UNIT 4(B)

With medical science and technology having conquered the major infectious diseases in all but the most impoverished countries of the world, cancer has become one of the most feared diseases of humanity. A large body of research has focused attention on the contribution of environmental carcinogens to the incidence of cancers in humans, and this, in turn, has sparked concern about the safety of chemicals that are introduced into the environment.

THE ANIMAL TEST(S)

The conventional test for carcinogenicity is the long-term rodent carcinogenicity bioassay described in Organisation for Economic Cooperation and Development (OECD) Test Guideline (TG) 451. The objective of this test is "to observe test animals for a major portion of their life span for the development of neoplastic lesions during or after exposure to various doses of a test substance by an appropriate route of administration." The study is usually conducted using two species – rats and mice of both sexes. The animals are dosed by oral, dermal, or inhalation exposures, based upon the expected type of human exposure. Dosing typically lasts around two years. Certain animal health features are monitored throughout the study, but the key assessment resides in the full pathological analysis of the animal tissues and organs when the study is terminated. Long-term animal bioassays provide the most widely accepted experimental evidence that a chemical is a carcinogen-at least in the animal species used in the test-and have traditionally been relied on for determining a chemical's carcinogenicity for regulatory decision making. However, these tests are very expensive (of the order of $0.5 million) and extrapolation of the results to dosages corresponding to expected human exposures is by no means certain. Similarity of molecular structure of a suspect chemical to that of known carcinogens is a rough indicator of potential carcinogenicity and may be used as a basis for undertaking carcinogenicity testing, but is certainly not definitive with respect to either carcinogenicityor noncarcinogenicity.

The use of short-term in vivo or in vitro tests with mammalian or bacterial cells is becoming increasingly important for screening large numbers of chemicals for potential carcinogenicity (Kolbye, 1980; Waters et al., 1980; McCann, 1982). The underlying theory is that most carcinogens are mutagens and that a substance that alters DNA and/or causes gross chromosomal aberrations in such cells must, in the absence of strong counter evidence, be taken as a potential carcinogen. The virtue of these tests is that they are relatively inexpensive. Their principal limitation is that although most carcinogens are mutagens, not all mutagens are carcinogens. Thus, one must be careful not to be misled by false positive, as well as by false. negative, results. The tendency now is toward subjecting a suspect chemical to a number of such tests in a structured testing scheme and weighing the evidence derived from the entire set of tests.

As suggested by the previous paragraphs, current emphasis in research on the detection of carcinogens by toxicological testing is in the direction of using combinations of short-term in vitro and in vivo tests to avoid having to rely solely on expensive long-term chronic studies for determining carcinogenicity. In vitro tests are designed to detect evidence of mutagenicity at the cellular or subcellular level. In vivo tests can also be performed at the cellular or subcellular level, although limited (or short-term) in vivo bioassays are used to detect oncogenic, or tumorogenic, effects in whole animals. Over 100 mutagenicity tests have been developed for the detection of mutagenic carcinogens. A number of these are currently under evaluation in EPA's Gene-Tox Program, among others. They are evaluated quantitatively in terms of their sensitivity, specificity, accuracy, and predictive value. Sensitivity is a measure of a test's ability to give a positive result for a carcinogen. A test having a sensitivity of 0.90 would give positive results for 90% of all carcinogens tested; only 10% would be false negatives. The specificity of a test measures its ability to give negative results for noncarcinogens; thus, a specificity of 0.85 means that of a group of noncarcinogens, 85% would give negative

results and only 15% of the results would be false positives. The accuracy of a test is the ratio of the number of correct results, for both carcinogens and noncarcinogens, to the total number of substances tested. The predictive value of a test is given by the ratio of the number of correct positive results to the total number of positive results obtained. The performance of a number of short-term tests and limited in vivo bioassays. By and large, the individual shortterm tests have different endpoints, but all are relevant to postulated mechanisms of carcinogenesis. The endpoint of all but one of the limited in vivo bioassays (ironresistant liver foci) is the actual development of a tumor. The term carcinogens, refers to substances that have been shown to be carcinogenic in animals. The carcinogens used in individual studies may have included one or more of the 12 known human carcinogens. Before reaching any conclusions based, several features about them warrant mentioning. Among the rapid short-term tests, the Ames Salmonella test has been investigated with many more substances than has any other single test. As a group, the limited in vivo bioassays have been investigated with far fewer substances than have the rapid short-term tests. In general, far fewer noncarcinogens have been tested than carcinogens; thus the number of potential false positives for any test has been constrained and the test's predictive value, as suggested by these data, may be too high. Nevertheless, the data disclose that a number of the tests that have been evaluated with over 25 carcinogens have sensitivities of 0.90, or greater, and rather high predictive values. Their specificities, while also high, are less credible because of the smaller number of noncarcinogens tested. In view of the many different types of carcinogens, it would not be reasonable to expect any individual short-term test to give a positive result for all carcinogens. Instead, it is being proposed by toxicologists that a suspected carcinogen be subjected to several tests, selected on the basis of the substance's most likely mode of carcinogenic action, and that the rapid short-term tests be supplemented by limited in vivo bioassays, which would be capable of disclosing the actual induction of tumors in whole animals.

REGULATORY REQUIREMENTS & TEST GUIDELINES

The UN Globally Harmonized System (GHS) classifies carcinogens under two categories based on the strength of the evidence: Category 1 chemicals are known or presumed human carcinogens (Category 1A if based on human data and 2A if based on animal data); Category 2 chemicals are suspected human carcinogens (UNECE, 2004, p. 167). According to GHS guidance, chemical-induced tumorigenesis involves genetic changes; thus, chemicals that are mutagenic in mammals may warrant being classified as carcinogens.

The GHS describes other "important factors" to be taken into consideration in carcinogen hazard classification, such as the location and number of tumors, tumor type and characteristics, responses in both sexes and/or multiple species, relevance of the mode of action to humans, and more. The OECD's guidance on these factors is provided in the 2001 ***Harmonized Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures*** (ENV/JM/MONO(2001)6), and in the 2005 ***Proposal for Guidance on How to Consider Important Factors in Classification of Carcinogenicity***(ENV/JM/HCL(2005)2/REV). The 2005 OECD guidance discusses various frameworks for assessing the "important factors" and states that "the weight of evidence analysis called for in GHS is an integrative approach which considers important factors in determining carcinogenic potential along with the strength of evidence analysis."

OECD TGs 451, 452, and 453 provide information for conducting carcinogenicity and chronic toxicity studies. The OECD ***Guidance Notes for Analysis and Evaluation of Chronic Toxicity and Carcinogenicity Studies***(ENV/JM/MONO(2002)19) "provides broad guidance on approaches to hazard assessment and on some of the problems and pitfalls that may arise during an assessment…."

The US Environmental Protection Agency's (EPA) revised its ***Guidelines for Carcinogen Risk Assessment***(EPA/630/P-03/001B) in 2005. The revised guidelines use five descriptors (Carcinogenic to

Humans, Likely to be Carcinogenic To Humans, Suggestive Evidence of Carcinogenic Potential, Inadequate Information to Assess Carcinogenic Potential, Not Likely to Be Carcinogenic to Humans) that are followed by a weight of evidence narrative to describe the carcinogenic potential of a substance. The EPA provides additional information on its Web page ***Evaluating Pesticides for Carcinogenic Potential***.

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (**ICH**) and drug regulatory authorities provide guidance on testing for the carcinogenic potential of new drugs. Long-term toxicity studies such as carcinogenicity testing are usually conducted concurrently with clinical trials. Jena, et al. (2005) provide a good overview of carcinogenicity testing for drug development.

The **International Agency for Research on Cancer** (IARC), part of the World Health Organization (WHO), provides ***Monographs on the Evaluation of Carcinogenic Risks to Humans*** and has evaluated the carcinogenic risk of more than 900 substances. "The IARC Monographs are recognized as an authoritative source of information" and may be used by national and international authorities in making risk assessments.

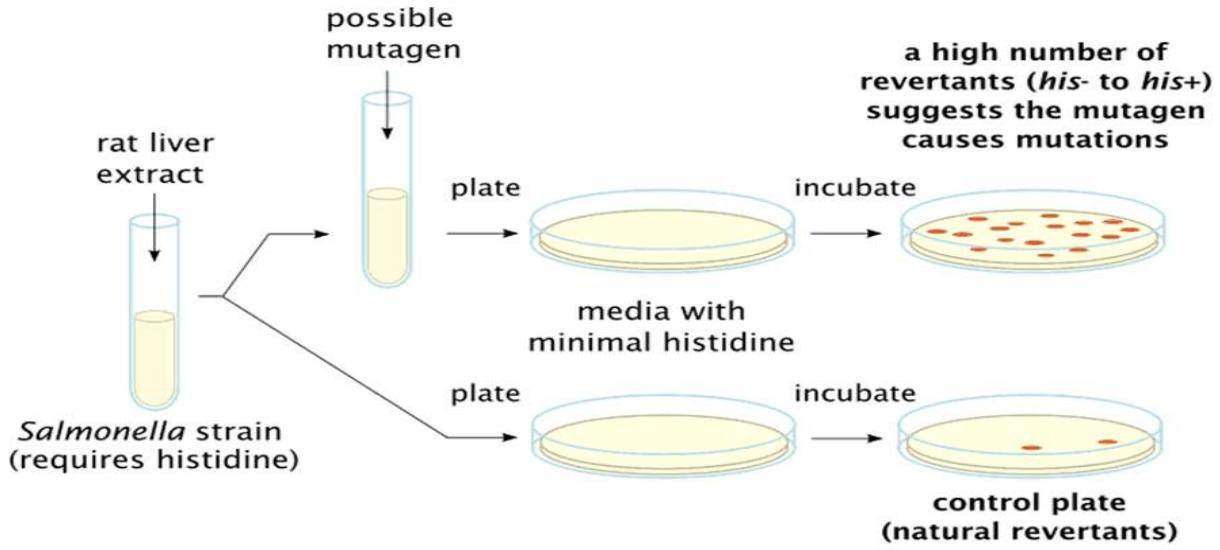
TEST SYSTEMS FOR CARCINOGENICITY ASSESSMENT

Numerous in vivo and in vitro experimental systems are available to assess the potential carcinogenicity of chemicals. The types of tests available to identify chemicals with carcinogenic potential can be classified into general categories, based on the duration required to conduct the test . Short-term tests are typically of the duration of days to a few weeks, intermediate-term tests last from weeks up to a year, while chronic long-term tests usually encompass 6 months to 2 years exposure to a chemical. These bioassays use bacterial and mammalian targets.

Short-term tests for mutagenicity were developed to identify potentially carcinogenic chemicals based on their ability to induce mutations in DNA either in vivo or in vitro. A variety of in vivo and in vitro short-term tests are available to test the potential carcinogenicity of a chemical. The majority of these tests measure the mutagenicity of chemicals as a surrogate for carcinogenicity.

Therefore, while they are usually very predictive of indirect acting and direct acting (if a metabolic source is provided), these tests routinely fail to detect nongenotoxic carcinogens. In Vitro Gene Mutation Assays The most widely used short-term test is the Ames assay (Ames *et al*., 1975). The relative simplicity and low cost of the test make it a valuable screening tool for mutagenic carcinogens. *Salmonella typhimurium* strains, deficient in DNA repair and unable to synthesize histidine, are used. In the presence of a mutagenic chemical, the defective histidine gene can be mutated back to a functional state (*back mutation*), resulting in a restoration of bacterial growth in a medium lacking histidine. The mutant colonies, which can make histidine, are referred to as "revertants." The Ames test in basic form can detect direct-acting genotoxic carcinogens. With the inclusion of a metabolic source, specifically the 9000g supernatent (S9) of a rat liver homogenate to promote metabolic conversion of the chemical, the Ames test can also detect indirect-acting genotoxic carcinogens. Figure 8-28 describes the standard method used for performing the Ames assay. Genetically unique strains of the *S. typhimurium* bacterium have been developed for determining specific mutational targets. Strains TA100 and TA1535 are able to detect point mutations, whereas strains TA98, TA1537, and TA1538 are able to detect frameshift mutations. Chemicals are typically tested at several dose levels (usually five or more) and the mutation frequency (number of revertants) is calculated. Activation-independent (e.g., sodium azide and methyl methanesulfonate) and activation-dependent (e.g., 2-aminoanthracene) positive controls are included in each assay. The mouse lymphoma assay is a mutagenicity assay used to determine whether a chemical is capable of inducing mutation in eukaryotic cells. Typically, mouse lymphoma L5178Y cells are used, and the ability of the cell cultures to acquire resistance to trifluorothymidine (the result of forward mutation at the thymidine kinase locus) is quantified. Another mammalian cell mutation assay, the

Chinese hamster ovary (CHO) test, is also commonly used to assess the potential mutagenicity of chemicals. This assay uses the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene as the end point. Cells are treated with the test chemical and then placed into suspension with selective medium for replication and fixation of induced mutations. Cells are then plated for colony growth, and after several days, colony numbers and colony size are recorded. The number of mutant colonies is a measure of the ability of the test chemical to induce a genetic change at the thymidine kinase or HGPRT loci in these transformed cells. As with the Ames assay, these assays are frequently performed in the presence of an exogenous metabolic source (e.g., irradiated epithelial cell feeder layer). Because not all carcinogens are mutagens and/or directly damage the DNA, the concordance with the chronic in vivo bioassay for these mutagenicity assays is relatively low.

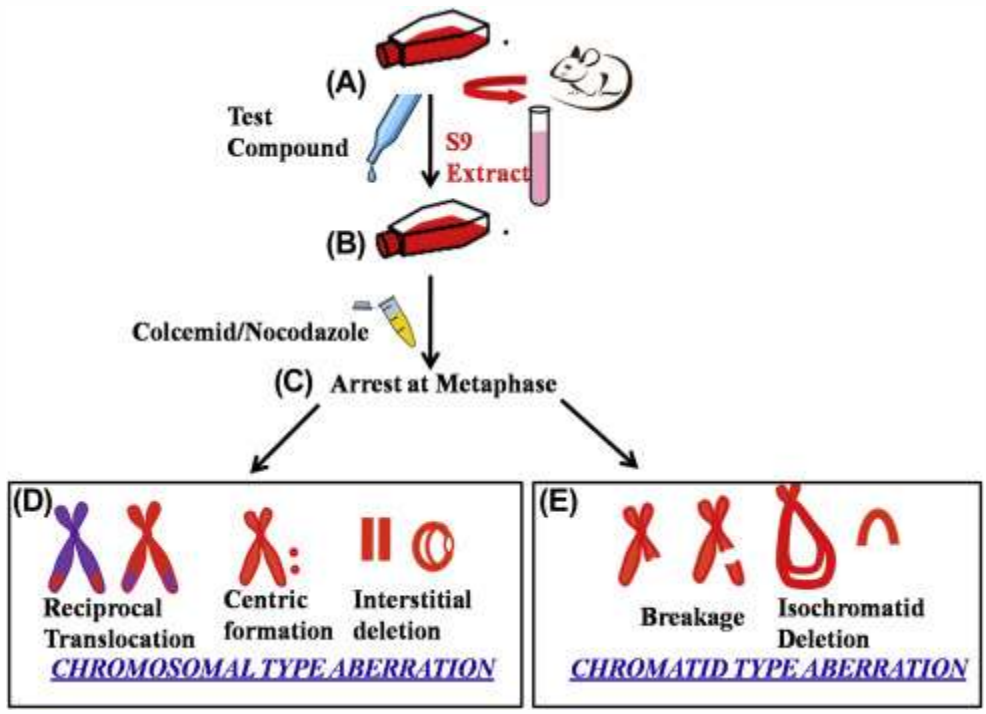


Chromosomal Alterations

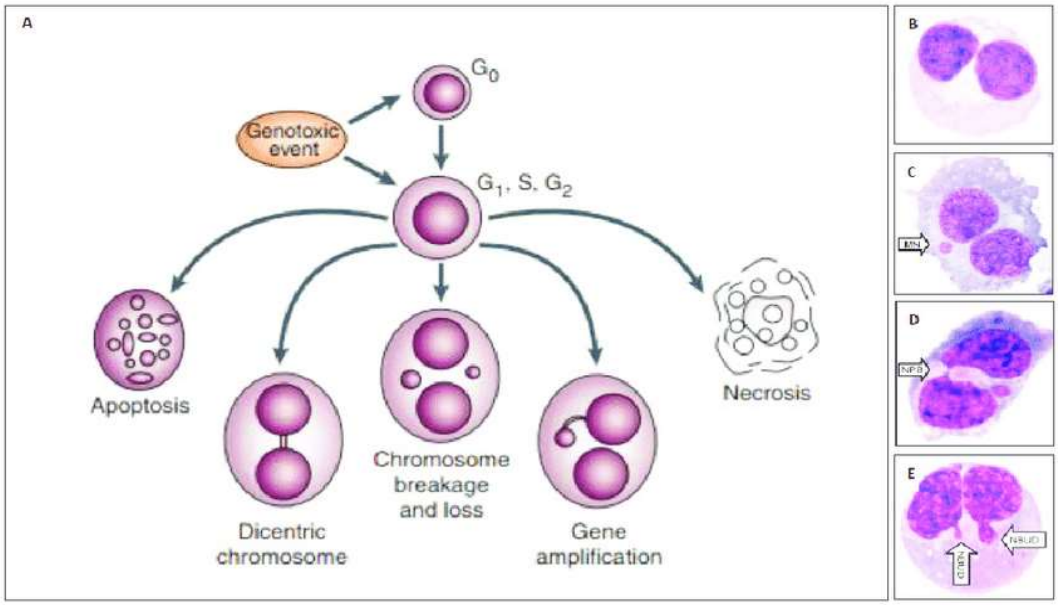
alterations are quite common in malignant neoplasms, as such the detection of chromosomal abnormalities by test chemicals is considered an excellent test for the assessment of carcinogenic potential. Both in vivo and in vitro assays are available to assess chromosomal alterations. In mammalian cell lines, most of the test systems used the same lines as used in the mutation assay (Galloway *et al*., 1985). To assess induction of chromosomal alterations, cells are harvested in their first mitotic division after the initiation of chemical exposure. Cells are
stained with Giemsa and scored for completeness of karyotype (21 +/− 2 chromosomes). The classes of aberrations recorded include breaks and terminal deletions, rearrangements and translocations, as well as despiralized chromosomes, and cells containing 10 or more aberrations.

The chromosomal aberration test is designed to evaluate the potential of a test compound to induce structural chromosomal abnormalities such as breaks and exchanges.The purpose of the in vitro or invivo chromosome aberration test is to identify agents that cause structural chromosome aberrations in cultured mammalian cells or bone marrow of experimental animal model . Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome-type aberrations also occur. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. However, this guideline is not designed to measure numerical aberrations and is not routinely used for that purpose. Chromosome aberrations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals. 2. The in vitro chromosome aberration test may employ cultures of cell lines or primary cell cultures. The cells used are selected on the basis of growth ability in culture, stability of the karyotype, chromosome number, chromosome diversity and spontaneous frequency of chromosome aberrations. At the present time, the available data suggest that it is important to

consider the p53 status, genetic (karyotype) stability, DNA repair capacity and origin (rodent versus human) of the cells chosen for testing (Pfuhler et al., 2011).

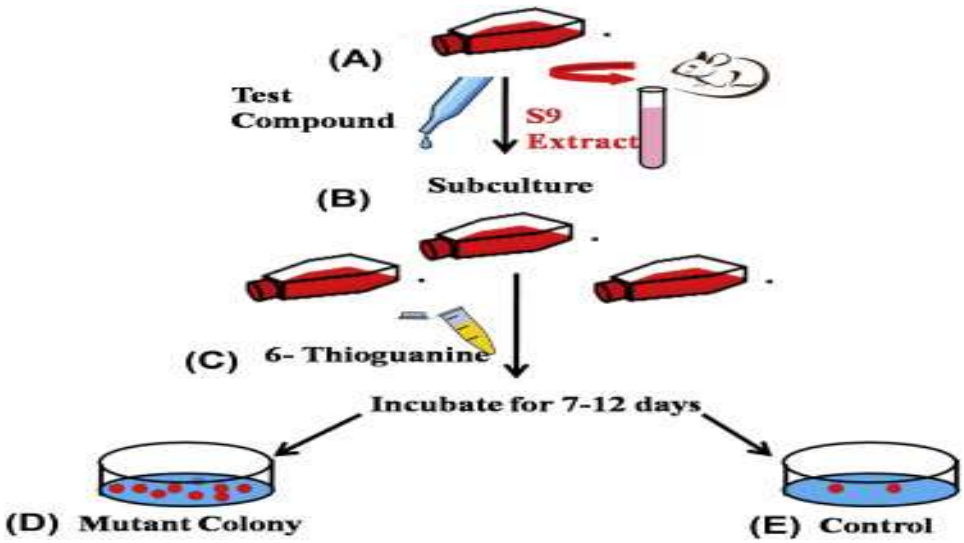


The micronucleus assay is similar to the *in vivo* aberration assay in that both measure chromosome alterations in treated mammals and, according to most regulatory guidelines, either can be used in the initial testing (Auletta *et al.*, 1993; Dearfield *et al.*, 1991). The micronucleus assay detects chromosome breakage and loss occurring following chemical treatment. Although micronuclei can be formed in any dividing tissue of any species following treatment, for regulatory purposes the assay is almost always conducted in the bone marrow or, less frequently, the peripheral blood erythrocytes of rodents (U.S. EPA, 1998f). As a bone marrow erythroblast develops into a newly formed RNA-containing (polychromatic) erythrocyte, the main nucleus is extruded. In a damaged cell, the micronucleus that has been formed remains behind in the anucleate cytoplasm. Using a stain such as acridine orange that differentially stains RNA and DNA, the DNA-containing micronucleus can easily be visualized in the cytoplasm of the newly formed RNA-containing erythrocytes. An increase in the frequency of micronuclei following treatment with a test chemical indicates that an increase in chromosome damage has occurred. The assay can be performed in one of two ways: with a single dose followed by two or more sampling times or with two or more sequential doses followed by a single harvest. As with the *in vivo* aberration assay, this *in vivo* assay allows normal metabolism, toxicokinetics, and DNA repair to occur. In addition, many humanand animal carcinogens when tested have shown positive results in this assay (Ashby and Paton, 1993).



Mammalian cell HPRT gene mutation assay:

The hypoxanthine phosphorybosyl transferase (HPRT) gene is on the X chromosome of mammalian cells, and it is used as a model gene to investigate gene mutations in mammalian cell lines. The assay can detect a wide range of chemicals capable of causing DNA damage that leads to gene mutation. The test follows a very similar methodology to the thymidine kinase (TK) mouse lymphoma assay (MLA), and both are included in the guidelines for mammalian gene mutation tests (OECD (1997) Organisation for Economic Co-operation and Development. Ninth addendum to the OECD Guidelines for the Testing of Chemicals. In Vitro Mammalian Cell Gene Mutation Test: 476). The HPRT methodology is such that mutations which destroy the functionality of the HPRT gene and or/protein are detected by positive selection using a toxic analogue, and HPRT (-) mutants are seen as viable colonies. Unlike bacterial reverse mutation assays, mammalian gene mutation assays respond to a broad spectrum of mutagens, since any mutation resulting in the ablation of gene expression/function produces a HPRT (-) mutant. Human cells are readily used, and mechanistic studies using the HPRT test methodology with modifications, such as knock-out cell lines for DNA repair, can provide details of the mode of action (MOA) of the test compound.



Chronic Testing for Carcinogenicity

The majority of in vivo carcinogenicity testing is performed in rodent models. The administration of chemicals in the diet, often for extended periods, for assessment of their safety and/or toxicity began in the 1930s (Sasaki and Yoshida, 1935). Animal testing
today remains a standard approach for determining the potential carcinogenic activity of xenobiotics. In addition to the lifetime exposure rodent models, organ-specific model systems, multistage models, and transgenic models are being developed and used in
carcinogen testing . Chronic (2-Year) Bioassay Two-year studies in laboratory rodents
remain the primary method by which chemicals or physical agents are identified as having the potential to be hazardous to humans. The most common rodents used are the rat and mouse. Typically the bioassays are conducted over the lifespan of the rodents
(2 years). Historically, selective rodent strains have been used in the chronic bioassay; however, each strain has both advantages and disadvantages.
For example, the National Toxicology Program (NTP) typically uses Fisher 344 (F344) rats and B6C3F1 mice. The F344 rat has a high incidence of testicular tumors and leukemias, whereas the B6C3F1 mouse is associated with a high background of liver
tumors .
In the chronic bioassay, two or three dose levels of a test chemical and a vehicle control are administered to 50 males and 50 females (mice and rats), beginning at 8 weeks of age, continuing throughout their lifespan. The route of administration can be via oral (gavage), dietary (mixed in feed), or inhalation (via inhalation chambers) exposure. Typically a number of short-term in vivo tests are conducted prior to the chronic bioassay to determine acute toxicity profiles, appropriate route of administration, and the maximum tolerated dose (MTD). Generally, the MTD is used to set the high dose in the
chronic study. The use of the MTD as the upper dose level has been questioned by many investigators, as it is recognized that the doses selected often represent doses that are considered unrealistically high for human exposure. Pharmacokinetics and metabolism at high dose are frequently unrepresentative of those at lower doses; in addition, a general relationship between toxicity and carcinogenicity cannot be drawn for all classes of chemicals. During the study, food consumption and bodyweight gain should be monitored, and the animals observed clinically on a regular basis, and at necropsy
the tumor number, location, and pathological diagnosis for each animal is thoroughly assessed

Transgenic Animals in Carcinogenicity Assessment

Due to the development of animal models with genetic alterations that invoke a susceptibility to carcinogenesis by chemical agents,
the use of transgenic and knockout animals in carcinogenicity assessment is gaining more popularity. The common models that have been used include the Tg.ACand rasH2 transgenic mice, and p53+/− and XPA−/− knockout mice (Gulezian *et al*., 2000). Recently, the feasibility of the use of these animal models as alternative assays for the 2-year chronic bioassay was assessed by the Health and Environmental Sciences Institute (HESI) branch of the International Life Sciences Institute (ILSI). In this assessment, 21 chemicals were evaluated, encompassing genotoxic, nongenotoxic, and noncarcinogenic chemicals. The conclusions drawn from the scientific review suggested that these models appear to have usefulness as screening models for assessment of chemical carcinogenicity; however, they do not provide definitive proof of potential human carcinogenicity. Further the scientific panel suggested that these models could be used in place of the mouse 2-year bioassay (Tennant *et al*., 1998; Cohen *et al*., 2001). Coupled with information on genotoxicity, particularly DNA reactivity, structure–activity relationships, results from other bioassays, and the results of other mechanistic investigations including toxicokinetics, metabolism, and mechanistic information, these alternate mouse models for carcinogenicity appear to be useful models for assessing the carcinogenicity of chemical agents.