GENETIC TOXICOLOGY

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M. Legator
Division of Biological and Medical Sciences, Brown University and Department of Medicine, Roger Williams General Hospital, Providence, Rhode Island 02908

S. Zimmering
Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island 02912

It has become more and more clear that as the incidence and severity of bacterial and parasitic diseases decline, impairments in man resulting from gene and chromosomal changes are exerting an increasingly serious impact on the health and economy of the human community. Moreover, the finding that some man-made chemicals in widespread use are mutagenic in subhuman experimental systems poses the possibility that such chemicals may constitute a potential genetic hazard for man, both for the contemporary generation through the production of gene mutations and chromosome breaks in somatic cells, and for future generations through the production of transmissible gene mutations and changes in chromosome structure and number. Expanded efforts in evaluating chemicals for mutagenicity are urgently needed, because many chemicals to which humans are exposed, which otherwise serve a useful function, have either not been tested in this regard or only fragmentary or conflicting data are available for them.

PRINCIPAL TYPES OF MUTATION OF IMPORTANCE IN MAN

Intragenic or Point Mutations

These are small microscopic alterations in DNA affecting only a very small number of nucleotides and supplying most of the genetic variation of evolutionary significance. The alteration, addition, or deletion of nucleotides can provide a variety of alleles at each locus. These alterations can be divided into two main subgroups; frameshifts and base-pair substitutions. Base-pair substitutions can be further divided into transitions and transversions. A transition is the replacement of a purine by a different purine or a pyrimidine by a different pyrimidine. A transversion involves substitution of a purine by a pyrimidine or a pyrimidine by a purine. Frameshift mutations usually involve deletions or additions of small numbers of

base pairs. By adding or deleting a base pair, the reading of every codon beyond the point of mutation (addition or deletion) is changed and is read incorrectly, i.e. the new codon either includes one of the bases originally present (addition) or excludes a base for a neighboring codon (deletion). Codons transcribed beyond the altered site now differ. The mutation will lead to the production of greatly altered proteins; see Fishbein, Flamm, & Falk for detailed discussion (1).

In humans a point mutation would be inherited as a single-gene (monogenic) difference from normal. This condition may be simulated where a small number of adjacently situated genes are deleted simultaneously. As summarized by McKusick & Chase (2) the number of abnormalities known to be associated with monogenic inheritance has increased to over 1000 in the past 15 years with an additional 1000 suggested for which proof is incomplete. The number of autosomal dominants has increased some 74%, autosomal recessives some 350%, and X-linked genes some 150%. The latter two results are due primarily to the expanded application and refinements of techniques employed in clinical biochemical genetics; almost without exception, disorders associated with enzyme deficiencies have been found to be due to recessive mutations, either autosomal or X-linked. It would appear likely, therefore, that in accord with virtually all organisms, the proportion of recessive to dominant mutations in humans will eventually prove to be very high. Under ordinary circumstances, independently arising recessive mutations rarely have the opportunity to become homozygous and hence be expressed (except for X-linked recessives which are expressed as hemizygotes as well); however, it has been shown in *Drosophila* that whether the homozygous effect of a recessive mutation is lethal (3, 4) or only mildly deleterious (5), the heterozygotes incur a disadvantage of about 2% or so, suggesting a correspondingly mild effect on viability and/or fertility, and therefore impeding transmission only negligibly for many generations. If the same holds for recessives in human populations, a large number of persons may be affected, which may lead to an amount of detrimental effect equal to or greater than a much more severe mutant.

Reciprocal Translocations

Reciprocal translocations arise where breakage in two (or more) nonhomologous chromosomes is followed by rejoining of appropriate ends resulting in monocentric chromosomes which are transported in regular manner at mitosis in F1. Such translocations are transmitted as dominants, may be maintained in the population for many generations, and as heterozygotes produce unbalanced chromosome sets at meiosis (either through adjacent segregations or loss of an element) resulting in embryonic death, or more important, where aneuploids are not lethal, in abnormalities in surviving progeny. Viable mosaic aneuploids may arise following loss of a small translocation element at mitosis—the earlier the loss the more severe the abnormality. That translocations constitute a genetic burden to man is evidenced from the recent survey of Friedrich & Nielsen (6) based on results of karyotype studies of some 31,000 newborn children suggesting that almost 0.2% are translocation carriers. Additionally, the presence of a heterozygous translocation may generate, through nonhomologous pairing, nondisjunction in unrelated chromosomes as

shown by Grell (7) in *Drosophila* and suggested by Grell & Valencia (8) and Grell (9) to explain several cases of concurrence within single human kinships of rare karyotype abnormalities, such as the association of a translocation with aneuploidy for an unrelated autosome (10, 11) and the far greater risk of trisomy in women heterozygous for a D/D Robertsonian translocation than in the general population (12).

Nondisjunction

Meiotic nondisjunction of homologous chromosomes leads to the production of aneuploid individuals having one chromosome in excess (trisomy) or lacking a chromosome (monosomy, also arising from chromosome loss; see below); nondisjunctional mosaics arise when homologues fail to separate at a mitotic division, aneuploidy being more severe the earlier the event occurs.

Because the very large majority of trisomics and monosomics in man are probably incompatible with intrauterine life and would result in dominant lethals, the condition would in most cases go undetected or result in spontaneous abortion, neither of which would impose a genetic burden on society. On the other hand, in view of recent estimates (6) that approximately 0.3–0.4% of newborn children are sex chromosome trisomics (XXX, XXY, XYY), 0.1–0.2% sex chromosome monosomics, (at least in part nondisjunction in origin), and 0.2% autosomal trisomics (principally G group), it is clear that nondisjunction-generated aneuploids pose a serious hazard to man.

A possible source of additional concern stems from the recent report of Clark & Sobels (13), which suggested that X-ray–induced nondisjunction in *Drosophila* females may rise linearly with dose in one (or more) stages of oogenesis, in sharp contrast to earlier findings of a threshold effect (14). Thus, in the case of environmental agents that may induce nondisjunction, the possibility exists that far greater damage may be expected if the former finding ultimately proves correct.

Chromosome Loss

Loss of a chromosome is detectable as the recovery of monosomic individuals. Loss may occur, for example, after a single chromosome (or chromatid) break, nonrestitution of the broken ends, and after fertilization and replication, failure of inclusion of the dicentric chromosome into either of the first cleavage nuclei (15). Where breaks occur (or are finalized) at meiosis affecting both chromatids of a dyad, and a dicentric chromosome is formed prior to anaphase II, nullosomic eggs or sperm are produced if the dicentric chromosome is excluded from either or both second anaphase cells. Finally, where a broken chromosome (the break originating in any one of a number of ways) is eliminated from a cell during mitotic division, or an unbroken chromosome "lags" and fails to be included in one of the daughter cells, a mosaic individual may be identified.

Whereas monosomy for virtually all chromosomes is lethal in utero, a notable exception, indicated above, is a significant frequency of surviving XO (Turner's) females. Additionally, there is a growing list of surviving autosomal monosomics, most mosaic for G monosomy, and associated with mild to severe congenital malfor-

mations (16). Thus, the presence in the environment of even those chemicals that produce or potentiate chromosome breakage without ensuing rearrangement (i.e. phenols and caffeine) could constitute a potential risk for future generations.

METHODOLOGIES IN MUTAGENICITY TESTING

In recent years, interest in the field of chemical mutagenesis has been stimulated by the development and refinement of procedures for evaluating active compounds in eukaryotes, and more specifically, in mammals. The desirability of utilizing mammalian systems is predicated on the presumed association between mammalian systems and man. An essential inference in all widely used toxicological procedures is that data can be extrapolated from animals to man; the same reasoning applies with equal force in the area of chemical mutagenesis. Mutagenesis, as an area of toxicology, emerged only when mammalian procedures were developed so that chemical effects could be measured in experimental animals. It is interesting to note that as procedures in mammals were used to detect chemical mutagens, test systems that were usually considered not as relevant to man, i.e. microorganisms, *Drosophila,* in vitro cell culture, were found, upon closer scrutiny, to offer greater potential for detecting chemical mutagens than had heretofore been recognized, i.e. *Drosophila,* or were modified so as to increase their relevance to man, i.e. in vitro microsomal enzyme systems.

The available methods can be conveniently classified as those that are capable of detecting direct and indirect acting mutagens. In turn, systems with the capability of detecting indirect acting compounds are those possessing the appropriate metabolic machinery to convert inactive substances to mutagenic forms and those not possessing this machinery but characterized by special genetic properties so that they serve as an "indicator organism," evaluating products produced in the intact host or any of its various organs for mutagenic activity. These procedures are illustrated in Table 1. Detailed discussions of all of these methods can be found in *Chemical Mutagens* edited by Hollaender (17) and in a methods manual currently in preparation (18).

Tests that Can Detect Direct Acting Compounds

All systems listed in Table 2 are suited to detect mutation events where a compound is direct acting or where the active compound is identified. Probably the most useful for rapid screening purposes are microorganisms and mammalian in vitro cell culture systems. The large population that can be raised, short generation time, simplicity, and low cost are characteristic of the majority of these testing procedures. In mutation research, we are interested in the alterations of a specific chemical, DNA, which in terms of reactivity, stability, and the mode of protein formation is essentially the same at all levels of biological organization. Because DNA and the types of genetic alterations inducible therein are similar in all organisms, credence is given to procedures in all test systems for characterizing known active compounds. On the other hand, microbial systems suffer in their inability to detect most

Table 1 Detection methods based on chemical activity

Procedure	Type of chemical detected
A. In vitro systems, i.e. phage, bacteria, yeast	Direct acting chemicals not requiring host metabolism
B. Microsomal activating system, in vitro	Selected compounds that can be activated by the in vitro microsomal techniques
C. Detection of host metabolites by indirect indicators, i.e. host-mediated assay	Direct acting chemicals as well as metabolites formed by a variety of routes in intact animal, specifically for chemicals that induce point mutations when microbial indicators are used
D. *Drosophila*	Chemicals metabolized in *Drosophila* in a manner similar to experimental animals
E. Studies in intact animals, i.e. repair, cytogenetic, dominant lethal, and mouse specific locus test	Potentially all mutagenic agents; however, with the exception of repair and specific locus tests, only chromosome breaking agents detected

mutagenic substances that may be active in man. Cellular uptake and distribution, metabolism, detoxification dosage, and method of administration of the chemical are among many factors that can profoundly affect the mutagenic process. Mammalian cells in cultures are sufficiently removed from man and intact mammals also to be of limited value for detecting all but direct acting compounds.

A wide range of microorganisms are available for characterizing chemical mutagens including *Neurospora,* various strain of yeast, and bacteria. The tester strains of *Salmonella typhimurium* developed by Ames (19) is probably the best developed and the most widely used microbial system in this field. These tester strains are mutants where the genetic alteration is known (base-pair substitutions or frameshift mutations) and so can be used to detect back mutations (reversion to prototrophy). The sensitivity of these tester strains has been markedly enhanced by the introduction of a deletion of one of the genes of the excision repair system and a mutant deficient in the lipopolysaccharide which coats the surface of these bacteria. The repair-deficient mutant greatly increases the sensitivity of the system, and the loss of the polysaccharide coat removes a barrier for the penetration of lipophilic chemicals. It has recently been shown that frameshift mutagens have a great deal of specificity as to the repetitive DNA sequence in which they are active, and tester strains have been and are being developed with known "hot spots." Although several tester strains have to be used in characterizing mutagenic agents by this system, the level of sophistication achieved with *S. typhimurium* probably makes this one of the best available microbial tests for the characterization of chemical mutagens.

Table 2 Genetic lesions detected by various systems (17)

Systems	Mutations			Chromosomal Aberrations				
	Forward and/or reverse	Specific loci (Multiple)	Recombination	Dominant lethal	Translocation	Deletions and duplications	Nondisjunction	Test systems for detecting metabolites
1. Microbial								
a. Prokaryote								
1. *S. typhimurium*	X							X
2. *E. coli*	X							X
b. Fungal								
1. Neurospora	X					X		X
2. Aspergillis	X	X	X				X	
3. Yeast	X	X	X	X			X	X
2. Plant								
a. *Vicia*					X	X	X	
b. *Tradescantia*	X				X	X	X	
3. Insects								
a. *Drosophila*	X	X	X	X	X	X	X	X
b. Habrobracon	X	X	X	X				
c. Bombyx	X	X						
4. In Vitro Mammalian cell systems								
a. Chinese hamster	X				X	X	X	X
b. Mouse lymphoma	X				X	X	X	X
5. In Vivo Mammalian systems								
a. Mouse		X		X	X	X	X	
b. Rat				X	X	X	X	
6. Man					X	X	X	

In Vitro Microsomal Enzyme Studies

Compounds that are metabolized by liver microsomal enzymes can be conveniently detected by the use of liver microsomal hydroxylase systems (20). Some aromatic amines, polycyclic hydrocarbons, and naturally occurring chemicals such as aflatoxin (21, 22) can be detected in this system. The most frequent indicator organism to be coupled with the microsomal activation procedure has been *S. typhimurium* but almost any indicator including other microorganisms, mammalian cells, or *Drosophila* could be used in conjunction with microsomal activation. This activation system is of distinct advantage in characterizing specific classes of chemical mutagens; however, it cannot reflect either quantitatively or qualitatively the dynamic processes that occur in an intact host. Even if this in vitro procedure could detect all metabolic products produced by microsomal enzymes, which it obviously cannot, metabolites produced by other routes would be overlooked. Compounds such as cycasin (23) which are activated by intestinal flora, the azo dyes (24), and chlorpurine (25) are examples of mutagenic agents that cannot be detected by microsomal activation methods. The simplicity and relatively low cost of this procedure are appealing, but it should be recognized that this method can only detect a limited spectrum of active compounds.

Screening, by Indirect Indicator, for Mutagenic Compounds Produced in the Intact Animal

These procedures utilize the intact animal either to activate (produce a mutagenic metabolite) or to indicate the elimination of mutagenic activity by the production of nonmutagenic metabolites. Bacteria are the most widely used indicator organisms, commonly used in screening for point mutations. A variety of other microorganisms as well as mammalian cells can be used also.

THE HOST-MEDIATED ASSAY The assay was introduced in 1969 (26), and various modifications have made this indirect method for detecting mutations a primary tool for characterizing mutagenic agents. In this assay, the animal during and after treatment with a potential chemical mutagen is injected with the indicator organisms in which mutation frequency can be measured. After a sufficient time period, the indicator organism is withdrawn from the animal and the induction of mutants is determined. The comparison between the mutagenic action of the compound on the indicator directly and in the host-mediated assay indicates whether (*a*) the host can detoxify the compound or (*b*) mutagenic products can be found as a result of host metabolism.

The majority of studies carried out with this procedure have relied on the addition of the indicator to the interperitoneal cavity of the treated animal. Several modifications of this method have been used in an attempt to detect mutagenic activity in various organs and tissues of the animal (27, 28).

ANALYSIS OF BLOOD AND URINE In a series of initial papers on the host-mediated assay (29, 30), the analysis of blood and urine in animals treated with Streptozotocin or Cytoxan® revealed the presence of mutagenic activity. This proce-

dure can be extremely useful expecially if information is available as to the pharmacokinetics of the compound. A variety of indicators can be used in this procedure, several samples can be tested, and, of special importance, this technique can be used to detect mutagenic substances in man. In urine analysis the use of specific enzymes to disassociate various conjugates has been described, and the method of concentrating both blood and urine prior to the evaluation of mutagenic substances has been reported (31, 32). Although these procedures can potentially detect active compounds wherever they are produced, they cannot conclusively demonstrate that the compound can induce mutations in animals. The possible repair of the genetic lesion by the host cannot be ascertained by these procedures.

Studies Conducted Directly in the Intact Animal

The procedures in animals are still limited. Methodologies exist for characterizing chromosomal aberrations, but no definitive procedures exist for detecting point mutations with the possible exception of the specific locus test. Chromosomal analysis, dominant lethal test, and translocation studies are all basically methods to determine chromosome alterations. In addition to cytogenetic alterations, the induction of genetic repair by chemicals altering DNA can also be determined in both somatic and germinal cells.

IN VIVO CYTOGENETIC STUDIES Cytogenetic studies can be carried out in almost all species including humans. It is the only procedure where the results of genetic damage can be directly observed through the light microscope. In animals, somatic as well as germ cells can be analyzed, and when carried out in vivo it is a meaningful selective assay. Chromosome alterations may be subdivided into three main categories: (*a*) numerical changes, (*b*) structural changes, recognizable at mitosis, most of which involve the formation of fragments, and (*c*) structural changes, recognizable during meiosis. The procedures for recognizing these alterations and a discussion of the utilization of cytogenetic analysis in a toxicological screen were reported by an ad hoc committee of the Environmental Mutagen Society (33). A procedure for examining chromosomes in vivo at anaphase rather than metaphase has been described (34). In this procedure, the animals are pretreated with colcemide (not colchicine) to collect mitotic figures, releasing the colcemide block, and allowing the mitotic cells to progress to anaphase ($\sim$ 1½ hr). The rapidity of this method should greatly facilitate the screening of potential mutagenic agents and further enhance the utility of cytogenetic screening for genetic damage.

Of special interest is the micronuclei procedure for evaluating chemically induced chromosomal abnormalities (35, 36). The principle behind this procedure is that mitotic cells with chromatin breaks or exchanges suffer from disturbances in the anaphase which cause bridge formation. The same is true if nondisjunction due to disturbances of the mitotic apparatus takes place. After telophase, a sizable proportion of the displaced chromatin is not included in the nuclei of the daughter cells and can be detected in the cytoplasm of these cells by suitable staining techniques. Weber & Legator compared trimethylphosphate in the micronuclei test and the standard metaphase analysis and found the micronuclei test to be more sensitive and yield response over a greater range of concentrations than the standard metaphase

analysis (37). At the present time the micronuclei test seems to be better suited for the detection of chromosome abnormalities than the anaphase procedure.

THE DOMINANT LETHAL TEST A dominant lethal mutation is defined as a dominant genetic change that is incompatible with the survival of the conceptus. In this procedure, male laboratory animals are dosed orally or systemically. The treated animals are then mated sequentially with groups of untreated females over a successive period of eight weeks. Females are scored for corpora lutea and implants comprising early deaths and living fetuses respectively.

HERITABLE TRANSLOCATION TEST In this procedure reciprocal translocations between nonhomologous chromosomes are observed in primary spermatocytes during diakinesis of the first meiotic division. The procedure can be used to detect reciprocal translocations that have been induced in spermatogonia if the meiotic chromosomes of treated animals are analyzed. The heritability of such translocations can be shown when F_1 and F_2 male progeny of treated males are analyzed. Depending on the breeding pattern it is possible to determine the sensitivity of pre- and postmeiotic stages of spermatogenesis. The detection of translocations in spermatogonia and their mutagenic effect on the progeny is extremely important, because the immature cells constitute a permanent population of cells whose alteration is far more significant for the resultant progeny than abnormalities found in later stages of spermatogenesis. Sterility and heritable semisterility are estimated in the progeny and correlated to the translocations. The value of this procedure will only be known after a sufficient number of chemicals are evaluated to determine the frequency of induced translocations so as to define the sensitivity of the procedure.

REPAIR STUDIES CARRIED OUT IN BOTH GERMINAL AND SOMATIC CELLS The final expression of a mutation lesion is an altered phenotype and is dependent on a number of factors including the type and extent of the mutation lesion and the ability or inability of the biological systems to repair or alter the modified DNA segment. Initial lesions in DNA, induced by either physical agents or chemicals, can either lead to permanent changes such as mutations or be removed by cellular repair processes. The significance of cellular repair processes is evident from studies of human genetic diseases that are characterized by inability to repair DNA damage. The induction of repair by specific agents could well be one of the most sensitive and earliest indicators of chemicals that have the ability to induce genetic lesions.

The term "repair replication" is used to describe the insertion of nucleotides into parental DNA strands and this process can occur at phases of the cell life cycle other than at the S phase. Methods for evaluating repair induction can be based on the demonstration of DNA replication occurring at periods other than the normal S phase. The rapidity of the repair process and the extent and the number of bases removed in areas adjacent to the damaged bases can also be used to classify the agent that induces repair.

SPECIFIC LOCUS TEST The specific locus test (38) consists of mating treated and untreated wild type mice to a strain of mice homozygous for known recessive genes. The recessive genes are readily expressed as visible phenotypes in the homozygous

state. If a mutation has occurred in any of the test loci in the germ cells of the treated animals it will be detected in the offspring. The value of this procedure in chemical mutagenesis is yet to be determined. The number of animals that must be employed in this procedure and the results with the few agents tested to date do not allow any firm conclusion about the utility of this method. Although there is a high probability that the specific locus mutations are point mutations, the qualitative similarity of the response of the specific locus mutations to that of translocations and chromosome exchanges in response to radiation suggests that the induced mutations may be the result of deletions arising from two adjacent breaks, rather than one event.

DROSOPHILA

Complementing the methods described are the several well-established genetic procedures available in the genetically versatile fruit fly, *Drosophila melanogaster.* These procedures have been employed to assay for a variety of gene and chromosome alterations produced in germ line cells, including dominant lethals, partial or whole chromosome loss, translocations, recessive lethals (gene or point mutations and small deletions), visible mutations at specific loci (more than a dozen may be simultaneously screened for) reflecting heterochromatic damage (*Minutes* and alterations at the *bobbed* locus), and nondisjunction. Furthermore, because types may be assayed (singly or in combination) in the entire array of germ line stages, cases of stage-specific mutation induction are identifiable (39–43). Finally, while *Drosophila* has long been one of the most utilitarian test organisms for evaluating the effects of direct acting mutagens, its ability to detect genetic damage induced by indirect acting mutagens, compounds requiring activation by mammalian liver microsomal enzyme systems (44, 45), has only recently been strongly documented in that (*a*) insects (including various diptera, orthoptera, and lepidoptera) carry out in vivo many of the same chemical reactions that occur in mammalian liver microsomes (46, 47), (*b*) compounds known to activate or inhibit these reactions in mammalian liver behave similarly in insects (48), and (*c*) many indirect acting carcinogens and mutagens activated by liver microsomes are highly mutagenic in *Drosophila* (49).

The four principal procedures generally employed in a *Drosophila* mutagenicity program are those detecting (*a*) sex-linked recessive lethals, (*b*) reciprocal (heritable) translocations, (*c*) nondisjunction, and (*d*) chromosome loss. These are described briefly in general terms below; for a full account of these and other procedures, the reader is directed to Abrahamson & Lewis (50).

TEST FOR SEX-LINKED RECESSIVE LETHALS This test serves to screen for deleterious recessive gene or point mutations and small deficiencies induced in germ line cells of the male or and where called for in the female as well. It is the most efficient in the battery of procedures available in *Drosophila* and is especially useful in identifying mutagens that produce point mutations but not chromosome aberrations i.e. phenols (51) and hycanthone (52); accordingly, it serves as the principal initial assay system in any *Drosophila* mutagenicity testing program. The procedure is

capable of detecting both complete and mosaic lethals. Testing for the latter is usually undertaken in view of the strong association of mosaicsm and chemical mutagenesis and marks one of the major differences between the effects of most chemical mutagens and that of X irradiation (first recognized by Auerbach (53) and confirmed by several other workers (54–56). Perhaps some 500 to 1000 loci are assayed simultaneously in this test.

TEST FOR TRANSLOCATIONS By virtue of its ability to identify reciprocal translocations involving the Y chromosome and the two major autosomes, chromosomes 2 and 3 (i.e. Y-2, Y-3, and 2–3 translocations; only translocations involving the microautosome, chromosome 4, are usually not screened for), the test can detect breakage and chromosome rearrangement in 95% of the chromosome material contained in a typical male *D. melanogaster* haploid set. The procedure serves, in conjunction with the test for chromosome loss, to evaluate the break-inducing ability of a compound and provides the best definitive information on the capacity of broken chromosomes to rejoin in appropriate ways to produce transmissible aberrations. It is common, using special strains, to test for recessive lethals and translocations in the same experiment.

TEST FOR NONDISJUNCTION While rates of primary nondisjunction in *Drosophila* females and males are virtually always measured for sex chromosomes only, if tested flies carry genetically marked but otherwise ordinary fourth chromosomes (the microautosome) and are mated with those carrying a special fourth chromosome (an attached-4), rates of primary nondisjunction for the sex chromosomes as well as the fourth chromosomes may be measured simultaneously. In this way, 50% of the chromosome complement may be simultaneously assayed for nondisjunction. This procedure tests the ability of a compound to induce meiotic nondisjunction and is especially useful where a compound provides no evidence of mutagenic activity as judged by tests assaying for the induction of gene mutations and chromosome breakage.

TEST FOR CHROMOSOME LOSS Simple procedures are available for testing for induced loss of the X chromosomes in the female, of X and Y chromosomes in the male, and of chromosome 4 in either sex, the loss reflecting production of breaks that fail to restitute. Additionally, where specially constructed Y chromosomes are used that carry wild type alleles of euchromatic genes ordinarily associated with X chromosomes or autosomes, loss of segments of this heterochromatic element may be detected, representing breaks that undergo rejoining. Tests for chromosome loss and nondisjunction are commonly carried out in the same experiment.

DETECTION AND CHARACTERIZATION OF CHEMICAL MUTAGENS

In a discussion on methodology a clear distinction should be made between detection of chemical mutagens in a screening program and characterization of the genetic lesion induced by the chemical. The most appropriate systems for character-

izing genetic lesions are those in which the genetics of the system has been extensively studied, and in this category one would place a variety of microbial systems as well as *Drosophila.*

In the initial screen for mutagenic activity the lesions need not be known, but a comprehensive initial screen should detect the full spectrum of genetic alterations. In Table 2 the capabilities of the specific test are described in terms of the genetic lesion that can be detected. In an in-depth study of chemical mutagens one would first select those test systems that have the capability of indicating the various type of DNA alteration produced by chemicals that are active per se or those that are activated by enzymes of the tissues or intestinal microbial flora of the host. In principle, then, with an unknown chemical one would start with the best available animal systems, including those tests that evaluate metabolic products of the intact host; these would include rodents and *Drosophila.* The integration of the results from these systems should identify a compound as a mutagen. The subsequent studies of an active compound would rely on refining procedures to isolate and identify the active compound and the subsequent characterization of the genetic lesion induced by the chemical under study. This approach is contrary to the normal tendency of initiating studies in in vitro systems and then advancing to animal tests. The in vitro system should be reserved for characterizing mutagens after they have been detected by tests that utilize the intact host. Unfortunately there is no one single test for detecting chemical mutagens, and a complete testing protocol would utilize a battery of tests carried out in the intact animal.

Priority for Testing Compounds

At first glance the task of screening environmental agents for mutagenicity seems overwhelming. No data are available for most of the hundreds of compounds introduced into our environment over the last 31 years. The appreciation of this formidable task led to a search for a simple, economic screen to detect mutagens. As stated heretofore it is not logical to utilize simple systems, such as in vitro procedures, to detect potentially active compounds; in fact the most meaningful screen would have to rely on a battery of tests carried out predominantly in intact animals. In view of these considerations, we must establish priorities for testing environmental agents and then proceed to screen the selected compounds in a meaningful manner. A comprehensive screen using a combination of available methods in animals including testing of metabolites produced in the intact host is still comparatively rapid and economical when compared to the conventional carcinogenicity screens.

The selection of compounds for testing should be based on the following criteria: (*a*) exposure of a large segment of our population, especially people of child-bearing age, (*b*) length of exposure, (*c*) persistence, and (*d*) structure-activity relationship.

The assigning of priorities to environmental agents and their subsequent in-depth screening and characterization offers the possibility of eliminating the most important deleterious environmental agents. In addition to limiting exposure to genetically active environmental agents, it is equally, or more, important to evaluate all new compounds for mutagenic activity prior to their introduction into the environment through commerce.

COMBINED TESTING PROGRAM IN RODENTS

Combined Testing Program (CTP) is a novel procedure for in-depth mutagenicity testing. In the preceding section a number of the procedures used for detecting mutagenic agents were described. We can now combine many of these procedures in a single experiment without sacrificing the efficiency or utility of the individual procedures. This approach has become feasible with the realization that the human population is exposed to most environmental agents on a chronic or subacute basis. In order to successfully combine many of the known tests for mutagenicity, the compound is administered in several divided doses over a period of time; typically, five administrations of the test compounds are given over a five day period. The multiple administration of the compound over a period of time removes the need for specific timing in any of the procedures employed. In a typical experiment the following studies can be carried out: (*a*) repair determination in germinal cells, (*b*) repair determination in bone marrow cells, (*c*) repair determination in lymphocytes, (*d*) metaphase analysis of lymphocytes, (*e*) micronuclei test, (*f*) metaphase analysis of germinal cells, (*g*) analysis of blood for active metabolites, (*h*) analysis of urine for active metabolites, and (*i*) the host-mediated assay. Additionally, an extra set of animals can be included in the test group and used for subsequent mating to determine dominant lethality. The data generated from such a comprehensive screen allow the direct correlation of all the various tests in the same animal under the same conditions. In a single experiment we have a comprehensive, detailed analysis of the potential mutagenic activity of the compound under investigation. In a typical CTP experiment only 10% of the animals are used than would be needed if each of these procedures were carried on individually. Because it is not feasible to rely on any single screen for mutagenic activity, this CTP approach may be the most practical, meaningful, and efficient approach to screening chemical mutagens. Where available, *Drosophila* procedures should be employed in conjunction with the test carried out to detect active metabolites in mammalian blood and urine. In view of recent evidence (57–59) suggesting the feasibility of using *Drosophila* as an indicator organism, definitive information can rapidly become available on specific classes of genetic alterations induced in germ line cells of a higher eukaryote animal by mutagenic substances found in mammalian body fluids (information unobtainable or obtained only with considerable difficulty from direct tests with mammals) and permit more meaningful inferences to be drawn concerning the kinds of genetic damage likely to be induced in mammalian germ line cells by these substances.

COMMERCIAL CHEMICALS WITH SUSPECTED MUTAGENIC ACTIVITY

The majority of research work in chemical mutagenesis has concentrated on the activity of known mutagenic agents, such as alkylating agents, in various test systems. As new procedures are developed it is logical to use these known mutagens to standardize the test procedures and to compare various methodologies. Also, known chemical mutagens are employed in an effort to uncover fundamental mecha-

nisms of action that can be applied generally to the field of chemically induced mutations. Unfortunately in contrast to mutagenic effects of radiation, few generalities can be deduced from studies with specific chemicals. By and large, each chemical (or class of chemicals) is unique, and information derived from specific studies with specific agents cannot usually be applied to other chemicals.

In this still young field, few studies have been conducted with environmental agents as to their potential mutagenic action and mechanism of action. The following six compounds are examples of commercial compounds where results of testing suggest mutagenic activity although sufficient definitive information is not available for a benefit-risk assessment.

Captan, An Agricultural Fungicide

Captan (N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide) is a fungicide widely used throughout the world. Besides commercial agricultural uses as a spray on fruits, vegetables, and flowers, it is also used by home gardeners (60). Additionally, it has been used in the treatment of seborrheic dermatitis in man, as a microbial inhibitor in soap preparations (61), and as a protectant in paints, plastics, leather, and fabrics (62). The mechanism of action of captan is believed to be the result of its reactivity with thiol groups; depending on the nature of the reacting thiol, captan can give rise to several endproducts (63). Gale et al (64) reported on the inhibition of incorporation of isotopically labeled precursors into DNA, RNA, and protein after exposure of ascites tumor cells to captan.

Although the compound shows limited toxicity by oral administration, a dose of several grams per kilogram for most laboratory animals is quite toxic when administered systemically (65).

Genetic effects of captan have been described from the end of the 1960s to the present time. The following observations have been reported: (*a*) 41% increase in chromatid breaks at a concentration of 10 ppm (66), (*b*) congenital malformation in chicken embryos (67), (*c*) an increase in mitotic gene conversion at two different loci with cytoplasmic-deficient respiratory mutants of *Saccharomyces cerevisiae* (68), (*d*) an increase in mutation to colcin resistance with *E. coli* with the effect more pronounced with repair-deficient strains (69), (*e*) no increase in mutations in the host-mediated assay using *S. typhimurium* G-46 (reported by Gabridge & Legator, 70), while at higher concentrations than used by Gabridge & Legator, an increase in mutations with *Serratia marcescens* as the indicator organisms (reported by Buselmaier et al 71), (*f*) in *Drosophila,* negative results with the sex-linked recessive lethal test (72).

The results with captan would indicate a direct acting mutagen, direct acting in the sense that host metabolism is not required for activation. Only fragmentary information is available from *Drosophila* and animal studies, but results that are available do not indicate mutagenic activity. It may well be that captan will serve as an example of an active mutagen that is detoxified in an intact host. The widespread use of this compound and the information available to date would place this compound in a high priority category for further animal studies.

Bisulfite, a Widely Used Food Preservative

Bisulfite is used as an additive in foods, beverages such as wine, pharmaceutical preparations, and is also the aqueous form of the common atmospheric pollutant, sulfur dioxide.

Shapiro et al (73) first reported the deamination of cytosine derivatives by sodium bisulfite and on the basis of this reaction suggested the induction of specific genetic lesions with this compound. The reaction occurs more rapidly at pH 5 than pH 7.0 (1% of pH 5.0). Mukai, Hawryluk & Shapiro (74) in reversion studies with *E. coli* found that sodium bisulfite specifically induces mutations in only those mutants that are cytosine-guanine at the mutant site, indicating specific mutations by the modification of cytosine. Summers & Drake (75) with bactereophage T_4 also demonstrated the specificity of bisulfite mutagenicity for G:C pairs. These authors concluded that the inactivation and mutagenicity are directly proportional to bisulfite concentrations and time of treatment. The compound appears, therefore, to be mutagenic in bacteria and in phage.

As in the case of captan the widespread use of this compound and in vitro mutagenic action would indicate a need for animal testing. It would be most appropriate to test this highly specific mutagen for the induction of somatic mutation as well as carcinogenicity in the stomach where the pH would be optimum for the chemical reaction of bisulfite.

Phenothiazine Antipsychotics, Triflupromazine and Thioridazine

There are nearly 20 commercially available phenothiazine antipsychotic drugs. The pharmacological actions of phenothiazines are numerous but their primary action is probably the depression of the subcortical area of the brain. In addition to the antipsychotic effect, some phenothiazine derivatives possess hypotensive, antispasmodic, sedative, antihistamic, antitussive, antipruritic, hypothermic, or weak electrolytic effects (76). The exact mechanism of action and the metabolic fate of these phenothiazines are not known, although well over 100 metabolites have been postulated or identified (77). One of the postulated mechanisms for the psychotropic activity of these drugs is the formation of a stable positive ion radical (78, 79).

The use of these agents is widespread; one of them, chlorpromazine, administered to date to well over 100 million individuals has been the most extensively studied of this group of compounds in regard to its potential as a mutagen. From in vitro studies, as well as in the host-mediated assay, chlorpromazine has been found to increase mitotic conversion in yeast (80). Experiments on the mutagenic and cytogenetic effects of the compounds in a Chinese hamster cell line have been reported by Garvert & Legator (81). In this investigation the chemical had to be photoactivated to increase the number of chromosome aberrations and mutation frequency. This study would suggest that the mutagenic activity of this compound, as well as the psychotropic activity, is probably the free radical-form such as that produced by photoactivation. Support for the suggestion that the mutagenic action of chlorpromazine may be due to a stable positive ion radical derives from the work of Ohnishi & McConnell (82) who reported intercalation of the free chlorpromazine

radical into DNA, and the work of Kahn & Davis (83) where chlorpromazine was found to form a complex mainly with single-strained DNA and with purine and pyrimidine bases with the exception of guanine.

In human cytogenetic studies, reports of the efficacy of chlorpromazine in inducing chromosome abnormalities in patients administered the drug have been mixed; Cohen et al (84) and Roman (85) reported failures to find an increase, whereas Nielsen et al (86) and Jenkins (87) have provided evidence of a significant elevation in the incidence of chromosome derangements in patients treated with the drug. The reason for this discrepancy is unknown.

Two other phenothiazines that are widely used but for which only meager mutagenicity data are available, are triflupromazine (TFP) and thioridazine. TFP has been reported to produce chromosome abnormalities in mammalian cell culture (88) and early deaths in mice but not in rats in the dominant lethal test (89). Thioridazine has been tested in *Drosophila* and reported in a note by Premlatha & Rao (90) to be mutagenic; from adult males allowed to feed on concentrations of 10 and 20 mg of thioridazine hydrochloride per 100 cc of food medium, they reported the induction of both sex-linked recessive lethals and translocations in all germ line states.

It would appear, then, that the cumulative weight of the evidence available on these phenothiazines suggests the possibility that they are mutagenic. In light of their widespread use, a far more definitive body of information on their potential mutagenic hazard should be provided.

Niridazole, a Schistosomicide

Schistosomiasis is a parasitic disease considered one of the most widespread syndromes known to man. It has been estimated that approximately 200 million of the world's population have been afflicted with this disease (91). A wide variety of chemicals have been tested for antischistosomal activity, but the ideal drug has yet to be discovered (92). Schistosomiasis occurs predominantly in developing countries in South America, the Middle East, and Africa.

In the late 1960s a new schistosomicide, hycanthone, was tested in clinical trials. This thioxanthone drug is a metabolite of a widely used commercial antischistosomal agent, leucanthone. In October 1971, it was estimated that over 300,000 individuals had been treated with the compound (93). Shortly after the introduction of this drug, it was reported to be mutagenic in a number of different test systems (*Salmonella,* Hartman et al 94; yeast, Meadows et al 95; *Drosophila,* Knaap & Kramers 96; and rat, Green et al 97). A WHO consultant group considering benefit-risk of hycanthone therapy voted to continue use of this compound (98). The most compelling reason for this decision was that no definitive information existed on the mutagenicity or carcinogenicity of the alternate schistosomicides. One of the alternative drugs, niridazole, is an effective and frequently used antischistosomal agent affecting the germ cells of both male and female worms (99).

Heterocyclic compounds bearing a nitro group have frequently been found to be carcinogenic and mutagenic as exemplified by the nitrofurano compounds. Niridazole, an ethylurea derivative, is an excellent schistosomicide and like other ni-

trofurazone derivatives inhibits spermatogenesis in experimental animals by a direct effect on the germinal epithelium of the testes. In both treated male and female human subjects a reduction in fertility has been noted, and in males there is a transient reduction in the number of spermatozoa in ejaculates. The antischistosomal activity is believed to be confined to the parent compound. High concentrations are found in the portal blood after oral administration, and the compound and its metabolites are eliminated equally in the urine and feces (99).

In tests with *S. typhimurium* histidine auxotroph the compound is active with tester strains TA 1538 (frameshift-, repair-, and lipopolysaccharide-deficient) and in *E. coli* induces gene duplication. Of special interest are the positive effects found in the host-mediated assay and the analysis of blood and urine after administration of the compound to mice (100). The compound was not active in the dominant lethal test nor in cytogenetic analysis in the rat or mouse (101, 102). At the present time this drug has been shown to induce point mutations, but no cytogenetic effects have as yet been demonstrated.

Saccharin, An Artificial Sweetener

For nearly 100 years saccharin has been widely used as a nonnutritive sweetening agent. During this period there have been numerous studies in animals, some contradictory, on the safety of this substance. In the majority of standard toxicological evaluations pertaining to fertility, reproduction, and fetal development, conducted primarily in rodents, no toxic effects that might limit the use of this compound have been reported. The seeming lack of animal toxicity, and its long-term use in the population are partly responsible for including saccharin in "generally recognized as safe" (GRAS) lists of food-additives. Lessel (103) summarized the data on the safety of saccharin in respect to evidence from chronic feeding studies and for induction of teratogenic and carcinogenic responses. While teratogenic effects were reported in mice by Tanaka (104), later studies of Lorke (105), Fritz & Hess (106), and Klotzsche (107) failed to confirm these earlier observations.

Carcinogenicity studies by Fitzhugh et al (108) in rats at levels of saccharin ranging from 0.1–5.0% in the diet, suggested an elevation in the incidence of lymphosarcoma at the 5% level; on the other hand, Lessel (109) found no significant increase in tumors after 24 months with rats fed for 18 months on 5% saccharin. In a recent study, Hicks et al (110) found saccharin to be cocarcinogenic in conjunction with a single dose of *n*-nitrosourea and speculated that saccharin may have a promoting action with cyclamates and other bladder carcinogens.

Information on the mutagenic potential of saccharin is fragmentary. In the mouse, preliminary studies by Epstein et al have failed to provide evidence of a dominant lethal effect (111). In submammalian species, Sax & Sax (112) showed that sodium saccharin produces an elevated incidence of chromosome breaks in cells of onion root tips, and Sram & Weidenhofferova (113) reported an increase (about 20 times control in sperm) in the frequency of sex-linked recessive lethals with sodium saccharin in tests with *Drosophila*. On the other hand, Samuel & Rao (114), using saccharin, but apparently not the sodium salt of the compound, failed to find evidence of a mutagenic effect in *Drosophila* as judged by results from tests for

sex-linked recessive lethals and translocations despite an identical route of administration and the use of doses some 80–120 times that employed by Sram & Weidenhofferova. It would appear therefore that in view of the widespread use of this artificial sweetener, and the as yet unresolved question of its potential as a mutagen, this compound clearly warrants additional investigation.

The examples cited above served to illustrate the following points: (*a*) among the food additives, drugs, pesticides, and industrial chemicals we will find chemicals that are mutagenic, (*b*) although in certain instances we can infer mutagenic activity on the basis of structure and known biological activity (i.e. nitrofurano) it will be difficult to predict mutagenic activity from chemical compounds (phenothiazines, captan), and (*c*) for the majority of the few chemicals where mutagenic activity has been demonstrated, sufficient data are not available (basically animal studies) to allow a benefit-risk assessment.

POPULATION MONITORING

In population monitoring, we can define two specific problems: (*a*) detecting overall changes in gene frequency and (*b*) determining the contribution of a specific agent or agents in altering the mutation rate.

Our ability to determine changes in the mutation frequency, much less ascertain a specific cause and effect for any single mutagenic chemical in the human population, is extremely limited. The long-term nature of the process, the number of factors that can influence mutation rate and natural selection, and any increase in genetic effects probably would be statistical rather than unique, are among the factors that tend to militate against our ability to monitor effectively the human population for mutagenic effects.

The major approaches to population monitoring are (*a*) biochemical, (*b*) cytogenetic, and (*c*) phenotypic. In the biochemical approach a genetically determined variant of a protein (variant allele) is sought. The most promising approach to detecting protein variants is by electrophoretic techniques where it may be possible to screen up to 9×10^4 loci per day (115). Cytogenetics, especially when automated, offers the possibility of screening large segments of our population including the newborn for chromosome abnormalities. Once baseline frequencies are established, monitoring for mutations could be initiated. The phenotypic approach is contingent upon the detection of autosomal phenotypes whose occurrence is due to mutations. The rarity of any single phenotypic sentinel, i.e. achondroplasia, Alpert's syndrome, and anurida, and the chances of faulty diagnosis limits the potential value of this approach. The biochemical and cytogenetic approaches therefore offer the greatest possibility for population monitoring. It is, however, doubtful that any of the available approaches will be productive in indicating a specific agent as a mutagen. The one area where meaningful human monitoring is entirely feasible would be in studies with particular subpopulations including high risk groups such as can be found in various chemical industries.

The Texas division of Dow Chemical Company, one of the largest chemical companies in this country, has embarked on an exemplary program that may well

be a prototype for industry to follow. The cytogenetic approach is used as a basis for identifying chemicals that may produce adverse genetic effects. New employees are cytogenetically analyzed when they are initially employed by the company, as well as at specified periods during the term of employment. Factors that may influence the cytogenetic findings including medical exposure to drugs, radiation exposure, infectious disease exposure, family history, patients' reproduction, etc, are determined prior to each cytogenetic work-up. A specific program has been developed to assess and correlate all the data generated. During the period of employment, exposure to a chromosome breaking agent can be ascertained and the necessary steps can be taken to eliminate the potential mutagen or to adequately protect the employee from exposure to the toxic agent. Additionally, a number of ancillary benefits have occurred as a result of this program. In the area of cytogenetics we need large field studies to determine the baseline information. A systematic collection of employees from large industrial concerns will help to establish a large data base. The collection of this type of information also serves to identify individuals who can profit from genetic counseling. The cost of this entire program is only a fraction of the cost of an overall industrial hygiene program. This special application of population monitoring will identify a certain percentage of specific mutagens, possibly valuable baseline data in the field of cytogenetics will be collected, and direct medical help can be offered to specific individuals included in this program.

CONCLUSION

Within the last six years the new area of genetic toxicology has come into being. The emergence of this area occurred as procedures were devised for detecting mutagenic activity predominantly in animals or in animal-derived material. Additionally, the development of the animal testing procedures was found to correlate, to a high degree, with results obtained from *Drosophila.* The optimum protocol for genetic toxicology would include a battery of tests carried out in animals or where the in vivo metabolic products of the animals are tested. *Drosophila* is a meaningful addition to the testing protocol. It is not wise at the present time to rely on any single procedure to indicate chemically induced genetic damage. Our ability to determine genetic damage accurately can only be assessed after quantitative data from a number of the best available systems are analyzed. Just as animal tests are essential for detecting mutagenic activity, mammalian cell cultures and various submammalian procedures are of value in characterizing the induced genetic lesion. The ability to characterize the induced change is a unique attribute of this area of toxicology.

The significance of the hazards posed by chemical mutagens to the welfare of our populations can not be determined with any degree of accuracy. It has been estimated that 15 million Americans suffer the consequences of birth defects of varying severity and that of this number 80% and therefore 12 million Americans carry genetic diseases wholly or partly due to alterations in gene or chromosomes. In a specific category of genetic syndromes, autosomal dominants, we can by pedigree analyze what percentage of the total number is due to mutations which have arisen

within this generation. Approximately one fifth of the analyzed dominant diseases are newly arisen mutations. It is not possible, however, by presently available techniques to determine what fraction of the newly arisen mutations, for this or any other category, is due to chemicals as opposed to radiation, viruses, or spontaneous mutations. It is safe to suggest that environmental agents play a major role as a causative factor in our overall genetic disease burden. With a minimal number of compounds tested in animals we have already identified several that can induce mutations. In succeeding years we will find that animal testing will serve as our first line of defense for detecting environmental mutagens, and, in all likelihood we will identify potential chemical mutagens in high risk exposure groups such as employees of certain industries. The high correlation between carcinogenic and mutagenic compounds (116) means that decisions concerning the elimination of genetically hazardous chemicals from our environment can be reached on the basis of results from currently employed procedures and that the validity of these decisions can be strengthened by the development of new procedures in the area of genetic toxicology.

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