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## ABSTRACT

In order to test the thousands of man-made chemicals in the environment for carcinogenic and genetic hazards, a multitude of short-term screening tests has been developed to complement long-term mammalian bioassays and epidemiological studies. These tests cover a broad spectrum of organisms, and include the use of naked and viral nucleic acids, bacteria, fungi, higher plants, insects in vitro mammalian cell cultures (cell transformation, cell-mediated mutagenesis, DNA repair, and chromosome aberration tests) and live mammals. Assay end points include effects on nucleic acids, DNA repair synthesis, point or gene mutation, structural and numerical chromosome aberrations, cytological alterations, and in vitro cell transformation. The present review describes and compares these assays. In addition, it discusses their historical development, the problems and limitations associated with their use, and their implementation in comprehensive testing programs. It is intended to provide overview and specific information to the laboratory that is in the process of establishing genetic toxicological systems. (The literature is reviewed to January 1978.)

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# I. INTRODUCTION

To date, about two million man-made chemical compounds have been introduced into the environment, with over 30,000 in commercial trade (47). Furthermore, it is estimated that to this total between 500 and 1000 new chemical species, involving significant human exposure, are added each year (214). These substances include cosmetics, food additives, drugs, and pesticides, in addition to household and industrial chemicals such as fibers, plastics, paints, solvents, and adhesives.

A rapidly growing concern regarding the wide use of these new chemicals is their potential long-term effects, particularly carcinogenic and genetic (30, 48, 58, 74, 170, 185, 238, 241). The International Agency for Research on Cancer (IARC) has estimated that at least 80% of all cancer has an environmental cause (see citations in ref. 30), and the concern that untoward genetic effects are caused by environmental agents (58, 74) has given rise to the rapidly expanding fields of environmental carcinogenesis and genetic toxicology (environmental mutagenesis). That the carcinogenicity of a chemical is a human health hazard is obvious; and chemical mutagenicity is now likewise accepted as a parameter for safety evaluation (252).

The expansion of these new fields of research and development has been only very recent, however. While carcinogenesis by chemical agents was first discovered 200 years ago with the reporting of scrotal cancer in young chimney sweeps caused by soot (202), it was not until 1930 that the first experimental induction of cancer with a pure chemical compound took place (172). Auerbach and Robson (11, 12) were the first to achieve experimental mutagenesis using a pure chemical agent (for review of chemical mutagenesis see ref. 13). Their objective was to develop methods for readily inducing mutations in order to study basic mechanisms, rather than to develop procedures to test and monitor compounds.

In the early fifties, the geneticist Herman Muller began to voice his concern about the possible mutagenic effects of the rapidly growing number of compounds being synthesized and used at the time (73), and in 1958, the Food Additives Amendment of the Food, Drug, and Cosmetic Act of 1938, known as the Delaney clause, was passed by Congress. This clause prohibited the use of food additives "found to induce cancer when ingested by man or

animal" (48). A report of the Food Protection Committee, Food and Nutrition Board, National Academy of Sciences, published in 1961, was one of the first to discuss actual methods for testing such additives for carcinogenicity (41).

In the midsixties, considerable concern regarding environmental mutagens was aroused with the discovery of "super mutagens" (60). These chemicals can produce high frequencies of mutations while allowing for high levels of survival of the cell or organism. It was thus realized that certain compounds could cause genetic damage to accumulate over generations while passing undetected through the traditional toxicological screening procedures based on cell survival.

The public health hazards of chemical carcinogens and mutagens were thus established by the late sixties, and the Mrak report (185), released in 1969, discussed the widespread testing of pesticides for carcinogenicity and recommended three methods (described below) for testing for mutagenic activity: the host-mediated assay, the dominant lethal test, and in vivo cytogenetic studies.

Since the late sixties, toxicologists and geneticists have joined forces to a significant degree (142), resulting in the vigorous expansion in the fields of environmental carcinogenesis and genetic toxicology. This development is reflected by the rapid increase in the number of publications dealing with chemical mutagenesis (262). In addition to an information retrieval service [the Environmental Mutagen Information Center (EMIC)] several societies, including the American, European, and Japanese Environmental Societies, have amalgamated to form the International Association of Environmental Mutagen Societies.

Despite these recent developments, testing for genetic toxicants remains an awesome task. Of the more than 16,000 chemicals listed in the Registry of Toxic Effects of Chemical Substances, mutagenic information has been recorded for less than one percent (48). In addition, of the 6,000 substances tested for carcinogenicity (through 1972), only 3,000 have been tested adequately, and of these about 500 may confidently be termed carcinogenic (214). The Toxic Substances Control Act (TOSCA) was passed in 1976 and authorizes the regulation of mutagens and carcinogens (251) [enforcement, however, seems to be a real problem (256)]. So the question becomes: how can we screen effectively and at reasonable cost for the hun-

dreds of compounds being introduced yearly, in addition to testing the tremendous number of compounds already widely dispersed?

Before the advent of short-term tests such toxicants were screened by whole-mammal studies or detected retrospectively by epidemiological studies. The latter have been able to pinpoint industrial carcinogens such as vinyl chloride and asbestos, but they detect the effects of these substances long after their introduction. Epidemiological studies are also very expensive, and their usefulness is limited in view of the complex facets of the variability in human populations. Long-term tests with whole mammals are also of limited value because of their long time span required (over three years), high cost (about $200,000), and skilled manpower requirements including experienced pathologists (214). In addition, correct extrapolation from high doses of single toxicants given to small animal populations in these experiments to the relatively large human populations that are exposed to low doses of a complex mixture of substances can be very difficult.

In view of these considerations, the recent surge in the interest in short-term tests for genetic toxicants and carcinogens is understandable (205). It is the purpose of this review to describe and evaluate such tests, as well as to make suggestions for their use. These tests have been chosen with the following criteria in mind: cost (about $50,000 or less), time (completed in less than two months), evidence for validity in toxicological testing, and ease of use. They are therefore comparable to the first level or the first tier of a complete testing program (27).

## II. END POINTS AND THE RELATIONSHIPS AMONG DNA DAMAGE, MUTATION, AND CANCER

The testing systems described below are characterized by one or more of the following end points: (a) effect on nucleic acids, (b) DNA repair synthesis, (c) point or gene mutation, (d) structural chromosomal aberration, (e) numberical chromosomal aberration, (f) cytological alteration, (g) *in vitro* cell transformation. While each end point is individually important in terms of long-term toxicity (see Table 1), a brief discussion of their interrelation is relevant.

Increasingly, correlations are being made between DNA damage/repair and cancer (30, 89). In addition, correlation has been demonstrated between chromosomal aberration and cancer (241). However, many compounds which are potent chromosome-damaging agents are also noncarcinogens, and tests with chromosome aberration end points have thus been criticized (238). In addition, while a strong correlation between chromosomal aberration and mutation seems obvious, much higher doses are often times necessary to induce the former (234), suggesting the direct relationship between these two end points is not as close as was previously considered (15).

A most important and controversial correlation to be made in recent years is that between mutation[1] and cancer (3, 36, 62, 63, 165-167, 179). Although the relationship between chronic toxicity and short-term effects has been examined in detail, interest in the relationship between mutagenesis and carcinogenesis has arisen largely out of the recent revitalization of the somatic mutation theory of cancer, originally proposed by Boveri (for historical review, see ref. 172), and the development of a large number of rapid mutagenicity tests designed to detect carcinogens (for example, ref. 4).

In the early fifties, a study was made to determine the mutagenicity of some chemical carcinogens, and little correlation was shown (55, 57). However, less was known at the time about the nature of mutation, or about the types of mutations that bacteria reflected. Research in the area continued, but only on a small scale.

[1] Mutation is often times used quite broadly to include chromosomal aberrations. In this review, the term is taken to mean, simply, point or gene mutation.

Three conferences, held in Honolulu (36), Brussels (179), and Seattle (63) (for a summary of these three conferences see ref. 62) have reviewed coordinated studies by researchers in the United States and Japan. The results indicate that at least 80% of the chemical carcinogens total are mutagens, and that less than 10% of compounds thought to be "noncarcinogens"[2] show mutagenic activity ("false positives") (57). Similarly Ames and his collaborators, testing about 300 chemicals with his Salmonella/microsome mutagenicity test, found 90% of the carcinogens are mutagens, and that 13% of the noncarcinogens are mutagens (166). These results and others have firmly established an empirical correlation between mutagenicity and carcinogenicity and have given impetus to the further development of mutagenicity testing for carcinogens. False positives have raised the question of the validity of long-term animal tests, in particular with regard to "weak carcinogens" and low dosage levels (110). However, the problem remains that while the majority of carcinogens are mutagens, a sufficient data base has not developed to determine the percentage of mutagens that are carcinogens (172).

Concerning the spectrum of test end points to be discussed in this review, two unanswered, yet fundamental, problems remain. First, which of these end points is most relevant to man? It is the opinion of Auerbach (14) that induction of point mutations and small deletions represent a sure test of genetic risk to human populations. Nevertheless, it must be kept in mind that nondisjunction in man is estimated to be associated with 40% of spontaneous abortions and 0.6% of abnormalities among live-born children (233). Obviously, more research is needed to determine the relevancy of end points. Second, how do the various end points interrelate? As noted above, correlations are poorly defined yet compelling, and, as emphasized by Sobels (234), studies on "comparative mutagenesis" must be given a high priority. It is not the purpose of this review to discuss the interrelation of these end points in detail--all are relevant to human long-term toxicity involving years and generations, and for that reason, all are considered.

[2] In view of the discussion above of long-term mammalian tests and "known" carcinogens, it may be quite difficult to define a chemical as a noncarcinogen.

## III. METABOLIC ACTIVATION

Substantial differences occur between the prokaryotic cell and the eukaryotic cell with regard to the organization of the genetic material into structural units and the operational control of these units. Nonetheless, the justification for the use of a tremendous variety of cells and organisms--from naked DNA to viruses to microorganisms to mammalian cells in vitro, to biopsy material--in short-term screening tests for genetic toxicology and carcinogenicity, is premised on the fact that the hereditary material, the nucleic acids, is basically the same from viruses to man. However, considerations of the complex pathways, including organ, age, sex, and species specificity through which chemicals may be activated or detoxified, are crucial. In predicting the fate of foreign compounds in living organisms, it is recognized that the use of isolated organs, tissue cultures, cell preparations, subcellular fractions, and isolated enzymes, while all quite valuable, will never be fully adequate (103, 142). Extrapolation to man requires the consideration of pharmacokinetics (the absorption, distribution, metabolism, and excretion of foreign compounds--ref. 103), a traditional part of toxicology. This need is made all the more apparent by the fact that most carcinogens require metabolic activation (241). Thus, using a variety of indicator organisms, in particular bacteriophage, Salmonella typhimurium, Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae, Neurospora crassa, Drosophilia, and various mammalian cell lines, three basic types of metabolic activation systems have been developed: (a) "in vivo" host-mediated assay (38, 52, 77, 102, 144, 165, 227); (b) "in vitro" host-mediated assay (26, 56, 87, 88, 91, 92, 112, 120, 142, 143, 158, 159, 163, 175, 177, 178); and, (c) internal activation--external exposure assay (4, 16, 56, 95, 101, 142, 156, 157, 159, 187, 193, 264). The specific tests are described below according to the indicator organisms, and general descriptions of the three systems run as follows:

### A) In Vivo Host Mediated Assay

This type of host-mediated assay procedure involves the chemical being activated while inside the host; the indicator organism is likewise exposed while within the host. The use of body fluids as a source metabolic activating systems has received the attention of both researchers and health

authorities; in the original form (99), a microbial organism (usually Salmonella typhimurium and Neurospora crassa--120, 142) is injected into the peritoneal cavity of mammals (usually mice--120, 143) which are then treated with mutagens. After a period of time (several hours to days, depending upon the indicator organism), the microorganisms are withdrawn, and the mutants are detected by standardized microbiological plating procedures. These results are compared to the effects of the compound on the microorganism directly to determine detoxification or activation by the host. However, the peritoneal cavity is a relatively insensitive site for metabolic activation and chemical activity, and so short-lived ultimate mutagens or carcinogens may not be detected. Thus, other modifications including putting the indicator organism closer to the male testes (87, 88) or the major site of metabolic activation, the liver (80, 112, 175, 178), have been developed. The liver test in which bacteriophage (178) or Escherichia coli K-12 strains (80, 112, 175) in particular, are injected intravenously, allows for longer exposure to chemicals and is considered more sensitive than the intraperitoneal cavity (87, 88). In addition, a "linear" host-mediated assay in which samples are taken at a number of time intervals, has been proposed as a means of more accurately assessing the true potency of a given compound (105, 151).

Although the in vivo host-mediated assay has successfully combined mammalian metabolism with sensitive microbial indicators, a number of shortcomings remain, including: (a) possible undesirable host reaction against the indicator organism, (b) the limited time span the indicator may be kept in the host, (c) site-specific activation and detoxification of the compound, and (d) differences in repair mechanisms between the indicator organism and host(159). In an ideal system the indicator organism would not be detected as foreign in the host and could be recovered from the liver; such a liver recovery system is now in the process of being developed (159).

B) In Vitro Host Mediated Assays

In this method the chemical is activated outside the host using the host's activating systems; the indicator organism is likewise exposed outside the host. The recent emphasis on this form of assay is reflected by the symposium (64) focusing on the combination of in vitro microsomal activating systems with microorganisms and mammalian cells in culture. Basi-

cally, in such tests the indicator organism is mixed directly with the activating system, containing microsomes and the drug. The organism is then scored for effects. This method eliminates the use of whole animals, and therefore avoids undesirable host reaction to the indicator organism. Also it makes possible the use or organ homogenates derived from amn (56), although this has proven to be difficult (8).

Malling first demonstrated the mutagenicity of metabolically activated carcinogens (157, cited by Bridges, ref. 30). Soon after, the Millers' laboratory (100, 101) developed a rat liver microsome system which was adopted by Ames, who was the first to combine rat liver microsomes with bacteria in agar (4). To prepare this rat liver subcellular fraction ("S-9"), the animal's microsomes are induced to produce activating enzymes by the polychlorinated biphenyl (PCB) mixture, Aroclor 1254, or other inducing agents. The liver is extracted from the animal and homogenized in 0.15 KCl and the homogenate is then centrifuged for ten minutes at 9000 x g to obtain the microsomal fraction. The liver is used because it is the tissue by far the richest in microsomal enzyme activity and because it contains the essential "mixed function oxidases," which include as the terminal electron acceptor cytochrome P-450. The homogenate is combined with cofactors, including NADH or NADPH, for proper activation.

There are, of course, serious limitations to the use of such activating fractions. Obviously only a limited number of relevant reactions may occur, the some reastions not occurring significantly in vivo may be detected (95). In addition, a wide range of factors affect the sensitivity of this microsomal method, including the amount of S-9 used (56), purity of the fraction (166), diet (53), and organ, sex, and species differences (263-265). Some maintain that this method is still more adequate than the intraperitoneal host-mediated assay (159), while others feel the latter is more appropriate for screening unknown compounds (95). However, there are some indications that the intrahepatic host-mediated assay is superior to either (95, 159, 175).

C) Internal Activation - External Exposure Assays

This host-mediated assay procedure has the chemical activated inside the host; after activation, the chemical is exposed to the indicator organism outside the host. Recent methods have employed samples of body fluids [expecially blood (80) and urine (77)] to be tested by indicator organisms such as

microorganisms and insects. Of these tests, the development of the urinary assay is the most useful (52, 77, 78, 144, 165, 227). Since some metabolities may be concentrated up to 1000-fold in the urine, this can be an extremely sensitive method; however, this very fact indicates that urinary metabolites may not reflect in a relevant manner the concentration of metabolites in the body, and, in addition, only long-lived metabolites can be detected (276). Another recently developed system involves subjecting bacteria or yeast (102) to lyophilized plant material after the plant is exposed to test agents for the purpose of producing ultimate mutagens.

# IV. SUBMAMMALIAN TESTS

The rapid expansion in the fields of environmental carcinogenesis is characterized largely by the proliferation of submammalian tests, which include use of cellular and viral nucleic acids, bacteria, yeasts and other fungi, higher plants, and insects (see reviews 30, 74, 238, 241, 243, 276). However, these tests have been sharply criticized (142, 210, 238). A number of unique features of human and other mammalian organisms (see part I; ref. 160) point to the limitations of submammalian tests and to the caution with which they must be interpreted. Even the strongest proponents of such tests point out that these tests presently allow only qualitative and not quantitative assessments of genetic risk (276).

Nevertheless, these tests still possess the distinct advantages which prompted their original development beginning in the 1960's: (a) they are quick, inexpensive, and relatively simple, (b) large sample populations may be used, (c) they are highly reproducible, (d) they are sensitive and may be used to detect, classify, and characterize genetic effects of a wide variety of compounds. In addition, the great variety of tests developed appear to cover the spectrum of genetic end points with the crucial problem of metabolic activation and detoxification being considered. Also, submammalian tests to date have been found to be the most effective in detecting carcinogens (62), and, using a battery of such tests, it appears possible to eliminate "false negatives" (234, 276). (A false negative is said to occur when a known carcinogen is not detected by a short-term test.) Thus, in spite of unresolved issues such as false positives, false negatives, relevancy of end points, etc., in view of the short-comings of alternative tests and considering the pressing need for rapid accumulation of information, this category of tests is receiving full attention for present implementation and future development; Table 1 is a summary and evaluation of useful and promising tests.

## A) Naked and Viral Nucleic Acids

For hereditary damage, the most direct assays involve the use of isolated nucleic acids. Many chemical and physical methods (35, 182, 242), including chromatography, electrophoresis, spectroscopy (ultraviolet absorption and fluorescence), radioactive labeling, various types of centrifugation, and electron microscopy, have been applied to examine the effects of compounds

on nucleic acids which have been separated from other cell components, released from protein complex, and finally purified. Phage or bacterial DNA is generally used.

Another method involves the use of isolated and transforming bacterial DNA, in particular that of Bacillus subtilis, Diplococcus pneumonia, and Haemophilus influenzae. Such transformation involves the transfer of genetic material from the ruptured (lysed) cells of one strain to a recipient strain which consequently develops characteristics of the donor strain. Experiments with this "transforming principle" provided final proof that DNA is the hereditary material. Procedures employing the transformation method for compound screening generally involve the exposure of isolated transforming DNA to a given compound, uptake of this DNA by the recipient strain, and a measure of the inactivation of the genetic activity or the introduction of mutations in the DNA (117, 155).

Viruses are unique in that they lack complete metabolic capabilities, cannot replicate in isolation, and come closest of all organisms to the nucleic acids of the unrepaired genome. The use of viruses in screening tests has been reviewed by Drake (72). One of the more prominent short-term tests using viruses is the prophage λ induction in Escherichia coli K-12 (116, 139, 181). In this test lysogenic bacteria, carrying the prophage as an integrated component of their genome, are exposed to the test agent. Disruption of this stable state results in the death of the bacterial cell (lysis) and in the proliferation of free infectious λ prophage particles. It is proposed this "prophage induction" indicates the mutagenicity and carcinogenicity of a compound. Recently this method has been improved by combining microsomal enzymes with three variants of the "inductest": (a) a qualitative spot test, (b) a quantitative test on plate, and (c) a quantitative test in liquid medium (181).

New short-term testing systems involving the use of viruses or viral components include the following end points to detect mutagens and carcinogens: (a) the increase in perturbations in the fidelity of DNA synthesis by a DNA polymerase from avian myeloblastosis virus (AMV) (used for metal salts in particular) (228); (b) inhibition of viral replication (QB RNA or ϕX174 DNA) when combined with E. coli spheroplasts (for polycyclic aromatic hydrocarbons and related compounds) (121); (c) stimulation of the rate of RNA synthesis by metal mutagens and carcinogens at concentrations that inhibit over-

all RNA synthesis using E. coli RNA polymerase with calf thymus DNA or phage T4 DNA templates (119). In addition, certain types of phage (λ, T4, and H) are totally nonpathogenic and survive for relatively long periods of time within the blood stream of their hosts. This may enhance their usefulness as indicator organisms in intrasanguineous host-mediated assays (178).

While the methods utilizing nucleic acids and viruses are the farthest removed from those based on the metabolic processes and chromosome structure of humans, they are the most rapid, economical, and sensitive. Nonetheless, they have not been emphasized as much as other methods in thort-term screening programs.

B) Bacteria

For the detection of mutagenicity of complex mixtures or particular compounds such as mutagens and carcinogens, fast and ultrasensitive bacterial assays utilizing in particular strains of Salmonella typhimurium, Escherichia coli, and Bacillus subtilis, have developed as powerful tools. The more important bacterial assay systems are characterized by two end points, mutation and growth inhibition. Basically two types of mutation deficiencies are used: (a) nutritional requirements: his$^-$ (histidine), trp$^-$ (tryptophan), nad$^-$ (nicotinic acid), arg$^-$ (Arginine), and lys$^-$ (lysine); and (b) DNA repair deficiencies: uvr$^-$ and exr$^-$ (excision repair deficient), and polA$^-$ (lack of polymerase I), and rec$^-$ (lack of ability to perform recombination repair). While the nutritional deficiencies are especially important in characterizing mutational end points, the DNA repair deficiencies often make mutational assays more sensitive in that the mutants are more responsive than the parent strain to DNA damage by chemicals. In addition to providing mutation end points, repair deficiencies also allow the comparison of the end point of growth inhibition or killing in the two strains by a test compound. Three types of experimental procedures are used for bacterial assays:

1. Spot test: Originally developed by Iyer and Szybalski (129), the test sample is applied to the center of a Petri dish containing bacteria. Later versions of the test also use microsomal enzymes. In Petri dishes with agar, the substance is either added directly to a center well, as crystals or microdrops, or a solution of the compound is soaked into a disc

which is placed on the agar surface ("paperdics" method). This procedure is extremely simple, tests a wide range of concentrations on a single petri plate, allows the detection of chemicals that act on replicating DNA (176). However, it is qualitative at best and produces false negatives for chemicals which cannot diffuse in the agar, which are insoluble in water, or which activate at a pH different from that of the medium. Thus, verification of this test with other assays is necessary.

2. Plate-incorporation assay: The only difference from the spot test is that the test compound, microsomes, and organisms are preincubated together and subsequently plated. Different concentrations of the sample must be tested in a concentration range of proportional response, so that this test may provide a more quantitative dose-response information than the spot test.

3. Liquid suspension: The test substance and organism are added to nutrient broth in tubes and are incubated for up to several days; the end point is then indicated by turbidity in the tubes or through plating on agar. This method may be more tedious, and many of the suspension procedures require temperatures too high for microsomal activation, yet several end points are simpler to score and a more thorough compound-organism mixture allows for the detection of weaker mutagens and carcinogens. An example of liquid suspension technique is the fluctuation test, originally developed by Luria and Delbück (152), used by Voogd et al. (261), and modified by Green et al. (109).

Bacterial tests are often categorized by the species employed or by a peculiar genetic characteristic of a strain. Several such systems are discussed in detail:

1) Salmonella typhimurium. The Ames test has been employed extensively (2-9, 22, 77, 136, 164-167). Currently being used in well over 1000 industrial, government, and academic laboratories worldwide, it is the most widely known and well documented of the bacterial tests. Ames and coworkers have published the results of testing some 300 compounds (166).

The test as originally described (8) used a nutritional auxotroph and assayed for reversion to prototrophy in minimal media. The original strains

were made more sensitive by the introduction of: a DNA repair deficiency (uvrB); a deficiency in the cell envelope lipopolysaccharide (rfa - "deep rough"), which results in increased permeability to larger molecules (154) such as polycyclic aromatic hydrocarbons; and certain plasmids. Depending on the strain used, base pair substitutions (TA1535, TA100) or frameshift (TA1537, TA1538, TA98) reverse mutations may be detected. The spot, plate-incorporation, or liquid assays may be used (9). For metabolic activation, the S-9 microsome mixture, as discussed above, has been developed specifically for this test (4), and additional modifications are the urinary assay (52, 77, 165), use of feces (38), and the host-mediated assay (120, 143, 177). While the Ames assay is limited to the detection of reverse mutations and is insensitive for many known mutagens and carcinogens, such as metals, chlorinated (diene) pesticides, azo dyes, and spindle poisons (e.g., colchicine), it remains as one of the more reliable tests for testing compounds individually (62, 206) and in complex mixtures (136). Already, some compounds now undergoing higher-level testing (such as certain hair dye components) were detected using the Ames test (6, 166). The sensitivity of Salmonella strains have been enhanced by incorporating R factor plasmids into the genomes (167), and new Salmonella strains are being developed to broaden the utility of this testing system (164).

2) Escherichia coli WP2 and related strains. Tests involving these widely used strains (32, 33, 106-109, 118, 127, 253) are similar to the Ames test in that reversion to prototrophy (in this case, from a tryptophan requirement) is utilized, and sensitive repair-deficient strains are employed. Again, spot, plate incorporation, or liquid assays may be used (107), and the modified fluctuation test developed by Green et al. (106, 109) seems particularly useful for detecting chemicals weak in activity and/or low in concentration (109). Also of interest is the application of whole liver cells instead of conventional microsome fractions (106). While these strains may be more sensitive for detecting certain compounds and may better characterize base-pair substitutions, they do not detect frameshift mutagens.

3) E. coli polA. Tests using this strain, originally developed by Slater et al. (229), are fundamentally different from (1) and (2) above in that growth inhibition, rather than mutation, is the final end point. Spot

("paperdisc") or more sensitive and quantitative liquid assays may be used (212), and polA$^-$ mutants, deficient in polymerase A-1 (used in DNA repair), are exposed to a given compound in tandem with the isogenic polA$^+$ parent strain. An increased relative growth inhibition in the former is taken to mean that relevant DNA damage is occurring. Intercalating agents and certain agents having bactericidal activity are negative in the Salmonella test and positive with the E. coli test, while the reverse holds true for compounds such as metronidazole and zathioprine (212). Thus, it is recommended that an E. coli test, while the reverse holds true for compounds such as metronidazole and zathioprine (212). Thus, it is recommended that an E. coli test be used in combination with Salmonella test.

4) Rec$^-$ bacteria. Strains containing the rec$^-$ mutation, principally of the E. coli and B. subtilis species, are important in that they are defective in different parts of the repair process from the above strains and may thus detect additional mutagens and carcinogens. Spot test procedures are used with these bacteria, and thus having growth inhibition end points similar to the test of Rosenkranz, they are subject to similar limitations.

The "rec-assay" using Bacillus subtilis M45 (rec$^-$) and H17 (rec$^+$) strains was originally developed by Kada and co-workers (133) and has been used to screen food additives and pesticides in particular (131-133, 226). Also, Nishioka, using a slightly modified "rec-assay," has been able to test certain metal compounds (189). For metabolic activation, the use of microsome fractions has not been reported, but the intraperitoneal host-mediated assay is used (133). Several strains of rec$^-$ E. coli are in use (126, 130, 246), and show similar responses as the Bacillus subtilis strains. They generally require higher doses, with the exception that nitrosamines may be detected at lower levels when rat liver microsomes are used (126).

5) E. coli K-12. Recently developed tests using this strain (80, 112, 176, 177, 186) show considerable promise because they detect both forward and reverse mutations using a single strain. Thus, the relative frequencies of these two mutation types may be compared, and forward mutations occurring at several locations on the genome may be detected. Both spot and liquid test, including microsomal fractions, can be used. The strain seems partic-

ularly suitable for the intrasanguineous host-mediated assay (80, 112, 176, 177). Compared to Salmonella, the K-12 strains both penetrate the hepatocytes more easily (112, 177) and shown a higher survival rate when withdrawn from the host (80, 177). Also, using these strains provides more sensitivity to certain compounds such as some nitro heterocyclics and dialkylnitrosamines (234). Excision-proficient strains of E. coli K-12 are particularly useful because they are capable of detecting certain DNA cross-linking mutagens (186).

6) Bacillus subtilis strains. As an organism for mutagenicity testing, B. subtilis has two potential advantages over S. typhimurium and E. coli: (1) being gram positive, it is more permeable to certain chemicals such as polycyclic aromatic hydrocarbons, and (2) spores may be stored and kept viable for many years. Several mutagenicity tests using this species have been developed including the one of McGregor and Sacks for detecting multigene forward mutations in the sporulating process (168). Tanooka (245) reports a procedure involving histidine reversion and another detecting relative growth inhibition of DNA repair-deficient mutants.

Collectively, bacterial testing systems detect a wide range of forward and reverse point mutations, but no one test may be singled out as best. Although the Salmonella test is the most popular, and has been shown to be the most reliable in certain comparative studies (201, 206), other studies have found certain B. subtilis (226) or E. coli (62) strains to be better. Thus, it must be concluded that a battery of tests is necessary, such an approach being crucial in eliminating false positives and false negatives (62, 201). In addition, while bacterial tests are being developed that detect genetic damage other than point mutations (for example, recombination), they must be integrated with other tests detecting other "high-order" genetic damage to be valid and comprehensive short-term screening procedures.

C) Eukaryotes

Since chemicals are known to cause chromosomal damage (21) testing systems have been developed that utilize eukaryotic cells, which are more characteristic of man. These systems have the additional advantage of detecting the whole spectrum of damage from point mutations and numberical chromosome aberrations (ploidy) to cell transformation, end points which

are related to both environmental carcinogenesis and mutagenesis. While a large number of organisms are used for such testing, the protozoan Tetrahymena pyriformis, is also used in liquid assay and examined cytologically for unequal division of DNA to daughter cells (174, 182) and for growth (184). Four groups of eukaryotes have been utilized most extensively: fungi, higher plants, insects, and mammals.

1) Fungi

a) Yeasts. Yeasts are single-celled eukaryotic microorganisms which may exist as stable haploid or diploid cells. Employing these organisms in short-term screening of chemicals has many of the same advantages as the use of bacteria. In addition, a greater diversity of end points may be detected, including forward and reverse mutations, mitotic or meiotic gene recombination and gene conversion, dominant and recessive lethal mutations, and nondisjunction (see Fig. 1, p. 316 of ref. 148). The two most widely used species are Schizosaccharomyces pombe and Saccharomyces cerevisiae (baker's yeast).

Use of S. pombe has been reviewed (148, 149, 150, 183), and the specific use of $P_1$, $P_2$, and $P_3$ strains to detect forward mutants, convertants, or recombinants is described by Loprieno (148). Of all the yeasts, S. cerevisiae has been reported in mutation studies more frequently than that of any other (39, 40, 111, 140, 150, 183, 195, 196, 203, 204, 223, 272-275). With a considerable number of strains in use, especially JD1, $D_3$, $D_4$, $D_5$, $D_6$, and $D_7$, a wide spectrum of genetic alterations can be detected, including forward mutations (39), reverse mutation (111, 196, 204, 274), and two types of mitotic recombination: reciprocal crossing-over (38, 233, 285, 286) and gene conversion (40, 196, 223, 271, 274). Using S. cerevisiae, Brusick (39) has developed a forward mutation system detecting several mutation types simultaneously, and Prakash et al. (203) have described a sensitive system capable of distinguishing among different types of reverse mutations; this system has yet to be implemented. Brusick et al. (40) have compared the $D_3$, $D_4$, and $D_5$ strains using four known mutagens, and concluded that $D_4$ could be used most economically and that $D_5$ would be the most reliable. Zimmerman (274) described the development of strain $D_7$ for the simultaneous detection of mitotic crossing-over, mitotic gene conversion, and reverse mutation. Zimmerman (275) also provides the most detailed report describing the metho-

dology of such testing, and Parry (196) has recently described a sensitive fluctuation test using strains JD1, $D_4$, and $D_7$ to detect mitotic gene conversion and reverse mutation. In addition, Parry et al. (195) describe the use of strain D-6 to detect nondisjuntion, Puglisi (204) reports on compounds having different effects during meiosis and mitosis, and Koske et al. (140) describe an assay using S. cerevisiae which is conceptually similar to those of Rosenkranz and Kada, measuring the differential killing of DNA repair-deficient mutants and their parental strain. Basically, spot and liquid assays are used, and all three types of metabolic activation, body fluids (227), host-mediated assay (87, 88, 177), and microsomal enzymes (40) have been applied to S. cerevisiae.

b) Other. Generally, tow other types of fungi are used: Aspergillus nidulans and Neurospora crassa.

(1) Aspergillus nidulans. This homothallic filamentous fungus, haploid or diploid, has recently been reviewed as a testing system by Roper (211). As with yeasts, a wide variety of end points may be detected, including point mutations, meiotic and mitotic crossing-over, nondisjunction, translocation and other chromosome aberrations, recessive lethals, and spindle poisoning. There are two systems which may simultaneously detect forward mutation to several distinct phenotypes: (1) strains which become capable of synthesizing methionine and (2) strains becoming resistant to the color-changing effect of 2-thioxanthine. Bignami et al. (20) describe a system useful for its detection of nondisjunction, in addition to crossing over. Again, spot and liquid suspension tests are generally used, and the use of body fluids and microsomal fractions, including an S-9 mixture from the plant Tradescantia, have been applied for metabolic activation.

(2) Neurospora Crassa. While this is a haploid organism, two-component heterokaryons (at least one haploid complement of two different genotypes in the same cytoplasm, but not in the same nucleus as in the case in diploid cells) may be produced (as in Aspergillus) which allow the organism to reflect many of the same genetic effects as diploid organisms (66). While recessive lethal damage over the active genome may be measured, and dominant and recessive lethal damage, in addition at the ad-3 region, characterized, these methods are time consuming and require considerable expertise (for review, see ref. 66). For screening purposes, strain 12 containing a heterokaryon heterozygous for two purple ad-3 genes, ad-3A and ad-3B, is used to detect

forward mutations and deletions (65, 66, 159). After the chemical is tested for cell killing, large flasks are inoculated with conidia and treated with the chemical at different survival levels. Untreated conidia serve as controls. A point mutation in either of the two loci or a deletion of one or both loci results in an adenine-requiring colony which, because it accumulates purple pigment in the mycelium, is readily distinguished from white wild types. This organism has been used with microsomal fractions (156, 157, 193) and in host-mediated assays (120, 158, 177).

2) Higher Plants

A wide variety of plants and plant cells have been used to monitor chemical mutagens, including Arabidopsis thaliana, barley (Hordeum vulgare), Crepis capillaris, Lilium, maize (Zea mays), onion (Allium), peas (Pisum sativum), soybean (Glycine max), tobacco (Nicotiana tabacum), tomato (Lycopersicon), Tradescantia paludosa, and Vicia faba (188, 255). Recent reviews are by Ehrenberg (79), and Nilan and Vig (188). Likewise, the types of genetic damage are diverse: intragenic and extragenic mutations (not well characterized), subchromatid aberrations and gaps, chromosome and chromatid structural alterations, somatic crossing-over and recombinations, nondisjunction (aneuploidy), and euploidy. The more important and promising plant testing systems may be divided as follows:

a) Multicellular. Generally, seed, pollen, or roots are treated. Plants from these sources are scored for somatic or genetic mutations, or mitotic or meiotic chromosome aberrations, in $M_1$ or $M_2$ generations. For example, spots appearing on the leaves of soybean varieties T219 and L65-1237 are considered to indicate somatic crossing-over, nondisjunction, segmental losses and/or point mutations (255). Other plants in which leaf spot somatic mosaicism is produced include tobacco, maize ($Yz_2$ locus), the garden pea, Arbaidopsis (188), and wheat and other polyploids (79). A plant having great potential for monitoring gaseous chemicals such as some air pollutants is Tradescantia. Heterozygous blue stamen hairs and petals are exposed to the test compound with pink and colorless cells are scored as mutants. It is thought that the color change indicates chromosome breakage, gene mutation, chromosome nondisjunction, or somatic crossing-over (235, 250).

In addition to somatic mosaicism, genetic mutations may be determined according to morphological alterations. Chlorophyll-deficient mutants are particularly useful because they are produced by many loci (most not yet mapped), and two particularly important plants for such testing are barley and Arabidopsis (79, 188).

Genetic damage may be detected through cytological examination of chromosomes in addition to morphological alterations. Mitotic cells in roots, embryonic shoots, microspores, and pollen tubes have been studies and Vicia faba (broad or horse bean), Allium cepa (common onion), and Allium proliferum (tree onion) root tips have been particularly useful (137). Meiotic cells may also be studies; one method involves fixing flower buds and then squashing them in acetocarmine stain to examine meiotic events (188).

b) Single Cell. The use of pollen and somatic-cell cultures seems to be particularly promising because they may provide information on plant mutagenesis comparable to that provided by microorganism and mammalian cell systems. In addition, whole plants may be generated and analyzed from these cells (188). Pollen is particularly advantageous because it is produced in large numbers and, being haploid, may express both dominant and recessive mutations (79). Two major characters studies are pollen self-incompatibility and waxiness. Microspore or pollen-tube mitosis may be studied cytologically for chromosome aberrations (188).

The advantages of plants are, then, that they detect a broad range of types of genetic damage, are inexpensive and relatively simple to use, and, especially in the case of seeds, can be subject to a broad range of environmental conditions. Ehrenberg has suggested future plant tests that may be particularly relevant to human exposure: examination of gene duplication, heterochromatin damage, and secondary chemical and physiocochemical changes following primary alkylation of DNA, as well as detection of weak mutagens and their multiple, additive, and synergistic effects (79).

The disadvantages of plant systems include the following: First, one-third of all angiosperms are polyploids and thus fundamentally different from mammals. Second, little is known about the actual nature of many induced genetic alterations. Third, and most important is the difficulty in

extrapolating from plant material to man. Little progress has been made in the problems of implementing the three major systems for metabolic activation. However, the fact that plants themselves may metabolically activate compounds, in particular components of fertilizers, pesticides, and herbicides, to ultimate mutagens or carcinogens recently has become recognized (276). Systems detecting these products are being developed. For example, in one test system using maize, pollen is scored, root tips are examined cytologically; in another system some treated plant material is lyophilyzed and tested in bacteria and yeast mutagenicity assays (102).

3) Insects

Three types of insects are predominant in mutagenesis testing: Drosophila (fruit fly), Habrobracon (parasitic wasp), and Bombyx mori (silkworm).

a) Drosophila. Drosophila is by far the most widely used insect for screening mutagens and carcinogens (1, 86, 144, 169, 231, 233, 254, 257-260); it is claimed that the most rapid and reliable short-term whole-animal tests utilize this organism (258, 270). One of the principal organisms used in both fundamental and applied genetics, it is extremely well characterized, and this makes possible screening for the total spectrum of genetic effects: forward and reverse mutations and small deletions, dominant or recessive mutations, translocation, crossing-over, partial or whole chromosome loss, and nondisjunction (for recent reviews, see refs. 260 and 270). Of the considerable number of tests available for screening purposes, the dominant lethal assay is the fastest, but it also detects many nongenetic effects (for a more detailed discussion, see ref. 260). This review will discuss only the most reliable and widely used screening test, the sex-linked recessive lethal assay (1).

In this test, originally developed by H. J. Müller, standard wild-type strains such as Canton-S or Oregon-R are used, and the procedure generally runs as follows (see Fig. 1, p. 265 of ref. 1): (1) treated males are crossed with females homozygous for a balance (IngB) chromosome lacking a recessive lethal mutant (see ref. 1 for explanation); (2) $F_1$ females heterozygous for the treated X chromosome and the balance chromosome are then mated with males from the same (parental female) stock; and (3) the $F_2$ generation is studied--cultures lacking normal-eyed (non-Bar) males reflect treated X chromosomes

carrying one or more sex-linked recessive lethal mutants. Taking into account the precision with which a great number of loci have been characterized on the four chromosome pairs the usefulness of Drosophila becomes apparent. Notably, it is also this organism that revealed discrepancies (discussed above) between the induction of mutations and chromosome aberrations (231, 234, 257, 260). While the problem of extrapolating to man always remains, Drosophila has correctly detected 55 indirectly acting carcinogens, and it is therefore capable of providing the same activation reactions as mammalian liver fractions (258) in addition to detecting short-lived activation products to some extent. Tests of interest which have been developed recently for Drosophila include one that may illuminate the mechanisms of nondisjunction and another that quickly detects high yields of chromosome aberrations in one generation (233).

While chemicals are generally administered to Drosophila through direct feeding or injection (the former generally being more effective than the latter), Vergburgt and Vogel (254) describe inhalation experiments involving exposure to vinyl chloride. Despite the danger of spiracle-closing by the test organisms (1), this route of exposure may prove particularly useful for assaying chemicals at concentrations and mixtures relevant to human exposure. Thus, large numbers of organisms having short generation times may be tested quickly and economically. Also the combination of Drosophila with mammalian tissue homogenates (270), intraperitoneal fluid, blood plasma, and urine (144) indicates the Drosophila test will soon become even more applicable to use in testing for carcinogens in humans. Drosophila seems to be the ideal organism for verifying the results of quicker bacterial assays and providing a screen for higher level testing. In light of the above, the suggestion should be seriously considered that while a chemical not mutagenic for Drosophila may be so for man, it should be assumed, until proven otherwise, that chemicals mutagenic for Drosophila are mutagenic for man.(1)

b) Habrobracon. This wasp reveals many of the same types of genetic damage as Drosophila, and its use for detection of these lesions has been reviewed by Smith and von Borstel (230). Topical applications of test agents, as well as the usual methods used in Drosophila, allow translocations, recessive lethal mutations, and dominant lethal mutations (particularly rapid and reliable) to be detected using sperm, cells in oogenesis, and eggs.

c) Bombyx mori. Particularly useful for testing weak mutagens, this silkworm has been used especially in a Japanese program screening food additives and other compounds (191, 248). A given compound is injected into wild-type females at a midpupal stage, and is thus incorporated into the developing oocyte. If egg color is changed, mutagenicity is indicated (248).

# V. MAMMALIAN TESTS

## A) In Vitro

The ultimate purpose of short-term screening with submammalian systems is to obtain chemico-biological information that can be extrapolated to higher mammalian organisms, particularly humans. Thus, it is important to understand the differences between mammalian and submammalian cells and organisms that are relevant to the end point responses of genetic toxicology. Malling (160) lists six unique features of human and other mammalian organisms: (1) their relatively high content of repetitive DNA, (2) mutagenically-active immunological mechanisms, (3) mutagenesis that may be induced by virus particles occurring only in mammalian cells, (4) unique susceptibility to particulate materials that may liberate and/or activate enzymes to interact with DNA, (5) unique DNA repair mechanisms, (6) differences within the organism as to cell type and stage. These different biological features restrict the validity of extrapolations from submammalian organisms to mammals including man. Thus, the role for mammalian test systems is apparent and real. A number of test organisms and end points are in use.

1) Cell Transformation. The advantages of studying oncogenesis in well-controlled tissue culture systems has long been recognized, and Berwald and Sachs were the first to develop a quantitative cell transformation assay (19). They showed that hamster embryo cells treated with polycyclic aromatic hydrocarbons piled up in cross patterns in contrast to control colonies and also gave rise to sarcomas when inoculated into hamsters. The great promise of this type of assay is reflected by the considerable number of recent improvements in its methodology (43, 44, 47, 67, 69, 70, 85, 115, 134, 161, 162, 206, 266--for reviews, see 42 and 114). Pienta's study of 58 compounds (200) showed a very high correlation between carcinogenicity and cell transformation. Other studies showed that transformation assays can detect carcinogens almost as well as bacterial mutagenicity tests (30, 68). A more recent study has demonstrated a transformation test that is claimed to be as reliable as bacterial assays (206).

However, cell transformation assays are not without shortcomings (for an excellent discussion, see ref. 96). Technically, cell lines may be hard to maintain and may show a high spontaneous rate of transformation. Also,

many experiments have been criticized because cells were treated for long periods of time before being "transformed" [Di Mayorca, however, reports a method involving short treatments followed by cloning (71)]. The most onerous problem, however, is determining what cell transformation is and distinguishing it from other phenomena. Freeman and Huebner (96) have defined transformation as "the acquisition of a series of abnormal phenotypic characteristics in a cell culture that exhibits normal characteristics under normal conditions," and list the following markers: "changes in morphology (cell type, colony type, loss of contact inhibition, loss of cell orientation, increased number of nucleoli, changes in nuclear-cytoplasmic ratio), increased saturation density, chromosomal aberration, loss of anchorage dependence, immortality in culture, and ability to produce tumors in animals." The last point is taken to be the most reliable indicator of carcinogenicity, and few transformed cells are characterized by all of the above criteria. In actual experiments, multilayer clone piles (loss of contact inhibition), or growth in soft agar commonly indicate transformation (266).

Typically, a cell transformation experiment will be performed as follows: cells are plated in Petri dishes (flasks are also used) in two concentrations, one to test for survival (cloning or plating efficiency--$\sim$ 100 cells) and another to detect transformation ($\sim 2 \times 10^2$ to $10^3$). On the following day the test agent, in solution and in different concentrations for different plates, is added. One to two days later, treated and control cells are washed with a medium change, and subsequent medium changes are made biweekly. Cells plated for survival are generally scored one to two weeks later. The time at which the cells are tested for transformation varies greatly, but is generally between 2 to 5 weeks. Usually cell transformation work is done with epithelial or fibroblast cell types.

a) Fibroblast cells. Most work and practically all technical advances have been with fibroblast and fibroblast-like cells. Hamster embryo cells, used in the original Berwald and Sachs assay, and developed recently by the group of Casto and DiPaolo, in the particular, are used widely (43, 44, 67, 69). DiPaolo and Casto add a virus, usually the oncogenic Simian adenovirus 7 (SA7), to the hamster cells before or after chemical treatment of the cells, and test for viral transformation (43). Much of their work involves studying synergistic effects of viruses, chemicals, and radiation in producing cancer (42, 67, 97). They have tested metals using this system (44), and have also

developed a "host mediated in vivo-in vitro" assay which incorporates metabolic activation by treating female Syrian hamsters in midgestation, excising the embryos, and culturing the embryonic cells to test for transformation (69). Two mouse fibroblast cell lines commonly used are Balb/3T3 (70, 115, 134) and C3H/10T1/2 (115, 161, 162). The latter seems particularly useful because of its low spontaneous transformation rate, and metabolic activation systems for this line include liver homogenates (115) and rat and mouse feeder cells (161). Rat embryo fibroblasts (67) and guinea pig fetal cells (85) have also been utilized, although the latter may need a long time for indication of transformation and cannot necessarily produce tumors when transformed.

b) Epithelial cells. Since a great majority (over 85%) of human cancers are carcinomas (from epithelial cells) rather than sarcomas (arising from fibroblast tissues), it seems necessary to develop systems for testing epithelial cell transformation. Such systems are difficult to construct, however, for the following reasons: (1) epithelial cell lines are difficult to maintain in a differentiated state; (2) there may be a long period between exposure to the chemical and actual transformation; (3) it is impossible to determine when morphological transformation has taken place, and a culture must be tested by inoculation into livers of living rodents. Generally, epithelial cells from the rat liver are used, but no such systems are yet suitable for routine screening (266). Another recently developed and promising cell transformation test not yet ready for screening purposes involves the detection of altered cytoskeletal patterns through anti-actin and anti-tubulin immunofluorescence (32, 98). Another promising epithelial cell system that may have application to carcinogenic screening has been developed from a cell line derived from a teratoma (209).

In summary, while cell transformation testing methodology must be validated and standardized (as to end points in particular), constant improvements will most certainly insure that this assay will be a vital and integral part of future screening programs.

2) Cell-mediated mutagenesis. Rapid developments in recent years in the field of mammalian somatic cell genetics have produced a number of short-term tests which may provide a valuable bridge between microorganism and whole mammal assays. The four principal types of end points used to detect

both forward and reverse mutations are morphological, biochemical, serological, and radiation-sensitive markers (46); those generally used for the two cell lines described below involve drug resistance and absence or presence of the enzyme HGPRT (hypoxanthine-guanine phosphoribosyl transferase), a system which Szybalski and Szybalska (244) first developed using the human cell line D98. This enzyme allows cells, through ribose phosphorylation, to utilize hypozanthine and guanine (and their analogs) such as the drug 8-azaguanine (8-azg). The HGPRT gene is particularly suitable because it is X-linked and thus functionally haploid (hemizygous) in mammalian cells.

The experimental design for cell mutagenesis testing is similar in many ways to that of cell transformation assays. Petri plates or liquid suspensions, including the fluctuation test, are utilized, and the compound is added to cells being tested for both survival or mutation, before or after plating or placement in suspension. In contrast to the transformation assays, cells may be treated for only one to several hours, the medium changed, and after an appropriate expression to allow for fixation and expression of the mutant phenotype, the selective agent (e.g., 8-azg) added for up to several days. In 8 to 10 days after treatment colonies are scored for mutations. Generally, the HGPRT locus is exploited through the use of two cell lines, Chinese Hamster V79 and mouse lymphoma L51787.

a) Chinese Hamster V79 Cells. Huberman has done much recent testing of polycyclic aromatic hydrocarbons requiring metabolic activation for mutagenicity and carcinogenicity (122-125); cells are scored for 8-azg resistance, and metabolic activation is adequately provided by lethally irradiated rodent embryo "feeder" cells. Three other markers, temperature resistance (123), ouabain resistance (10, 123), and 6-thioguanine (6-TG) resistance (141, 192), are also being employed. Arlett et al. have compared ouabain and 8-azg resistance, finding general agreement between the two end points, but better detection of many weak mutagens, with less intrinsic variability, by the former. They conclude that the two systems should complement one another (10). Krahn and Heidelberger (141), testing polycyclic aromatic hydrocarbons and aflatoxins, have included the S-9 fraction to detect 6-TG resistance, and O'Neill and Hsie have quantified the detection of this form of resistance (192).

b) Mouse lymphoma L51787. Clive et al. describe the use of the HGPRT locus with this cell line (49), compare its utility to that of the enzyme thymidine kinase (TK) locus (49, 50), and conclude the latter is more suitable. The TK locus detects weak mutagens (49), possibly even threshold levels, and involves considerably less labor (266). Fischer and his group (91, 92) have long been developing a host-mediated assay with this cell line, using resistance to methotrexate, arabinoside, and high concentrations of thymidine to detect forward mutations.

3) DNA repair. The correlations among primary DNA damage, mutagenesis, chromosome aberrations, and carcinogenesis have been discussed above. The fact that almost all DNA-damaging agents with the exception of some intercalators and metal carcinogens show evidence of a repair effect (208) indicates that the development of tests for DNA repair capacity may have relevance to tests for genetic damage and carcinogenesis. Another line of evidence for the importance of repair is the fact the cells of individuals having the hereditary disease xeroderma pigmentosum (XP) (which causes sensitivity to UV and is correlated with a high incidence of skin cancer) are deficient in the repair of UV-induced DNA damage (208, 222). Most rapid DNA repair assays utilize human cells. Stich (238) emphasizes that while microbiological systems and Drosophila are better for actually detecting mutagenic and carcinogenic compounds, these assays should logically be developed to assess the actual hazards of these compounds to human health. Regan and Setlow (208) have reviewed the following DNA repair assays: unscheduled DNA synthesis (UDS), thymidine uptake, repair replication, exicision of UV-induced pyrimidine dimers, and photolysis of 5-bromodeoxyuridine. This review will concentrate on describing and evaluating the most widely used system, unscheduled DNA synthesis.

The UDS assay, originally developed by Rasmussen and Painter (207), has been updated by Stich and co-workers (215, 236, 238, 240). The procedure, described by San and Stich (215), is described as follows: cells from normal humans or from patients with diseases such as XP are first subjected to survival tests to determine the proper concentration of compound to be added. For the DNA repair assay, their division is inhibited so that normal DNA synthesis is not confused with repair synthesis. Different concentrations of the chemical are added, generally for 1-1/2 to 5 hours, and tritiated

thymidine ($^3$HTdR) is added simultaneously with, or immediately following, chemical exposure. Autoradiograms are prepared (this may take several days) which reveal heavily labeled nuclei in the S phase and lightly labeled (5 to 40 silver grains per diploid nucleus above background) nuclei which represent nonscheduled repair synthesis of DNA. This test has been validated with over 100 compounds for detecting carcinogens (240). Further, it does not detect substituting compounds (e.g., BUdR), or intercalating agents (e.g., ethidium bromide) which are mutagens but supposedly not carcinogens (238). Tonomura and Sasaki, using normal and XP human cells, have demonstrated a good correlation between chromosome aberration and unscheduled DNA synthesis (249).

The UDS assay is promising because small numbers of cells can be used, a broad range of DNA damage is detected, and cultured cells, biopsy material, or peripheral lymphocytes (146) may be used. This ability to test a variety of materials, in particular, makes possible the assessments of human exposure and the comparisons of different human populations and subpopulations. Nevertheless, this test also has its shortcomings. Technically, it may take time to determine the proper concentration and time exposure of the compound to be applied. The possible modes of DNA-compound interactions triggering repair are as yet unknown. Unscheduled DNA synthesis may be affected by other factors such as inhibition of repair enzymes, a gene defect, a change in $^3$HTdR uptake. Finally, there reamins, even with human cells, the problem of metabolic activation. Human cells in vitro may activate only certain compounds, and for this reason both the S-9 mixtures (215) and the host-mediated assay (237) have been combined with unscheduled DNA synthesis assays.

The UDS assay is often used in combination with a more direct method of determining DNA damage, such as DNA fragmentation (239, 240) or alkaline sucrose gradients (197). The UDS assay is, however, less sensitive, very qualitative, and can give false results (238). Two of the other more important repair assays are repair replication (208) and 5-bromodeoxyuridine photolysis (208,222). Painter has recently developed a novel assay in which a decrease in DNA synthesis in human HeLa cells, as measured by thymidine uptake following treatment by a test chemical, reflects damage to DNA (194).

Although many DNA repair tests are currently operable, most need further refinement, and the only such assay being used in a number of screening programs is the unscheduled DNA synthesis test.

4) Chromosome aberrations. Short-term screening tests detecting chromosome aberration may be made in vitro or in vivo. Recent reviews are by Cohen and Hirschhorn (51), Evans (84), Russell (213), and Savage (217). A great variety of aberrations, numerical and structural (both chromatid and chromosome types), can be detected at different parts of the cell cycle and at nuclear division, principally at metaphase, anaphase, and interphase. (1) Metaphase: This is traditionally the phase in which chromosomes are scored, and provides detailed information on the types of chromosomal abnormalities and on ascertaining which part of the cell cycle is being affected. However, this classification of aberrations is time consuming and requires experienced personnel even when expensive automated image processing devices are employed. Sister chromatid exchanges (SCE) (described below) are detected in this phase and are sensitive indicators of damage. (2) Anaphase: Although discriminating poorly between aberration types, observations at this phase are fast and simple and may detect effects on the spindle apparatus. Analysis at this phase detects only a fraction of the aberrations detected in metaphase, and mistakes in scoring can easily be made (for example, mistaking a lagging short chromosome for a fragment). (3) Interphase: Micronuclei, described and evaluated below with the other in vivo bone marrow tests, may be detected in this phase.

Despite the fact in vivo and in vitro short-term chromosome aberration screening tests are among the most commonly employed, they do have significant disadvantages. It has already been noted that (1) scoring is generally only qualitative, and it may be difficult to determine certain aberrations, and (2) these methods may be very insensitive, and do not necessarily correlate well with other end points, showing many false negatives in mutagenicity testing, and many false positives in carcinogenicity testing. The use of abnormal aneuploid cell lines, such as HeLa cells, has been criticized by some workers (268).

Rapid in vitro cytological testing may be divided into two categories according to the type aberrations scored: gross chromosome aberrations and sister chromatid exchanges.

a) Gross chromosome aberrations. Two types of cells are most frequently used, Chinese hamster (128, 271) and small lymphocytes in peripheral blood (190, 271). Ishidate and Odashima describe a series of experiments in which a subline of a Chinese hamster fibroblast cell line is subjected to 134

chemicals; slide preparations are made 24 and 48 hours after treatment, and metaphases are analyzed (128). Small lymphocytes of peripheral blood are the most widely used cell type in both in vitro and in vivo cytogenetic assays. Zharkov and Yakovenko (269) note that the use of these cells in vitro is quite simple, can be combined with a wide range of concentrations of chemicals, in contrast to in vivo testing, and, in the case of short-term cultures, is a good model of human tissue. They make several recommendations for the use of this method, and conclude that since these cells cannot detect clastogens requiring metabolic activation, they should be complemented by in vivo bone marrow assays. [Any agent which can cause chromosome breaks and aberrations is termed a clastogen by Shaw (224)].

b) Sister chromatid exchange. As noted above, sister chromatid exchange, a reciprocal and symmetrical exchange at homogenous loci between sister chromatids, is quicker and perhaps easier to detect than other metaphase changes. The pehnomenon was originally discovered in plant somatic cells by Taylor (247), and the assay has developed to the point of being more sensitive and quantitative than the other metaphase assays, and can be used both in vitro and in vivo. In in vitro tests, Chinese hamster (198) or human cells have generally been used. More recent techniques do not use autoradiography and radioisotopes: treated cells are grown in a medium containing 5-bromodeoxyuridine for two rounds of replication so that one chromatid is substituted unifilarly while the other is busstituted bifilarly. In the fluorochrome plus Giemsa (FP6) technique of Perry and Wolff (199), these stained strains are then applied to the "cold" culture medium, with the result that the two chromatids stain differentially and SCE is detected.

Despite its numerous advantages the SCE assay still required validation. It must be remembered that SCE is but one type of aberration, and it remains to be determined how well it correlates with the majority of chromatid and chromosome aberrations.

B. In Vivo

A considerable number of in vivo tests, notably the dominant lethal (17), specific locus mutation (45), and heritable translocation (145) assays, are used in testing for genetic damage and cancer, but are not dealt with in this review because they are too expensive and time consuming to be considered for short-term screening purposes. In contrast to the in vitro methods, which have been developed to screen new compounds or detect carcinogenic or genetic

hazards in complex mixtures such as air pollution, short-term *in vivo* assays measuring chromosome aberrations are employed to monitor human populations for genetic damage.

For example, Kilian and Picciano (138) describe methods for monitoring industrial populations through blood sampling of individuals at regular intervals, followed by metaphase analysis of whole blood samples, and the use of computers to analyze results. Generally, however, when blood samples are taken, cells from the lymphopoietic system (84, 147) or, especially, small leukocytes of the peripheral blood (18, 180, 190) are analyzed. When used for monitoring purposes, such analyses involve many complications, such as difficulties in assessing dosage, metabolic activation or inactivation, the problems of suitable controls, and the synergistic effects found in the complex mixtures of compounds in the environment.

*In vivo* tests developed to detect specific clastogens have been provided with various forms of metabolic activation. Brewen describes a host-mediated cytogenetic assay in which freshly drawn human peripheral lymphocytes are prepared and slipped into the peritoneal cavity of a rodent host, which is then treated intravenously or intraperitoneally with the test compound. Two to three days later the cells are harvested from the sacrificed host and metaphase cells are analyzed (26). Lilly *et al.* describes a procedure in which a rat is given the test substance through intraperitoneal injection, killed 6 hours later, and lymphocytes withdrawn and put into culture. The metaphase chromosomes of lymphocytes are subsequently analyzed, and the use of the SCE method is suggested (147).

Bone marrow cells may also be separated from human or other mammalian bone to be studies cytogenetically. As with lymphocytes, metaphase (84, 216, 218) and anaphase (84, 218) analyses may be performed, and a particularly interesting assay, developed by Schmid (219, 220), involves the detection of interphase micronuclei. The source of these micronuclei is chromatin that, lagging in anaphase, is included in a daughter cell. This material may form secondary nuclei. These, or "Howell-Jolly bodies," are best detected in young erythrocytes which have just expelled their nuclei. The test runs as follows: the mammal, usually a mouse, is given the compound, and 30 hours after the administration the animal is sacrificed, marrow is taken from the femora, and the material is prepared for visual scoring. This method is quick and easy to implement, and in addition, has the following advantages:

detection of damage is not limited to mitotic cells, many cells can be scored, and detection of impairment of the spindle apparatus is possible. Limitations of the micronucleus test are that compounds not reaching the bone marrow cells are not detected and that the test has not been in use long enough to determine its correlation to metaphase and anaphase analyses. Goodman and co-workers have compared the mutagenic effects of nitrofurans by the micronucleus, Salmonella, and cytogenetic tests (104).

Other pertinent in vivo cytogenetic tests include the well-known miotic fluid test and meitoic cell (spermatocyte and oocyte) analysis (51). The measurement of DNA repair synthesis and DNA fragmentation, as discussed above, also provides in vivo genetic end points.

There are many rapid in vivo tests characterized by indirect indicators. For example, the implant (206) and mouse sebaceous gland tests (24) have been developed to detect carcinogens. A promising test developed by Bruce is the sperm abnormality assay (37). Important because it detects activity within the germ cells in vivo, it correlates well with micronucleus and Ames assays (113). Shaw's chromosome banding techniques have been used to identify presence of genetic diseases (225), and the concept of identifying a mutagenic effect in this way is an intriguing possibility.

Finally, although this review has concentrated on tests involving biological specimens and substances, tests based purely on indications that are not biological in origin have been suggested. The concept is based on the hypothesis that the carcinogenic compounds have certain chemical properties that allow them to produce a carcinogenic response in a biological system (see refs. 155, 171, 173). Purchase and co-workers (206) found acridine and iodine color tests and a piperidine alkylation test to be unsuitable, although they gave no specific reason for their judgment. On the other hand, they found that the test of Williams and Rabin (267) correlated well with known carcinogens and noncarcinogens. This test is based on the tendency of carcinogens to degrade when placed in association with hormone-dependent membranes containing polysomes.

# VI. DISCUSSION

Having viewed the advantages and disadvantages of the more prominent and promising short-term screening assays, overall problems and limitations in their use can be assessed. The following is a brief discussion of some of the major problems:

1) Metabolic activation and mutagen specificity. This problem has already been mentioned in detail above. It may be added to the presentation above that in addition to the above metabolic activating systems, Sugimura et al. (243) have suggested the use of a "cocktail mixture" of enzymes from various sources to provide more adequate activation and the use of mixtures of S-9 from different organs to determine organ specificity.

2) End point and test correlations. Of both basic and applied interest, correlations (as well as validations of individual systems) must be made through more comparative experiments involving a greater variety of compounds. To this end, Sobel's modified "parallelogram," in which different end points are compared at different concentrations (234), may be applied.

3) Correlating observed and relevant effects. Auerbach's opinion that point mutations and small deletions are the most relevant forms of genetic damage (14) and the importance of nondisjunction are discussed above. Progress toward the solution of this problem requires further development of short-term assays and research assessing relevant mechanisms and pathways of genetic damage.

4) False positives and false negatives. It is important to eliminate as much as possible both these types of spurious results. False positives may incorrectly indict useful chemicals. Purchase et al. (206) have shown how in the testing of 1000 chemicals, 10 of which are carcinogens, an assay 90% accurate will give 108 positives (of which only nine would be carcinogens), thus giving 99 false positive results. The consequences of the false negative are even more grave, in that further testing seems not to be indicated when in fact it should be done. The elimination of both false positives and false negatives must be brought about through an improvement of

not just one, but a whole battery of short-term screening tests, and proper verification with the longer-term whole mammal tests and epidemiological studies.

5) Quantitative determination of dosage effect. To help in establishing regulatory guidelines, dose-effect curves for chemicals must be established so that basic units may be used in determining exposure hazards. Two such units, the rate-doubling concentration and the rem-equivalent-chemical have been suggested (74). Both have been roundly criticized (14, 232) because present assay systems, in particular the short-term tests discussed above, provide only qualitative information concerning the effects of compounds.

6) Chemical synergism and antagonism. The interactions of chemicals in complex mixtures (81, 82, 83) must be considered. For example, an inactive chemical may activate or enhance the effects of another, or a chemical may inactivate a normally active chemical.

7) Test improvement. Short-term screening tests for mutagens and carcinogens must be improved and expanded in several ways. First, they must be able to detect a greater variety of compounds, including, for example, hormones, plastic films, etc. (243). Second, they must be expanded to detect a broader spectrum of end points. Examples include cytogenetic damage and, in submammalian testing especially, structural and numerical chromosome aberrations (276). Thirdly, the relationship of mutagenesis as detected by short-term tests to teratogenesis must be investigated (135). Finally, short-term test must be expanded in their application. The utility of these tests for assessing human hazards is continually emphasized (59), but less mention has been made of their use in testing for the impact of chemicals on other organisms and whole ecosystems.

The proper place of these short-term screening tests for genetic damage and cancer tests in overall testing schemes should be reemphasized. These assays are only qualitative indicators of compounds of potential harm and thus are implemented as primary screens in programs testing large numbers of chemicals. Negative results cannot be taken to mean that a given compound is safe, and positive results should be used only to establish priorities for longer-term testing and epidemiological studies.

The Japanese-American cooperative program (62) mentioned above went far in comparing and validating these test, and there are a large number of programs currently under way which are improving these short-term assays. Considerable research is taking place in academic, private, and government institutions. For example, Dr. Virginia Dunkel, of the National Cancer Institues, is coordinating a program to validate several of these tests (76). Also, the National Center for Toxicological Research is involved with test methodologies (93). A number of industries are using short-term tests both to test new chemicals and to monitor for worker exposure, and many institutions have developed capabilities to implement such tests on a contract basis (94).

The different governmental agencies which write the use of these assays into regulatory policy have acted inconsistently in the past. While the Environmental Protection Agency (EPA) and the National Institute of Environmental Health Science (NIEHS) are preparing for a large-scale use of such assays, the Food and Drug Administration (FDA) is somewhat more cautious, and the Occupational Safety and Health Administration (OSHA) is paying little heed to such testing (94).

It is clear that no one perfect test exists and that a series of tests measuring different end points is desirable (61). Many integrative schemes have been proposed to carry out the task of testing comprehensively both the chemicals currently being used and new ones being developes (23, 24, 25, 27, 28, 31, 48, 54, 221). Bridges' "three-tier" system is the most well known (27, 28, 29, 31), and many more will undoubtedly be proposed. As discussed above, different testing programs have different goals, and the tests most appropriate for the aims of the specific program should be considered. Since the development of these short-term assays if progressing rapidly, a program should remain flexible enough to change assays where indicated.

# VII. CONCLUSION

Short-term screening assays for genetic and related damage possess numerous limitations. Particularly important are the problems of metabolic activation, extrapolation to the levels and mixtures of compounds to which humans are exposed, and assaying for the relevant types of damage. Despite these shortcomings, the vital role these assays may play in a comprehensive screening program is emphasized. Only through the continued use and improvement of these short-term assays will it be possible to screen the thousands of man-made chemicals in the environment and detect those compounds which are toxic, mutagenic, and carcinogenic and which have an impact on the irreplaceable genetic constituency as well as on the health and well-being of humans and the entire ecosystem of the planet.

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TABLE 1. Comparison and evaluation of short-term test systems.

| | TEST SYSTEM | END POINTS | ADVANTAGES | DISADVANTAGES |
|---|---|---|---|---|
| I. | DNA | Physical changes in DNA. | Quick; quantitative. Variety of methods can be used to detect physical changes in DNA. Some subtle physical changes in DNA can be converted to recognizable easily measurable end points. | Only a biological test can tell conclusively whether any particular DNA alteration is mutagenic (but all biological responses are not equivalent). Physical tests can give indications (only) of possible mutagenic action. |
| | | DNA transformation. | Transformation is stable and allows a precise assay. Mutagens can be detected and quantitative values assigned to their relative strengths. Very low transformation frequencies can be evaluated under certain conditions. | |
| | | Bacteriophage genetics (can also be RNA or DNA). | Easy to culture; short generation times. Rapid, reliable, very inexpensive. Many different strains possessing various genetic markers are available. Very simple genetic material. Can reproduce like cells. | Different strains have different drawbacks: some types of mutations cannot be detected in certain strains. Processes that produce mutations in some organisms may cause lethality in some bacteriophage. May lack some cellular functions, e.g., DNA repair. |
| II. | Bacteria | Mutagenesis. Growth rate changes/ lethality. Effects on sporulation. | Sensitive; quick; determine need for metabolic activation; inexpensive; easy to handle. Growth inhibition/lethality tests assume that the factor that causes mutation also sensitizes to toxicity. This is often true. | Certain cells are insensitive to certain classes of compounds. Limited to certain kinds of genetic lesions. Prokaryotic may be genetically different from higher cells. Most mutagenic systems measure only reverse mutations. |
| III. | Fungi | Forward/reverse mutations. Mitotic crossing over. Mitotic gene conversion. Specific locus test. Nondisjunction. Recombination. Various chromosomal aberrations. | Sensitive; measure many end points; eukaryotic; inexpensive. Good all-around system in that several end points can be measured by the same organism in the same test application. | Tests can be very time consuming and tedious. |
| IV. | Plants | Forward/reverse mutations. Chromosomal aberrations. | | Tests can be very slow. |
| V. | Insects | Forward/reverse mutations. Specific loci. Dominant lethal test. Translocation. Deletions/duplications. Nondisjunction. Recombination and crossing over. Heterochromatic deficiencies. Deletions, translocations. | Process microsomal enzyme activating systems. Can screen many genes. A complete system. | Takes up to 6 weeks to complete a test. Expensive. |
| VI. | Mammalian- in vitro | Cell transformation. Forward/ reverse mutation. Translocation/ deletions/nondisjunction. Unscheduled DNA synthesis, DNA repair. | Cells are easier to handle than animals. | Skilled workers needed. Expensive. Activating systems may have to be provided. |
| VII. | Mammalian - in vivo | Specific locus. Dominant lethal. Translocations. Deletions/duplications. Nondisjunction. Micronucleus. Sperm abnormality. | Shows the response of the entire animal system, therefore better approximating the human response. | Weeks to months to obtain results; may require especially well-trained manpower (biological and statistical). |