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SYSTEMATIC CARCINOGEN TESTING THROUGH THE DECISION POINT APPROACH

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INTRODUCTION

In recent years, great progress has been made in developing means to evaluate the hazardous potential of chemicals. A major achievement has been the introduction of techniques for the rapid identification of mutagenic or carcinogenic potential, and a number of excellent monographs or reviews have dealt with this subject (1–13). General agreement now appears to exist that a battery of tests is required for toxicological evaluations in mass-screening programs (14–19). Over 100 short-term tests for detecting potential chemical carcinogens and mutagens are available (9), and the critical issue in developing a battery of tests is to formulate appropriate criteria for selecting a battery of tests.

One of the first proposals for the systematic application of short-term tests for the detection of carcinogens and mutagens was that of Bridges (20), in which a three-tiered protocol involving submammalian tests, whole mammal tests, and finally, in vivo tests for risk assessment was recommended. A similar approach was that of Flamm (21) for mutagenicity testing, noting that any single genetic test could not detect all genetic events of concern to man. Whereas Bridges favored tests for chromosomal damage, Flamm and in a later modification Green (22) recommended the dominant lethal test. Neither of these early proposals utilized a DNA damage test. A hierarchical approach described by Bora (14) does include a DNA damage test, and, in addition, recommends host-mediated systems. Thus,

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all these proposals include tests involving effects in whole organisms. The rationale for tests of this type has recently been reviewed by Rinkus & Legator (8).

With the awareness that testing schemes were becoming increasingly complex and expensive, recent efforts have been initiated to reduce the number of tests to an essential core (15, 17–19, 23).

Examination of the foregoing proposals reveals that most tiers or batteries are structured around seven tests: bacterial mutagenesis, eukaryote mutagenesis, Drosophila mutagenesis, mammalian cell mutagenesis, DNA damage, chromosome damage, and malignant transformation. The emphasis on specific tests appears to be mainly a function of whether the proposed scheme is directed toward only mutagen testing, or carcinogen and mutagen detection. For example, all of the tests, except transformation, are listed by the International Commission for Protection Against Environmental Mutagens and Carcinogens (24) as among the most widely used tests for mutagen screening.

Thus, there is substantial consensus regarding the most useful tests for screening for mutagenicity and carcinogenicity. As yet, however, this consensus is reflected in current testing requirements to only a limited degree. This subject has recently been reviewed by Prival (25).

CRITERIA FOR A BATTERY

The philosophy underlying a battery is that a group of tests should be performed before a decision is made regarding the potential hazard of the chemical. Thus, a battery corresponds to the initial "detection" phase that is part of most tiers (20, 21). The crucial difference with a battery, however, is that it attempts to combine "detection" and the next step of a tier, "confirmation" in one stage. Implicit in this approach 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 battery approach thus formally incorporates the concept that no decision on potential hazard should be made without the minimum data offered by the battery.

The choice of tests to constitute a battery will vary depending upon whether the goal is to define potential mutagens or carcinogens. Little is known about the validity of mutagenicity batteries because few chemicals have been shown to be mutagenic to germ cells in experimental animals, and no chemicals are known to produce human germinal mutations. Thus, at present, such batteries must be constructed to identify the broadest possible spectrum of genetic damage (26). In contrast, carcinogenicity batteries can be verified against in vivo data, albeit with an important qualification. Several lines of evidence now indicate that carcinogens may operate by a

variety of mechanisms (23, 27–31). Among these, effects involving DNA damage can be readily detected in short-term tests. Other oncogenic mechanisms of a nongenetic nature are clearly not detected in tests with a genetic end-point. Some tests such as malignant transformation and sister chromatid exchange, which can be produced by effects other than a direct attack on DNA, may be capable of detecting non-DNA-damaging carcinogens. In addition, efforts are being made to develop in vitro tests for identifying agents that operate as tumor promoters (32–35). As yet, however, none of these approaches is sufficiently validated for routine inclusion in a battery. Therefore, in the use of batteries for identification of carcinogens, it must be recognized that a whole category of chemicals (to be discussed) 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. First, the tests should be reliable and of clear biologic significance. This means that they should truly measure what they purport to measure and that the end point should have conceptual relevance to mutagenicity or carcinogenicity. Second, a battery should seek to maximize the metabolic parameters provided by all tests. As an example, tests with intact cell metabolism should be included to extend the metabolism obtained with the commonly used exogenous enzyme preparations. This may be of particular importance in view of the artifactual enhancement of activation over detoxification that is known to be characteristic of enzyme preparations (36–40). Moreover, the DNA adducts formed by activation through microsomes differ from those produced by intact cell metabolism (41).

Adhering to these concepts, a battery of short-term tests was proposed by Weisburger & Williams (23, 42) as part of a "decision point approach."

THE DECISION POINT APPROACH

As a model for systematic approach to the evaluation of carcinogenicity, we present the current status of the "Decision Point Approach" which was first described in 1978 (42).

The Decision Point Approach consists of five sequential steps in the evaluation of the potential carcinogenicity of chemicals. This approach was formulated to incorporate into chemical evaluation several newer developments in chemical carcinogenesis. Of prime importance among these was the concept that chemicals could produce an increase in the tumor incidence in exposed animals; that is, they could be carcinogenic by several distinct mechanisms each having different theoretical and practical implications. One of these mechanisms, proposed by Miller & Miller (43), was through the generation of an electrophilic reactant which would react cova-

lently with cellular macromolecules. The work in several laboratories, notably the Millers, Brookes and Lawley, Magee and Swann, and Ames (see, 23,43–46) has strongly indicated that DNA is in fact the critical cellular target. However, in addition to chemicals of this type, others lacking this property are nevertheless carcinogenic or oncogenic. Among chemicals of the latter type are plastics, hormones, immunosuppressants, cytotoxic agents, cocarcinogens, and promoters. Thus, we suggested that chemical carcinogens could be divided into two main categories, based upon their capacity to damage DNA. Carcinogens that reacted covalently with DNA were categorized as genotoxic, while those lacking this property and probably acting by other mechanisms, were categorized as epigenetic (23). The genotoxic category thereby contains the classic organic carcinogens that damage DNA either through direct chemical reactivity or following metabolism by enzyme systems [Table 1, from (23)]. In addition, the availability of some evidence for DNA damage or alteration by inorganic carcinogens led to their placement in this category. In contrast, the second category, epigenetic carcinogens, is composed of agents that have not been found to damage DNA but rather appear to act through other indirect mechanisms (Table 1).

The decision point approach takes these two categories or types of carcinogens into account in two ways; (*a*) a battery of short-term tests is constructed in an effort to include systems that may respond to epigenetic as well as genotoxic carcinogens; and (*b*) it is recognized that all forms of subchronic testing may fail to detect chemicals that can induce tumors in animals under specific conditions upon chronic administration.

Other elements of the decision point approach are the use of a battery of short-term tests that either may 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 when an already completed chronic test series has yielded ambiguous results.

The decision point approach, therefore, is a systematic approach to the reliable evaluation of the potential carcinogenicity of chemicals which provides a framework in which to minimize the necessary testing in chemical evaluation, and at the same time, develop an understanding of the mechanism of action of a test chemical.

An outline of the decision point approach is given in Table 2. It involves a systematic stepwise progression of tests. A critical evaluation of information obtained and its significance in relation to the testing objective is performed at the end of each stage. A decision is made 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 quantitative—high, medium, or low—effects.

Table 1 Classes of carcinogenic chemicals[a]

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

[a] From reference (23).

Structure of Chemical

For a number of reasons, the evaluation begins with a consideration of structure. Of principal importance is the fact that predictions as to whether or not a given chemical might be carcinogenic can be made with fair success within certain classes of chemicals (29, 47–51). This is particularly true in the case of chemicals of a type that includes known carcinogens. For example, within the large series of azo dyes, Miller & Miller, as well as

Table 2 Decision point approach to carcinogen testing[a]

A. Structure of chemical
B. Battery of in vitro short-term tests
C. Limited in vivo bioassays
D. Chronic bioassay
E. Final evaluation

[a] From reference (42).

Yoshida, Kinosita, Druckrey, and Schmähl have provided data on carcinogenicity versus structure. 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. On the basis of this knowledge alone, for example, it is not likely that pure FD and C Red No. 2 or FD and C Red No. 40 that bear such deactivation substituents on both sides of the azo bond would be carcinogenic (23). 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 also in man. This is because the 1-isomers do not undergo to a significant extent a required metabolic activation reaction, namely, N-hydroxylation, but are rapidly detoxified by ring hydroxylation. Structure must always be considered in relationship to species metabolic parameters (27, 52). The guinea pig, for example, in contrast to rodents or man, has only limited amounts of the necessary enzymes to carry out N-hydroxylation, and yields almost exclusively detoxified metabolites. Therefore, the arylamines so far tested are not carcinogenic in this species. Other examples of species selectivity based upon metabolic capability are well documented (27, 29, 49, 52, 53).

Information on structure and metabolism also provides a guide to the selection among limited bioassays at stage C (limited in vivo bioassays) and, as more information accrues, may eventually contribute to selection of specific short-term tests at stage B (battery of in vitro short-term tests).

In Vitro Short-Term Tests

The status of in vitro assays is that no individual test adequately studied has detected all carcinogens tested. Thus, from this practical consideration alone a battery of tests is desirable. However, the importance of a battery becomes obvious upon consideration of the complexity of metabolism and mechanism of action of chemical carcinogens. As indicated earlier, carcino-

gens can be classified as genotoxic or epigenetic. Most in vitro tests identify genetic effects and thus would detect only genotoxic carcinogens. If epigenetic carcinogens are to be detected, additional tests will have to be developed. Known species differences in response to carcinogens can be related to a large extent (but not exclusively) to metabolism and thus, tests with different metabolic capabilities are important.

A screening battery must include microbial mutagenesis tests, developed mainly by Ames, deSerres, Malling, Rosenkranz, Matsushima, and Sugimura [see (2, 9) for details], because these have been the most sensitive, effective, and readily performed screening tests thus far (5–7). In deciding what other tests to include, it is important to consider the contribution of the proposed test in terms of metabolic capability, reliability, and biologic significance of the end point. The bacterial mutagenesis tests require a mammalian enzyme preparation to provide for metabolism of procarcinogens and hence, any test that is dependent upon an enzyme preparation does not expand the metabolic capability of the battery, since this factor is often the key limiting element of a test.

Mutagenesis of mammalian cells is also included because it is a definitive end point like bacterial mutagenesis but involves effects on the more highly organized eukaryotic genome (54, 55). Moreover, differences in the mutagenic response between microbial and mammalian cells have been observed (56).

Tests for DNA damage or chromosome effects provide further evidence of the ability of a chemical to alter genetic material. Proposed indicators for DNA damage tests have included DNA fragmentation (57), inhibition of DNA synthesis (58), and DNA repair (59). Measurement of DNA fragmentation, although showing an excellent correlation with carcinogenicity, has a conceptual disadvantage in that DNA degradation can occur as a result of cell death (56). Inhibition of DNA synthesis has been suggested to offer an advantage in its ability to detect intercalating agents (57). This is not judged to be a substantial advantage because pure intercalators are a limited group of chemicals comprising at best weak carcinogens (23) and DNA synthesis is also inhibited by noncarcinogenic intercalators. Furthermore, the intercalating agents with reactive groupings induce DNA repair.

DNA repair is a specific response to DNA damage and, unlike DNA fragmentation and inhibition, cannot be attributed to toxicity (60). Therefore, a DNA repair test offers an end point of high specificity, biologic significance, and is included in preference to these other assays.

A chromosomal test is included in the battery to provide detection of chemical effects at the highest level of genetic organization (8). In addition, such tests may detect nongenotoxic agents that operate through effects on DNA replication, chromosome separation, etc.

Cell transformation is included because this alteration is potentially the most directly relevant to carcinogenesis. However, much more needs to be done to clarify the significance and limitations of this end point.

BACTERIAL MUTAGENESIS Valuable bacterial screening tests have been developed in the laboratories of Ames (61) and Rosenkranz (62). The Ames test measures back mutation to histidine independence of histidine mutants of *Salmonella typhymurium* and can be conducted with strains that have been modified to make them highly susceptible to mutagenesis making them sensitive indicators. The test developed by Rosenkranz and associates utilizes DNA repair-deficient *Escherichia coli* and measures their enhanced susceptibility to cell killing by mutagens. In this system, a chemical that interacts with DNA is more toxic to the repair-deficient strain than to wild-type *E. coli* because the mutant strain cannot repair the damage. Thus, by measurement of relative toxicity an indication of DNA interaction is obtained. These tests depend upon mammalian enzyme preparations for metabolism of carcinogens. The capability of the Ames test to detect certain carcinogens has been enhanced by application of preincubation of the compound and the biochemical activation system with the test organism (63).

MAMMALIAN MUTAGENICITY TESTS The three mutational assays in mammalian cells that have been most widely used for carcinogen screening are resistance to purine analogues (64–66), bromodeoxyuridine (BUdR) (67), or ouabain (68). Of these, purine analogue resistance is the most popular. In this assay, mutants lacking the purine salvage pathway enzyme hypoxanthine-guanine phosphoribosyl transferase are identified by their resistance to toxic purine analogues such as 8-azaguanine or 6-thioguanine which kill cells that utilize the analogues. This assay has the advantage over ouabain resistance in that it involves a nonessential function, unlike the membrane ATPase system affected by ouabain, and thus there are no lethal mutants. Its advantage over the measurement of thymidine kinase–deficient mutants by resistance to BUdR is that the gene for hypoxanthine-guanine phosphoribosyl transferase is on the X chromosome rather than a somatic chromosome, as with thymidine kinase. Hence, there is only one functional copy in each cell. Consequently mutations in wild-type cells can be measured, whereas a heterozygous mutant is required for measurable mutation to homozygous thymidine kinase deficiency and BUdR resistance.

The target cells used in purine analogue resistance assays have almost all been fibroblast-like, such as the V79 and CHO lines. The hamster-derived CHO line, and perhaps the V79, possess a limited capacity for repairing O ^{6}-guanine alkylation (69) like hamster in vivo, and therefore, these lines would be expected to display greater sensitivity to chemicals producing this adduct than would repair-competent lines such as those derived from rat.

In addition, fibroblast lines have displayed little ability to activate carcinogens, other than polycyclic aromatic hydrocarbons (65). This deficiency has been overcome in severed systems by providing exogenous metabolism mediated by either cocultivated cells (65, 70) or enzyme preparations (64). The latter again provides no extension in metabolic capability over that used for bacterial systems. However, the use of freshly isolated hepatocytes as a feeder system (70) offers additional possibilities since the metabolism of hepatocytes has been shown to be different from that of liver enzyme preparations (39, 41). Another interesting development is the finding that liver epithelial cultures can be mutated by activation-dependent carcinogens (66) and may therefore provide an additional system with intact cell metabolism.

DNA REPAIR The covalent interaction of chemicals with DNA provokes an enzymatic repair of the damaged regions of DNA known as excision repair. Two types of excision repair have been described: base removal and nucleotide removal [cf Roberts in (46); Laval & Laval in (13)]. The first step differs in each of these, but both processes result in incision of the strand of DNA near the point of damage and an excision of a stretch of DNA containing the damaged region. The gap created by excision is filled by resynthesis of a patch using the opposite strand as a template and then the patch is closed by a ligase. Several of these steps could be measured as an indication of repair, but the resynthesis of the patch is most widely used to monitor repair in screening systems. Repair synthesis can be measured in a variety of ways (60, 71, 72), but incorporation of radioactive precursors as DNA is the most simple. Autoradiographic measurement of repair synthesis has the advantage over liquid scintillation counting in that it excludes cells in replicative synthesis, whereas these are part of the background with liquid scintillation counting. In addition, with liquid scintillation counting, increases in incorporation can result from changes in uptake or the pool size of precursors without any repair occurring. Furthermore, autoradiography affords a determination of the percentage of cells in the affected population that responds. Two deficiencies with most repair assays are that they require suppression of replicative DNA synthesis if continuously dividing lines are being used, and that they depend on exogenous metabolizing systems to provide biotransformation of chemicals. Both of these complications are overcome in the hepatocyte primary culture/DNA repair assay of Williams (73) which uses freshly isolated nondividing liver cells that can metabolize carcinogens and respond with DNA repair synthesis measured autoradiographically. This assay has demonstrated substantial sensitivity and reliability with activation-dependent procarcinogens (73, 74). It also offers the advantages of expanded metabolic capability and biologic significance of the

end point. Thus, it is a valuable addition, with distinct metabolic capability and indicator end-point, to bacterial mutagenesis assays in a screening battery.

CHROMOSOME TESTS Chromosome tests are of conceptual importance because they reveal damage at a higher level of genetic organization than do mutagenesis assays. There has been difficulty, however, in developing means of objective analysis of many chromosomal alterations. Measurement of sister chromatid exchanges (SCEs) overcomes this problem and has shown sensitivity to carcinogens not readily detected in other in vitro assays (3, 75). Therefore, determination of SCEs is presently recommended for a chromosomal level test. The resulting essential extension of the information base with SCE will be useful to delineate further the value and limitations of this relatively new approach.

CELL TRANSFORMATION The first reliable system for transformation of cultured mammalian cells was introduced by Sachs and associates (76). This system utilizing hamster fibroblasts was subsequently developed into a colony assay for quantitative studies by DiPaolo (77) and has been adapted as screening test by Pienta et al (78). In addition, a quantitative focus assay for transformation using mouse cells has been devised in the laboratory of Heidelberger (79) and a quantitative assay for growth of BHK cells in soft agar has been developed by Styles (15). The correlation between transformation and malignancy appears to be good in these systems, but a subject of concern is their high frequency of induced transformation. Nevertheless, they provide a useful indication of the potential carcinogenicity of chemicals either through genotoxic or epigenetic mechanisms and will almost certainly assume a major role in screening in the future. Another approach under development is the use of cell systems carrying oncogenic viruses as a more sensitive means of detecting transforming chemicals. Also, because human cancers usually involve epithelial tissues, transformation in epithelial systems is actively being pursued.

Summary of Rapid In Vitro Tests

The five assays recommended thus far (bacterial mutagenesis, mammalian mutagenicity tests, DNA repair, chromosome tests, and cell transformation) provide a basis for decision making at this stage. A survey of literature data on the application of the recommended test reveals a high degree of sensitivity and specificity for this battery (Table 3).

If clear-cut evidence of genotoxicity in more than one test has been obtained, the chemical is highly suspect. Indeed, positive results in the Ames Test and that of Williams are an almost certain indication of carcinogenicity. Confirmation of carcinogenicity may be sought in the limited

Table 3 Results in "decision point" battery with prototype compounds[a]

Class and chemical	Carcino-genicity	Salmonella/ microsome[b]	Mammalian mutagenesis	HPC/DNA repair	SCE[b]	Transfor-mation
Polycyclic aromatic hydrocarbon						
Benzo(a)pyrene	+	+	+[c–e]	+	+	+[g]
Benzo(e)pyrene	+/–	+	+[c, d], –[e]	–		+/–[g]
Pyrene	–	–	–[d, e]	–		–[g]
7,12-Dimethylbenz(a)anthracene	+	+	+[d, e]	+		+[f, g]
Anthracene	–	–		–		–[f, g]
Mycotoxin						
Aflatoxin B_1	+	+	+[d, e]	+	+	+[f, g]
Aflatoxin G_2	–		–[e]	–		
Mitomycin C	+	+	+[c]	+	+	
Aromatic amine and amide						
2-Acetylaminofluorene	+	+	+[c–e]	+	+	+[f, g]
2-Aminofluorene	+	+	+[e]	+	+	+[g]
Fluorene			–[d]	–	–	
Aniline	w[h]	–		–		–[f, g]
Aza-aromatic						
Quinoline	+	+	+[d]	+		
Isoquinoline	–			–		
Aminoazo dye						
3'-Methyl-4-dimethylaminoazo-benzene	+	+	+[e]	+		+[g]
2'-Methyl-4-dimethylaminoazo-benzene	+	+		+		–[g]
4-Aminoazobenzene	w	+	–[e]	+		+[f], –[g]
Nitrosamine						
Dimethylnitrosamine	+	+	+[c, d]	+	+	+[f, g]
Dimethylformamide	–		–[e]	–		–[f, g]
Nitrosopyrrolidine	+	+	+[d, e]	+		
Hydrazinc						
1,2-Dimethylhydrazine	+	–				+[g]
Hydrazine	+	+				+[f, g]
Hydralazine	+	+		+	+	
Phthalazine	–			–		
Pyrrolizindine alkaloid						
Lasiocarpine	+	+		+		
Monocrotaline	+	–		+		
Pyrrole	–			–		
Miscellaneous						
Urethane	+	–			–	+[f], –[g]
Ethionine	+	–				+[f, g]
Safrole	+	+				+[f, g]

[a] From Williams in reference (30).
[b] Hollstein et al (9).
[c] Clive (67): mouse lymphoma/$TK^{+/-} \rightarrow TK^{-/-}$.
[d] Hsie (64): CHO/HGPRT.
[e] Tong & Williams (66): ARL/HGPRT.
[f] Styles (15): BHK.
[g] Pienta (78): HEC.
[h] weak.

in vivo bioassays without the necessity of resorting to the more costly and time-consuming chronic bioassay.

Evidence of genotoxicity in only one test must be evaluated with caution. In particular, 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 must be evaluated only in the light of the chemical structure and metabolism. For equivocal results, further testing in the limited in vivo bioassays is indicated.

If all the preceding test systems yield no indication of genotoxicity, the priority for further testing depends on two criteria: (*a*) the structure and known physiological properties (e.g. hormone) of the material and (*b*) 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 relevant course of action (47–51). 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 promoters which are negative in tests for genotoxicity operate by complex and as yet poorly understood mechanisms. Thus, it is not certain that the limited in vivo bioassays would yield any results with such materials. Therefore the standard chronic bioassay is necessary 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 requiring the large investment associated with a chronic bioassay.

The testing of metal ions in rapid bioassay tests may take advantage of the concept proposed by Loeb (80) that such ions affect the fidelity of enzymes concerned with DNA synthesis. Obviously, the nature of the metal ion, of which there are only a limited number, would provide the necessary insight as to the need for testing such a material further and what kind of assay would most likely reveal adverse effects.

Compounds with hormone-like properties do exist outside of the strict androgen and estrogen type of material. Such chemicals are potential cancer risks mainly because they interfere with the normal physiological endocrine balance (23). More research on ways and means to test quickly 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.

The potential of halogenated polycyclic hydrocarbons to act as promoters in the production of liver tumors has been discussed in detail (23, 35). As yet, the structural requirements for promoting activity are poorly understood outside the class of phorbol esters, and these agents can be identified only in initiation-promotion protocols in limited in vivo bioassays or in chronic bioassay.

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 without the necessity of undertaking chronic bioassay.

A number of tests for in vivo genotoxicity have been developed; these include the dominant lethal test, specific locus test, heritable translocation test, host-mediated mutagenicity, chromosomal damage, testicular DNA synthesis inhibition, sebaceous gland suppression, and DNA fragmentation or repair in various organs. A chemical that is negative in all the in vitro genotoxicity tests is unlikely to be positive in any one of these in vivo tests, with possible exception of chemicals activated to genotoxic metabolites by host bacteria. Therefore, at present, little basis exists for recommending one of these. Furthermore, a positive result in one of these in vivo tests would not be conclusive evidence of carcinogenicity and thus would serve only as a further indication of the need for chronic bioassay, which, as discussed, has been the only recourse for suspect chemicals that are negative or equivocal in the in vitro tests. Such in vivo tests therefore serve primarily to establish priorities for chronic bioassay of chemicals negative in in vitro tests.

Thus, at this stage, 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. 30–40 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.

SKIN TUMOR INDUCTION IN MICE The carcinogenicity of a limited number of chemicals and crude products can be revealed readily upon continuous application to the skin of mice, producing papillomas or carcinomas, or upon subcutaneous injection, yielding sarcomas (48). 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. Arylamines and related carcinogens by themselves usually do not provide a positive response on mouse skin. Some exceptions are 2-anthramine and 3-methyl-2-naphthylamine, which are active in this system perhaps because these chemicals are converted to active epoxy intermediates, in the same manner as the polycyclic aromatic hydrocarbons (81). Some arylamines and urethane also provide a positive indication, but only upon promotion with phorbol ester.

PULMONARY TUMOR INDUCTION IN MICE Andervont & Shimkin (see 82) pioneered with the model involving the development of lung tumors in specific sensitive strains of mice, especially the A/Heston strain and related strains such as A/J. A singular advantage of the assay system is that, in addition to an end point measuring the percentage 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. Another useful aspect of this assay is that significant results are obtained in as short a time as 30–35 weeks, and sometimes faster. Extension of the test for a longer period is not desirable since the incidence of pulmonary tumors in control animals increases rapidly after 35 weeks, and thus the test loses sensitivity. A negative result in this test system, however, does not signify safety, inasmuch as not all classes of chemical carcinogens induce lung tumors.

BREAST CANCER INDUCTION IN FEMALE SPRAGUE-DAWLEY RATS Shay discovered and Huggins (83) elegantly extended the finding that polycyclic hydrocarbons rapidly induced cancer in the mammary gland of young female random-bred Wistar and, to a greater degree, in Sprague-Dawley rats. With powerful carcinogens, especially select polycyclic hydrocarbons, arylamines, or nitrosoureas, a positive result is obtained rapidly in less than nine months. As in the case of lung tumor induction in mice, the multiplicity of mammary tumors provides an additional quantitative criterion to denote relative strength of the carcinogenic stimulus. As is true with lung tumors in mice, a positive response with a chemical 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, Weisburger & Williams (42) proposed rodent liver tumors for limited in vivo bioassay. This concept is still valid (84, 85), but in recent years, research in a number of laboratories has established that during liver carcinogenesis several distinct hepatocellular lesions precede the development of carcinomas (86, 87). 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, glucose-6-phosphatase, and adenosine triphosphatase which have been used for their histochemical detection (86–88). Another important marker for foci that permits histochemical identification is their resistance to iron accumulation (89). This latter property is more sensitive than the enzyme abnormalities under some conditions and also, unlike the enzyme abnormalities, characterizes rat and mouse liver lesions. Therefore, induction of iron-resistant altered foci in mouse or rat liver can be used as a limited bioassay (23). With known carcinogens, foci have been detected within three weeks of carcinogen exposure and in high numbers by 12 to 16 weeks of 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 (90). Few carcinogens have yet been submitted to this regimen, 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 [cf Weisburger & Williams in (53)] this test should possess substantial sensitivity.

Another approach to detecting altered foci employs their resistance to the cytotoxic effect of carcinogens (87). In this approach, administration of the test chemical is followed by exposure to *N*-2-fluorenylacetamide and partial hepatectomy. The *N*-2-fluorenylacetamide is metabolized by normal liver cells and affects them so that they cannot proliferate in response to the partial hepatectomy. In contrast, the cells in liver altered by the test chemical proliferate and become extremely conspicuous. A sufficient data base is not available to recommend one approach for screening over the other, and future developments in this active field may lead to significant improvements in these approaches. The recent finding by Williams et al (91) that phenobarbital enhances γ-glutamyl transpeptidase activity in preneoplastic and neoplastic liver lesions may provide a technique whereby this liver tumor promoter could be used to make foci more readily detectable for this marker.

Summary of Limited In Vivo Bioassays

The delineation of positive results in a battery of rapid in vitro bioassay tests reliably indicating genotoxicity and also a positive result in a limited in vivo bioassay, namely skin painting, pulmonary tumor induction in mice, breast cancer induction in rats, or induction of liver altered foci, would make a product highly suspect as a potential carcinogenic risk. This is true especially if these results were obtained with moderate dosages and more so, if there was evidence of a good dose response, particularly as regards the multiplicity of the lung or mammary gland tumors.

In the absence of genotoxicity, it is possible to test for promoting activity on mouse skin initiated with small doses of, for example, benzo(a)pyrene or 7,12-dimethylbenz(a)anthracene. 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 revealed by administration following pretreatment with limited amounts of a bladder carcinogen (92–94).

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

The classes of compounds active in limited in vivo bioassays are listed in Table 4.

Chronic Bioassay

Chronic bioassay is used in the decision point approach as a last resort for confirming questionable results in the more limited testing or in the case of compounds that are negative in the preceding stages of testing, but where

Table 4 Carcinogens active in limited in vivo bioassays

Skin tumors in mice
As complete carcinogens
Polycyclic aromatic and heterocyclic hydrocarbons
Direct acting alkylating agents
Alkylnitrosoureas
As initiators with a promoter
Polycyclic aromatic hydrocarbons
Certain arylamines
Urethane
As promoters or cocarcinogens with initiation
Phorbol esters
Anthralin
Catechol
Phenol
Pulmonary tumors in mice
Polycyclic aromatic hydrocarbons
Urethane
Alkylnitrosamides and alkylnitrosamines
Alkylating agents
Aziridines
Hydrazines
Arylamines (poor)
Breast cancer in female Sprague-Dawley rats
Polycyclic aromatic hydrocarbons
Arylamines
Alkylnitrosoureas
Altered foci in rodent liver
Rats
Polycyclic aromatic hydrocarbons
Arylamines, certain aminoazo dyes, heterocyclic amines
Nitrosamines
Urethane
Ethionine
Aflatoxin
Safrole
Mice
Safrole
Hamsters
Nitrosamines

extensive human exposure is likely, or for the acquisition of data on possible carcinogenicity through epigenetic mechanisms. In the latter situation, multispecies and dose response data are most important if the data are to be applied to risk assessment.

The conduct of chronic bioassay has been described in a number of review articles [23; cf Weisburger in (49, 95)].

Final Evaluation

If the decision point approach has led to chronic bioassays, then fairly definitive data on carcinogenicity would be obtained. However, the results of the in vitro short-term tests *must* be considered for evaluation of possible mechanisms of action and risk extrapolation to humans. Convincing positive results in the in vitro tests coupled with documented in vivo carcinogenicity permits classification of the chemical as a genotoxic carcinogen. It would, therefore, be anticipated that the chemical could display the properties characteristic of such carcinogens which include the ability under some circumstances to be effective as a single 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 types of agents [cf Weisburger in (27)].

If, on the other hand, no convincing evidence for genotoxicity is obtained, but the chemical is carcinogenic in certain animal bioassays, 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 that represent 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 and is probably quite different for different classes of carcinogens. They may involve chronic tissue injury, immunosuppressive effects, hormonal imbalances, blocks in differentiation, promotion of preexisting altered cells, or processes not yet known. Regardless, most types of epigenetic carcinogens share the characteristic of being active only at high, sustained doses, and up to a certain point, the lesion induced may be reversible. Thus, these types of carcinogens 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

We now recognize a number of distinct types of carcinogens and mutagens depending on chemical structure such as polycyclic aromatic hydrocarbons, arylamines, nitrosamines, halohydrocarbons, and the like. These agents differ from each other as to effectiveness and target organ affected in cancer

causation in humans and in animal models. In many cases this is now understood as a function of biochemical activation leading to the ultimate carcinogen or mutagen, in contrast to detoxification products. For example, benzo(a)pyrene is a much more powerful carcinogen than benz(a)anthracene, 2-fluorenylamine is more active than 4-biphenylamine, and short chain-length aliphatic nitrosamines are more active than long chain-length compounds. Because of distinct ratios of activation over detoxification metabolites obtained in vitro compared to in vivo, these qualitative and quantitative structure-activity relationships do not always hold in studies involving in vitro effects such as mutagenicity assays. In particular, most of the biochemical systems used to convert promutagens to the active electrophilic metabolite are deficient in detoxification ability, thus accounting in part for the lack of correlation in specific instances.

The primary objective of mutagenicity and carcinogenicity testing is to provide a reliable, sound data base for risk assessment to humans of environmental chemicals and situations with respect to somatic aspects such as neoplastic disease or germ cell aspects such as genetic diseases. Therefore, it is essential to use the test approach described in a manner such that data are generated which can indeed be used for objective, reliable definition of potential adverse effects. This goal necessarily must involve consideration of quantitative potency as well as qualitative yes or no answers. It is quite evident that the protective measures needed for the liver carcinogen aflatoxin B_1 (active at 1 ppb) are distinct from those required for the liver carcinogens safrole (active at 2000 ppm) or acetamide (active at 12,500 ppm). The same consideration applies even in a complex world where the general public as well as specific occupational groups are exposed to varied environmental influences and hazards. It is beyond the scope of this review dealing with decision points in carcinogen and mutagen testing to review in detail the quantitative aspects of this field. It can be said that the current mathematical evaluations of dose-response studies have been based on very few carefully conducted animal bioassays. In fact, a large number of mathematical models have been based on a single experimental series involving subcutaneous injection of polycyclic aromatic hydrocarbons which, because of their relative insolubility and thus slow absorption from the injection site, would exhibit a much broader dose response curve (active over 3–5 log units) than rapidly absorbed agents such as aromatic amines or nitrosamines. For example, we (see 81) noted that even with the powerfully carcinogenic *N*-2-fluorenylacetamide, a lowering of the dose by only one log unit, a factor of 10, converts a very powerful carcinogenic stimulus (200 ppm) to a vitually inactive dose rate (20 ppm). On a larger scale, in the case of cigarette smoke, an individual smoking 40 standard cigarettes per day has

a fairly high risk of disease, whereas with 4 cigarettes per day the risk would be minimal. The reduction again is only by a factor of 10. Thus, quantitative aspects are most important if the goal of risk elimination and thus disease prevention is to be approached in a realistic manner (96). In fact, it is probably true that the high level testing in animal bioassays, instituted by the Weisburgers (see 49), may mimic the reality of established carcinogenic human exposures. As we just discussed, the situation with respect to disease stemming from life-style factors such as cigarette smoking reveals risk only at high exposure. As another example, in the occupational setting, Scott (97) described the high contamination of workers exposed to aromatic amines in relation to bladder cancer, or more recently, the cases of workers exposed to vinyl chloride in which only the reactor cleaners with high exposure have developed angiosarcoma so far (98).

In summary, the decision point approach provides a framework for systematic evaluation of the potential hazards of chemicals, which indicates the need for and can be integrated with other elements in toxicity testing (99). It is designed to yield a stepwise progression of data acquisition. An evaluation carefully conducted of this systematic program should provide a qualitative and a semiquantitative sequential data base, which need not necessarily entail an expensive and extensive long-term bioassay, and which provides an effective tool for the protection of the public against environmental cancer and mutagenic risks.

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