3. Breast cancer induction in rats
	4. Induction of altered foci in rodent liver
	5. Tests for promoting effects
D	Chronic bioassay
E	Final evaluation

Carcinogens that react covalently with DNA are categorized as genotoxic, while those lacking this property and probably acting by other mechanisms, are designated as epigenetic. The genotoxic category contains the classic organic carcinogens that damage DNA either through direct chemical reactivity or following metabolic activation by specific enzyme systems (Fig. 1). In addition, in view of some evidence for DNA damage or alteration, inorganic carcinogens were tentatively placed in this category (*15*).

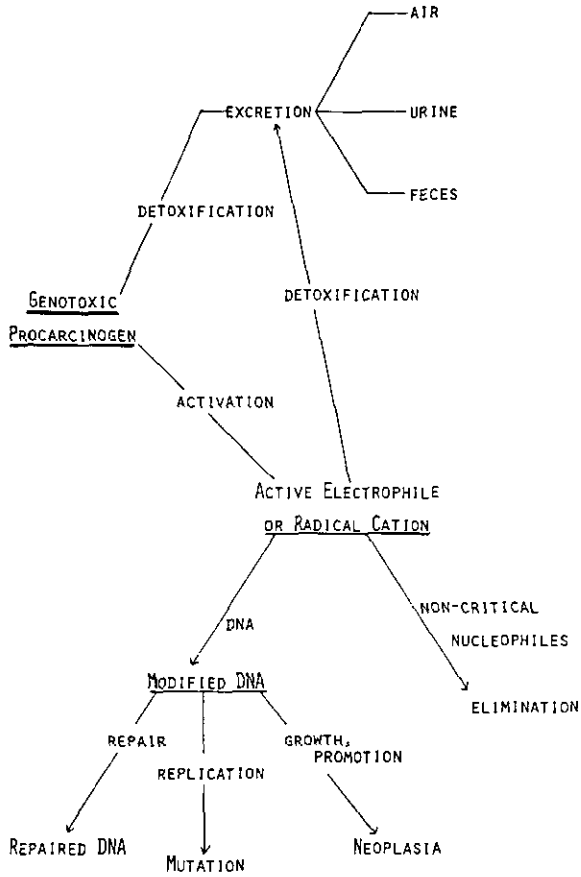

FIGURE 1. Diverse reactions of genotoxic carcinogens. A procarcinogen undergoes biochemical host-mediated activation to ultimate carcinogen, a reactive electrophile or radical cation. Usually this activation step involves only a small portion of a dose of procarcinogen, most of which is detoxified by specific biochemical reactions, leading to excretion. The potency of a carcinogen depends on the ratio of activation/detoxification, itself a function of host and environmental variables such as species, strain, sex, age, diet, enzyme modifiers, and the like. The ultimate carcinogen can also be detoxified or react with cellular macromolecules not associated with carcinogenesis or mutagenesis. Thus, only a minute fraction of the initial dose of procarcinogen is usually productively involved in the complex processes yielding neoplasm.

In contrast, the second category, epigenetic carcinogens, is composed of those agents that have not been found to damage DNA but rather appear to act through other indirect mechanisms. This category contains several classes of agents such as plastics, cytotoxic agents, hormones, immunosuppressants, cocarcinogens and promoters, that operate by distinctly nongenotoxic mechanisms.

The decision point approach distinguishes between these two categories of carcinogens by testing in a battery of short-term tests that includes systems which reliably detect genotoxic carcinogens as well as others which may respond to epigenetic agents. Implicit in this approach is that fact that all forms of subchronic testing may fail to detect substances which can induce tumors in animals under specific conditions upon chronic administration. The battery of short-term tests may either eliminate the need for further testing of the chemical or may enable the verification of carcinogenic potential in one of four limited *in vivo* bioassays for carcinogenicity. The battery also adds essential information for data evaluation and risk analysis when an already completed chronic bioassay has yielded ambiguous results.

The decision point approach, therefore is a systematic approach to the reliable evaluation of potential carcinogenicity that provides a framework in which to minimize the necessary testing for evaluating a chemical, without loss of the capacity for acquiring essential information from which to draw correct conclusions. At the same time, the system through which data are collected provides an understanding of the mechanism of action of a chemical that is essential to reliable risk extrapolation.

The decision point approach involves a systematic stepwise progression of tests through five stages. At the end of each phase, a critical evaluation of the information obtained and its significance in relation to the testing objective is performed. A decision is made as to whether the data available are sufficient to reach a definitive conclusion or whether a higher level of tests is required. Attention is paid to qualitative—yes or no— answers, and to semiquantitative—high, medium or low—effects. The following outline has been described in detail with full literature citation (*5*, *12*).

Structure of Chemical

The evaluation of potential carcinogenicity begins with a consideration of structure. Predictions as to whether or not a given chemical might be carcinogenic can be made with some success within certain classes of chemicals, especially in the case of chemicals of a type that includes known carcinogens. For example, within the extensive series of azo dyes, Miller and Miller, as well as Yoshida, Kinosita, Druckrey, and Schmähl, have provided extensive data on carcinogenicity as a function of structure. Carcinogens of this type often have amino groups in the para position of a benzene ring. Inclusion of relatively polar substituents such as sulfonic acid abolishes carcinogenicity. On the basis of this knowledge alone, it is not likely that pure FD & C Red No. 2 or FD & C Red No. 40, which bear such deactivation substituents on both sides of the azo bond, would be carcinogenic, and they have been found

not mutagenic. On the other hand, more complex tetrazo dyes that include a potentially carcinogenic benzidine residue, available on biochemical reduction, are carcinogenic. Within the arylamine type of chemicals, *ortho*-substituted (next to the vicinal ring) polynuclear arylamines such as 1-naphthylamine or 1-fluorenylamine are not carcinogenic, whereas the 2-isomers are powerfully active in rodents, and 2-naphthylamine is active also in humans. This is because the 1-isomers do not undergo the required metabolic activation reaction of *N*-hydroxylation to any significant extent, mainly because they are rapidly detoxified by ring hydroxylation and type II conjugation reactions.

Structure must always be consideed in relation to species metabolic parameters. The guinea pig, for example, in contrast to rodents or man, has only limited amounts of the necessary enzymes needed to carry out *N*-hydroxylation and thus yields detoxified metabolites almost exclusively. Therefore, the arylamines so far tested are not carcinogenic in this species. Other examples of species selectivity based upon metabolic capability are well documented. More data are needed on the metabolic activation and detoxification of important new heterocyclic carcinogens or aliphatic carcinogens such as ethylene dibromide or 2-nitropropane. Data dealing with structure and metabolism also yield a guide to the selection among limited bioassays at stage C (see below) and, as more information accrues, may contribute to the choice of specific short-term tests at stage B.

Battery of *in Vitro* Short-Term Tests

Current views are that critical decisions can be made only with a battery of such assays for toxicological evaluations. The key element in the design of an appropriate battery of tests is the formulation of relevant criteria for selectivity of the best, most effective, and economical combination of tests. Also, since testing schemes have become exceedingly complex and expensive, it is important to reduce the number of tests to an essential core without loss of necessary information.

No decision should be made regarding the potential risk attached to chemical exposure by any route until the entire data base is available from a battery consisting of a group of selected tests. Implicit in this philosophy is the concept that all available short-term tests may yield false-positive or false-negative results that require parallel data for proper interpretation.

The selection of which tests will constitute a battery depends in part upon whether the goal is to detect potential mutagens or carcinogens. Little is known about the validity of mutagenicity batteries because few chemicals have been shown to be germ cell mutagens in experimental animals, and no chemicals are known now that produce human germinal mutations. Therefore, it would seem prudent to design such batteries so as to identify the broadest possible spectrum of genetic damage. In contrast, carcinogenicity batteries can be verified against *in vivo* data, albeit with an important qualification. As indicated, evidence now supports the concept that carcinogens operate by a variety of mechanisms. Among these, DNA damage and its biological consequences such as mutagenesis can be readily detected in short-term tests. It is important to realize that other oncogenic mechanisms of a nongenetic nature are clearly not detectable in tests with such genetic endpoints. Some tests such as malignant transformation and sister chromatid exchange, the results of which seem in some cases to be produced by effects other than a direct attack on DNA, may be capable of detecting non-DNA- damaging agents. In addition, efforts are being made to develop *in vitro* tests for identifying agents that operate as tumor promoters (*16-19*).

As yet, however, none of the approaches for nongenotoxic chemicals is sufficiently validated for route inclusion in a battery. Therefore, in using batteries to identify carcinogens, it must be recognized that an entire class of chemicals containing such agents as saccharin, hormones, certain organochlorine compounds and pesticides and several pharmaceuticals may not be detected.

Several other principles should guide the construction of a battery. Importantly, the tests should be reliable and of clear biologic significance, and the battery should seek to maximize the metabolic parameters provided by all tests. In particular, tests with intact cell metabolism should be included to extend the metabolic competence obtained with the commonly used exogenous enzyme preparations. This may be of particular importance in view of the differences in metabolism between subcellular and cellular systems, in particular, the artifactual enhancement of activation over detoxification for certain classes of compounds that is known to be characteristic of subcellular fractions and enzyme preparations.

Adhering to these concepts, a battery of short-term tests was proposed by Weisburger and Williams (*4,12,20*) as part of the "decision point approach." This battery includes a microbial mutagenesis test, because, thus far, such tests have been the most sensitive, effective, and readily performed screening tests. In deciding what other tests to in-

clude, it is important to consider what the candidate test could contribute in terms of metabolic capability and reliability and biologic significance of the endpoint. The bacterial mutagenesis tests require a mammalian enzyme preparation to metabolize procarcinogens, and hence, any other test that is dependent upon such an enzyme preparation does not expand the battery's metabolic capability, which is usually the key limiting component of a test series. Thus, tests utilizing other indicators of DNA change, but the same enzymic activation system, may be similar in their capability simply because their limitations are inherent in the metabolic properties. Such redundancies should be avoided by using whole cell systems where possible.

Mutagenesis of mammalian cells is included in the battery because it has a definitive endpoint, as has bacterial mutagenesis, but involves effects on the more highly organized eukaryotic genome. Moreover, differences in the mutagenic response between microbial and mammalian cells have been observed.

DNA repair is a specific response to DNA damage and, unlike other indicators such as DNA fragmentation and inhibition of DNA synthesis, cannot be attributed to toxicity. Therefore, a DNA repair test offers an endpoint of high specificity and biologic significance. Moreover, the DNA repair test of Williams using intact hepatocytes provides a whole cell system in the battery.

A chromosomal test is included in the battery to detect chemical effects at the highest level of genetic organization. Sister chromatid exchange is currently preferred because of its greater sensitivity compared to chromosome aberrations.

Cell transformation is included as an optional part of the battery because this alteration is potentially the most directly relevant to carcinogenesis. Moreover, certain chemicals, without any other evidence of genotoxicity, have yielded a positive response in cell transformation, and thus this endpoint may be sensitive to epigenetic agents. However, reliable transformation assays are not widely available, and more experience is needed to clarify the significance and limitations of this endpoint.

Decision Point 1: Summary of Rapid *in Vitro* Tests

In summary, these include tests for: (1) bacterial mutagenesis, (2) mammalian mutagenicity tests, (3) DNA damage, (4) chromosome effects, (5) cell transformation. There are detailed reviews for the individual tests of each type in the battery (*21-23*).

The steps recommended thus far, namely structure-activity relations and a sequence of rapid *in vitro* tests, provide a basis for preliminary decision making. A survey of literature data on the application of the recommended tests has revealed a high degree of sensitivity and specificity for this battery.

If clearcut evidence of genotoxicity in more than one test has been obtained, the chemical is highly suspect. Confirmation of carcinogenicity may then be sought in the limited *in vivo* bioassays. This sequence avoids the necessity of resorting to the more costly and time-consuming chronic bioassay, probably without loss of capability to reach reliable conclusions.

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 not reliably carcinogenic. Also, positive results have been obtained with synthetic phenolic compounds or natural products with phenolic structures like flavones. *In vivo*, such compounds are likely to be conjugated and excreted readily. Their carcinogenicity, thus, would depend on *in vivo* splitting of such conjugates, which may occur more readily in laboratory rodents than in man. Therefore, positive evidence of bacterial mutagenesis only must be evaluated in the light of the chemical structure and metabolism. Similar caution is required when the sole evidence rests on tests for SCE or cell transformation. Chemicals that are not obviously genotoxic by other criteria have sometimes yielded positive results in one or the other of such tests.

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., hormone) 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 direct obvious guidance on the proper course of action. Organic chemicals with structures suggesting possible sites of activation may reveal their carcinogenicity in limited *in vivo* bioassays. On the other hand, chemicals such as solid-state materials, hormones, possibly some metal ions, and organochlorine compounds that are negative in tests for genotoxicity operate by complex and as yet poorly understood mechanisms, which in many cases appear to involve tumor-enhancing effects. Thus, it is not certain that the limited *in vivo* bioassays would yield any positive results with such materials. Therefore, either specific promotional assays or the standard chronic bioassay are necessary at this time to detect any potential activity with these agents in relation to realistic human exposure conditions. It is indeed urgent to develop reliable means to detect such materials readily without re-

quiring the large investment associated with a chronic bioassay, especially if large numbers of people have potential exposure to more than trace amounts.

The testing of metal ions in rapid bioassay tests may take advantage of the concept proposed by Loeb (*15*) that such ions affect the fidelity of enzymes concerned with DNA synthesis. Obviously, the nature of the metal ions, of which there are only a limited number, would provide the necessary insight as to the need for testing such a material further and as to what kind of assay would most likely reveal adverse effects.

Compounds with hormonelike properties other than the strict androgen and estrogen types do exist. Such chemicals are potential cancer risks mainly because they interfere with the normal physiological endocrine balance (*24*). More research on ways and means to quickly test for such properties is required. It is known for example, that certain drugs lead to release of prolactin or other hormones from the pituitary gland. Chronic intake of such drugs causing a permanently higher serum and tissue peptide hormone level might, in turn, alter the relative ratio of other hormones. At this time, any material with such properties needs to 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 needs to take into account the normal diurnal, monthly and even seasonal cycles of the endocrine system and whether the test would have led to interference in this balanced, rhythmic system. It is essential to consider dosage and include in any bioassay a number of dose levels, including any prevailing environmental or proposed use levels.

The potential of polychlorinated cyclic hydrocarbons to act as promoters in the production of liver tumors has been discussed in detail (*25*). As yet, the structural requirements for promoting activity are poorly understood, outside the class of phorbol esters, and these promoting agents can be identified only in initiation-promotion protocols in limited *in vivo* bioassays or in chronic bioassay. New *in vitro* systems for the detection of promoters (*18,19,25*) may be promising also to delineate the effects of such chemicals.

The implications of the absence of convincing data for genotoxicity, but a positive response in chronic bioassays, are discussed under the final evaluation.

Limited *in Vivo* Bioassays

This stage of evaluation employs tests that will provide further evidence of potential hazard of chemicals positive for genotoxicity in the battery of *in vitro* tests without the necessity of undertaking an extensive chronic bioassay.

Thus, the *in vivo* tests recommended are those that will provide definitive evidence of carcinogenicity, including cocarcinogenicity and promotion, in a relatively short period (i.e., 30 weeks or less). Unlike the *in vitro* tests, these are not applied as a battery, but rather used selectively according to the information available on the chemical. A positive response is a significant finding, especially for agents that are genotoxic by the criteria defined above. On the other hand, a negative finding in the limited *in vivo* bioassays does not signify safety.

Skin Tumor Induction in Mice

The carcinogenicity of certain chemicals and crude products can be noted readily upon continuous application to the skin of mice, producing papillomas or carcinomas, or upon subcutaneous injection, yielding sarcomas. Also, activity as initiating agents can be rapidly determined by the concurrent or sequential application of a promoter, such as one of the phorbol esters. Tars from coal, petroleum, or tobaccos are active in such systems, as are 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 resulting in initiation, especially in the presence of cocarcinogens or promoters in the crude products. On the other hand, such mixtures rarely yield visceral tumors such as those in the liver, mainly because the liver can detoxify these chemicals quickly. However, lung and lymphoid tumors in sensitive mouse strains can be secondary tumor sites.

Mouse skin is useful, primarily, therefore, for chemicals such as polycyclic hydrocarbons, and also direct-acting chemical carcinogens such as sulfur or nitrogen mustard, bis(chloromethyl) ether, propiolactone, and alkylnitrosoureas.

Pulmonary Tumor Induction in Mice

Andervont and Shimkin pioneered with the model involving the development of lung tumors in specific sensitive strains of mice, especially the A/Heston strain and related strains like A/J (*1,12*). A singular advantage of this assay system is that, in addition to an endpoint measuring the percent of animals with tumor compared to controls, the multiplicity of tumors is an additional parameter expressing the "strength" of any carcinogenic action. Most chemicals that are active in this system are also carcinogenic in other longer, chronic animal tests. Sig-

nificant results are obtained in as short a time as 30-35 weeks, and sometimes faster.

Breast Cancer Induction in Female Sprague-Dawley Rats

Shay discovered, and Huggins extended, the finding that polycyclic hydrocarbons rapidly induced cancer in the mammary gland of young female random-bred Wistar rats and, to a greater degree, in Sprague-Dawley rats (*1,4*). With powerful carcinogens, especially select polycyclic hydrocarbons, arylamines, or nitrosoureas, a positive result is obtained in less than nine months. The multiplicity of mammary tumors provides an additional quantitative criterion. As with lung tumors in mice, a positive response in this system has usually been confirmed in other animal bioassay models. A negative response, however, does not prove lack of potential carcinogenicity.

Altered Foci Induction in Rodent Liver

In the first version of the decision point approach, production of rodent liver tumors was proposed for limited *in vivo* bioassay. This concept is still valid, but in recent years research in a number of laboratories has established that during liver carcinogenesis several distinct hepatocellular lesions precede and are related to the development of carcinomas. The earliest of these, the altered focus, when sufficiently developed can be demonstrated in routine histologic tissue sections. However, altered foci are abnormal in a number of properties that permit their reliable and objective identification at early stages by more sensitive techniques. Altered foci in rat liver display abnormalities in the enzymes γ-glutamyl transpeptidase (GGT), glucose-6- phosphatase, and adenosine triphosphatase which have been used for their histochemical detection. Another important marker for foci that permits histochemical identification is their resistance to iron accumulation. This latter property is more sensitive than the enzyme abnormalities and also, unlike the enzyme abnormalities, characterizes hamster and mouse liver lesions. Thus, the induction of altered foci in rodent liver can be used as a limited bioassay.

With known carcinogens, foci have been detected as early as with three weeks of carcinogen exposure, and in high numbers by 12 to 16 weeks exposure. Therefore, the recommended approach is that of 12 weeks exposure to the test chemical with injection of subcutaneous iron during the last two weeks to produce the iron load that delineates the foci resistant to iron accumulation. The multiplicity of the foci can be used for quantitative, or at least semiquantitative estimation of relative potency, when tests have been conducted under controlled identical conditions. A positive control serves as a standard reference point. Few carcinogens have yet been submitted to this technique, but based upon current knowledge of the pathogenesis of liver cancer, this is anticipated to be a highly reliable test for liver carcinogens. Since the liver is the target for so many carcinogens because of its metabolic capability (*24*), this test should possess substantial sensitivity.

Tests for Promoting Effects

In the absence of genotoxicity, it is possible to test for one type of epigenetic effect in limited *in vivo* bioassays, namely for promoting activity. For example, mouse skin initiated with small doses of genotoxic carcinogens such as benzo(a)pyrene or 7,12-dimethylbenz(a)anthracene responds readily to certain tumor promoters. A material exhibiting endocrine properties likewise may show an effect in modifying breast cancer induction in animals given limited amounts of methylnitrosourea as an initiating dose. Similarly, promoters of urinary bladder cancer may be discovered by pretreatment with limited amounts of a carcinogen specific for the bladder (*10,11,26*). Similar systems can be developed for virtually all organs, although one of potential general utility is the liver system (*27*).

Decision Point 2: Summary of Limited *in Vivo* Bioassays

The presence of two positive results in a battery of rapid *in vitro* bioassay tests reliably indicating genotoxicity, and also, a definite positive result in the limited *in vivo* bioassays would make a substance highly suspect as a potential carcinogenic risk to humans. This is true especially if these results were obtained with moderate dosages; more so, if there was evidence of a good dose response, particularly as regards the multiplicity of the lung or mammary gland tumors.

Proven activity in more than one of the limited *in vivo* bioassays may be considered unequivocal, qualitative evidence of carcinogenicity. A wide variety of structural types of chemicals are active in one or more of these systems.

Chronic Bioassay

In the decision point approach, chronic bioassay is used to confirm questionable results in the more limited testing, or as a last resort measure to test compounds negative in the preceding stages of testing but where extensive human exposure is likely,

or to acquire data on possible carcinogenicity through epigenetic mechanisms. In this last situation, multispecies and dose response data are most important if the data are to be applied to risk assessment. Likewise, in this instance, if such an agent is suspected of affecting a specific organ, much time and expense can be saved by a short course with a genotoxic carcinogen for that organ, preceding the test of the epigenetic agent.

Chronic bioassays, especially at three to five dose levels, are extraordinarily expensive. For this reason, and in view of the fact that mice are more likely to exhibit positive responses with agents operating through epigenetic mechanisms, it may be preferable to initiate such studies on dose response only in mice. However, in the rare case where both mice and rats are used, it will still be important to utilize sufficient numbers of animals and dose levels to enable the delineation of a dose-response effect. In this instance, it will be important to have the dose levels sufficiently spaced. One recommendation might be to determine the maximally tolerated dose and to use that plus lower doses such as 1/3, 1/9, 1/27, and 1/81, thus essentially covering two log units. With compounds where a human exposure level can be estimated, it may be useful to include that level, and possibly three times that level, as part of such a study. The conduct of chronic bioassay has been described in a number of review articles (*1,4*).

An important point is the estimation of the maximally tolerated dose in a subacute or prolonged study. With relatively toxic compounds, the maximally tolerated dose will readily be found in subchronic studies. With compounds that are not highly toxic, arbitrary dose levels should be selected which are consonant with the expected human exposure. Thus, where humans are likely to have a relatively low exposure rate, an arbitrary top dose of 0.5 or 1% in the diet or equivalent amounts through other routes of intake would be sufficient. Where humans might consume relatively large amounts, a proportionately higher arbitrary top dose should be selected.

In multidose tests, consideration should be given to utilizing larger numbers of animals at the lower dose levels so as to have better statistically valid comparisons between the controls and the lower-dose experimental groups where a lower response would be expected to ensue.

Decision Point 3: Final Evaluation

If the decision point approach has led to a chronic bioassay, then fairly definitive data on carcinogenicity would be obtained. However, the results of the *in vitro* short-term tests must be considered in the evaluation of possible mechanisms for action and risk extrapolation to humans. Convincing positive results in the *in vitro* tests, coupled with documented *in vivo* carcinogenicity, permit classification of the chemical as a genotoxic carcinogen.

Genotoxic carcinogens share a number of properties, including: the ability under some circumstances to be effective as a single, large dose; cumulative effects; and synergism, or at least additive effects with other genotoxic carcinogens. Genotoxic carcinogens, therefore, represent clear qualitative hazards to humans, and the level of exposure permitted must be rigorously evaluated and controlled. Along those lines, no distinction should be made between naturally occurring and synthetic carcinogens. In fact, there is growing evidence that the majority of human cancers stem from exposure to the former type of agents (*28-30*).

If, on the other hand, no convincing evidence for genotoxicity is obtained, then the possibility exists that the chemical is an epigenetic carcinogen. The strength of this conclusion depends upon the relevance of the *in vitro* tests. For example, the finding that certain stable organochlorine pesticides do not display genotoxic effects in liver cell systems, which are identical to the *in vivo* target cell for these carcinogens, strongly supports the interpretation that these carcinogens may act by epigenetic mechanisms. The nature of these mechanisms is poorly understood at present, is probably quite different for different classes of carcinogens, and may involve chronic tissue injury, immunosuppressive effects, hormonal imbalances, blocks in differentiation, promotion of pre-existing altered cells, or processes not yet known. A large number of carcinogens are now known or suspected to exert their oncogenic effects through promoting activity and therefore, the limited *in vivo* bioassay and *in vitro* systems for promoters are of increasing importance. Most types of epigenetic carcinogens share the characteristic of being active only at high, sustained doses, and up to a certain point, their effects may be reversible. Thus, this type of carcinogen may represent only quantitative hazards to humans, and safe levels of exposure may be established by carrying out proper toxicologic dose-response studies.

Quantitative Aspects and Health Risk Analysis

The preceding stepwise decision point approach leads to a qualitative "yes or no" answer to the question of whether a given substance or a mixture, as it might occur naturally or as a result of industrial operations, constitutes a potential cancer risk. The sequence of steps furthermore provides an indi-

cation of possible mechanisms of action as regards the important question of whether the substance has genotoxic properties or whether it participates in the overall complex sequence of steps involved in cancer causation through nongenotoxic, epigenetic actions. It is apparent from the modern conceptual development presented in this review that a distinction between substances that are genotoxic and those that are not is of key importance.

However, with each class, genotoxic carcinogens and epigenetic agents, distinctions can and should be made in relation to quantitative aspects. With respect to genotoxic carcinogens, animal experiments performed under similar conditions reveal that dietary intake of 100 ppb of aflatoxin B_1 leads to the high incidence of hepatocellular carcinoma in rats in less than a year—and indeed this chemical can induce liver cancer at levels as low as 1 ppb—while, in contrast, safrole at dietary levels of 5000 ppm yields liver cancer after a longer latent period. It is obvious that the health risk analysis for two such chemicals requires a different perspective in which exposure to aflatoxin B_1 would be viewed as a greater risk than safrole.

With agents that operate via nongenotoxic epigenetic mechanisms, current concepts show that the properties of such agents are quite different from those that operate by genotoxic mechanisms. First of all, their action appears to be reversible, so that intermittent exposure of animals or humans does not appear to constitute a risk if the intervals between exposures are sufficiently long for reversal of effects. Indeed, if the action mimicks that of normal diurnal or otherwise periodic cycling, as is true for hormones, it may actually stabilize the physiological system at specific dose levels. For example, low doses of hormone, as in some current formulations of oral contraceptives taken to mimic the rhythmicity of the normal menstrual cycle, may serve to regularise the physiological hormonal pattern, thus, approximating the optimal endocrine pattern. Thus, physiologic doses given within a physiologic rhythm are not likely to present abnormal risks. On the other hand, higher dosages or continuing exposures via long-lasting implants or injections most likely would be a risk since endocrine balances would be upset.

While classic promoters for mouse skin such as phorbol esters (the active ingredients of croton oil) have been studied for many years, it is unfortunate that there are few quantitative data on the effect of other epigenetic agents potentially involved in the carcinogenic process. Thus, there have not been many carefully conducted dose-response studies or investigations dealing with the functioning of such agents. As noted previously, this is a gap that needs to be filled. Of great relevance and practical importance, are studies on the precise mechanism of action for each kind of promoter as regards the organotropism and the molecular events associated with each kind of agent, whether they be hormones with effects on the endocrine system, bile acids as they affect the intestinal tract, or pesticides, drugs, artificial sweeteners or even the essential amino acid L-tryptophan, as they might affect certain other organs such as the liver or the urinary bladder.

Health Risk Analysis for Genotoxic Carcinogens

There have been relatively few dose-response studies with diverse genotoxic carcinogens. The classic study of Bryan and Shimkin (*31*) has been utilized by mathematical statisticians to formulate the theoretical shape of dose-response curves, especially in extrapolations to low level effects. The experimental design involved the subcutaneous injection of three polycyclic aromatic hydrocarbons in mice with the evaluation based on the detection of sarcoma at the point of injection. With all three hydrocarbons, the dose-response curve assumes an S-shaped pattern typically seen in virtually all pharmacological dose-response studies. In pharmacology, it is possible with such curves to readily determine a no-effect level. Indeed, the actual data in the report by Bryan and Shimkin show that with the three carcinogens there were several doses at the low end which yielded no increase in tumors. Nonetheless, because of the possible errors involved and the relatively small number of animals used, mathematical theory suggests that the overall response might involve a one-hit linear model with no threshold (*32,33*). Over the last 30 years, this experiment has been interpreted in a number of different ways, and it is the formulation of Cornfield (*34*) that seems to approximate the actual experimental values best.

Health risk analysis, in our opinion, needs to take into account the possible mechanisms of action of carcinogens, especially procarcinogens, that require metabolic activation and undergo detoxification not only at the level of the procarcinogen itself but also of the reactive electrophile, the ultimate carcinogen. Indeed, the latter can undergo reaction with nonspecific cellular nucleophiles. Even DNA itself or the chromosomal apparatus as a whole may not uniformly react at significant points with regard to cancer induction. Thus, from the point of view of the long sequence, starting with administration of a procarcinogen leading to the specific cancer-inducing molecular interaction, there are many nonproductive side reactions which leads us to suggest that at very low dosages even with genotoxic

agents there may indeed be practical, effective threshold no-effect levels.

A number of more recent experiments, involving more readily absorbed and excreted carcinogens, in contrast to the polycyclic hydrocarbons used by Bryan and Shimkin, that remain at the site of application for a long time, point even more to the possibility that there might be no-effect levels.

In the large-scale study conducted by the National Center for Toxicological Research (NCTR) involving dosages ranging from 150 ppm to 30 ppm of *N*-2-fluorenylacetamide (or 2-acetylaminofluorene), fed to mice, two organs were affected: the urinary bladder and the liver (*35*). With tumors in the urinary bladder, a definite no-effect level was found. However, for liver tumors, especially for the groups of animals that remained alive through 33 months, a straight-line response was calculated from 150 to 30 ppm. It was further suggested that this response corresponded to a linear model going through zero. The experimental design aimed for a 1% response, and for that reason large numbers of animals were used at levels of 30, 40 and 45 ppm; as might be expected, the response of this complex biological system was unable to discriminate among such closely spaced doses. An intriguing question is what the response might have been if the spacing of doses at the lower end were greater and went, for example, to 3 or even 1 ppm. We believe such levels would have shown no appreciable effect even in large samples. Such exposure levels to this chemical would have been readily measurable chemically (*36*) and correspond to human exposures which have been the subject of concern.

Recently, a large-scale, dose-response experiment was conducted in Great Britain with dimethylnitrosamine and diethylnitrosamine (*37*). Dimethylnitrosamine produced liver tumors, whereas diethylnitrosamine produced cancer in the liver and in the esophagus. It was found that the process leading to cancer in the esophagus demonstrated a threshold or no-effect level. In a preliminary mathematical evaluation of the response as regards liver carcinogenesis, the interpretation being made by pooling data for both chemicals, both sexes, and all liver lesions, there was evidence of a linear response even through the lowest level used, 33 ppb. However, examination of the actual individual results by chemical, by sex, and by lesions, rather than from the pooled data, would seem to indicate that the occurrence of tumors or lesions in the liver is random and found in controls or treated groups in the three lowest dose levels, namely 33, 66 and 132 ppb. The lowest effective level, as revealed by inspection of the actual data, would be 264 ppb and possibly even 528 ppb. Thus, the interpretation of this experiment requires further expert review and discussion in relation to health risk analysis. Regardless, the point is that even with genotoxic carcinogens in a large-scale, well-conducted experiment there is practical and presumptive evidence, even though no mathematical evidence, for a no-effect level.

The experimental evidence for no-effect levels for genotoxic carcinogens, of course, can be challenged by the contention that the data obtained are a function of the group size. Thus, even with relatively large-scale experiments such as those described above, the question has been raised whether the apparent practical no-effect level would disappear if the number of animals were to be increased, for example, tenfold or a hundredfold. Such tests obviously are impractical. Nonetheless, decisions need to be made in health risk analysis based on practical rather than theoretical considerations. For example, the United States Food and Drug Administration (FDA) has ruled that a food crop such as corn or peanuts that contains less than 20 ppb of aflatoxin B_1 can be marketed. This mycotoxin is a powerful carcinogen, and under laboratory conditions 20 ppb is an effective carcinogenic dose, albeit the customary dietary level for high yield, short latent period tumor induction is 100-500 ppb (*38*). This naturally occurring toxin has induced liver cancer not only in the customary laboratory rodents but also in nonhuman primates. Thus it seems likely that this agent would cause cancer in humans at that site. In tropical areas such as parts of Africa, China, and certain other parts of Asia, where the dietary level of aflatoxin is approximately 5-10 ppm or 200-500 times higher than the action level in the Western world, hepatocellular carcinoma is at present a major human neoplasm. On the other hand, in the United States and other Western countries where people have no doubt consumed foods contaminated with this powerful carcinogen (discovered only in 1960 and controlled a few years later), primary liver cancer is a relatively rare disease. This not only justifies the action of the FDA in establishing an action level for aflatoxin B_1, but it also suggests that there are practical no-effect levels or at least no-effect situations with regard to even powerful carcinogens such as aflatoxin B_1.

The conclusion is that, while more research and better tools to delineate low-dose actions of genotoxic carcinogens are needed, it is also essential to minimize human exposure to agents known to be genotoxic. This is further imperative because of possible additive effects and the potential of situations leading to tumor enhancement. Nonetheless, there are now rigorous tests to establish whether or not a given substance is genotoxic. Furthermore, it is possible to evaluate in semiquantitative terms the rela-

tive strength of the expression of this genotoxic effect under *in vivo* conditions. At that point, rational decisions can be made as to the kind of data needed to evaluate rigorously the risk of the presence of such agents in the human environment. Such considerations need to be applied as much to naturally occurring substances—some of which unquestionably account for the current incidence of human cancer—as to synthetic chemicals.

Health Risk Analysis for Tumor Promoters

Health risk analysis dealing for tumor promoters recognizes the fact that these agents operate under mechanisms quite distinct from those applicable to genotoxic agents. Their action is reversible and presumably highly dose-dependent. Unfortunately, there have not been many studies on dose-response with such agents. Where they were tested, it was usually in the context of a carcinogen bioassay with limited dose levels and dose ranges. Under these conditions, it was apparent, however, that activity was seen mainly at very high dose levels with a sharp drop-off occurring as doses were lowered even relatively slightly. Such findings were made when testing, for example, saccharin and some chlorinated hydrocarbons in the mouse liver tumor system. It will be important to further delineate such effects of dose on response in specifically designed experiments with a genotoxic initiator, and the promoter or epigenetic agent given at a number of dose levels including one or two in the range of practical exposure. In the instance of colon cancer, where bile acids and bile acid concentrations appear to be the relevant measures of promoting stimuli, reduction of the concentration of total bile acids to about one-third either by lowering the intake of dietary fat or by increasing the amount of dietary fiber which increases stool bulk, converts a high risk situation to one of lowered risk (*7*).

As we have noted, the concept of promotion in chemical carcinogenesis is almost 50 years old. However, the focus of many of the early workers was on the mechanism of action of croton oil and of phorbol esters. The possibility of receptors for such compounds being present in certain cells has been postulated, so that this kind of promoter may operate by specific mechanisms not necessarily pertaining to other agents such as saccharin or certain of the chlorinated hydrocarbons such as DDT. In the case of saccharin, a beginning has been made to delineate a dose-response curve. In a model study, Cohen et al. (*10*) demonstrated that saccharin, given after a short course of FANFT, can act as a promoter when given at 5% (50,000 ppm) in the diet. By utilizing a different urinary bladder carcinogen, dibutylnitrosamine, a dose-response curve was delineated (*26*). It was found that for male and female rats, levels lower than 10,000 ppm at 1% in the diet had no effect. Likewise, despite a sizable national and international effort, epidemiological studies have failed to demonstrate any effect of saccharin use in humans, especially when corrected for confounding factors such as cigarette smoking (*39*). In consideration of the concepts developed in this paper, it will be necessary to develop novel epidemiological strategems in order to study the effect of agents in the human environment that operate through promoting rather than genotoxic mechanisms.

Along these lines it seems likely at this juncture that a number of hepatocarcinogens, such as trichlorethylene, tetrachlorethane, perchlorethylene and above all DDT, are not genotoxic carcinogens

Table 3. Effect of DES or estradiol on mammary tumor induction in MT virus+ or MTV- mice in 52 weeks.[a]

Agent (ppb)	No. of mice	Marked ovarian atrophy	Mammary: Hyperplastic alv. nodules	Mammary: Adenocarcinomas
C3H/HeJ (MTV+) mice				
0	47	20	0	4
10 DES	32	31	3	0
100 DES	38	53	5	8
500 DES	48	100	14	7
100 E_2	35	15	0	0
1000 E_2	36	29	3	6
5000 E_2	48	91	9	8
C3HeB/FeJ (MTV-) mice				
0	18	11	0	0
10 DES	39	0	0	0
100 DES	18	33	0	0
500 DES	37	90	0	0

[a] From Highman et al. (*43*).

but can act as promoters. There is virtually no reliable information as to dose response with these chlorinated hydrocarbons studied as promoters. The exception is a single study by Peraino et al. (*40*) of liver tumor promotion by phenobarbital. In accordance with the view that promoters would exhibit a steep dose-response curve with a definite threshold, there is no evidence at this time that these chemicals have had any effect in enhancing human tumor formation even though some people were exposed in the course of their occupation to a number of such chemicals such as perchlorethylene or DDT. There have been a number of detailed epidemiological studies of workmen involved in the manufacture of DDT and of applicators of DDT who most likely were exposed to higher levels than the general population; no evidence of excess cancers was noted (*41,42*).

Hormones, especially estrogen, have been classified as promoters as well. A recent study by Highman et al. (*43*) shows that, in mice bearing the mammary tumor virus which acts as the genotoxic event to yield cancers, DES or equi-estrogenic amounts of the naturally occurring estradiol had a dose-related effect in mammary carcinogenesis (Table 3). Of great importance in the light of the concepts discussed, is the fact that in mice free of MTV, and thus presumably with a normal genetic structure, DES at any level did not produce mammary carcinogenesis. This study illustrates the point that promoters are not carcinogenic by themselves but require a specific antecedent gene change mediated by an appropriate genotoxic reactant which can be chemical, viral, or physical.

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