MUTAGENICITY STUDIES WITH BENZENE

by
JAMES PAUL LYON

B.S., Idaho State University, 1964
M.S., University of Kansas, 1967

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

COMPARATIVE PHARMACOLOGY AND TOXICOLOGY

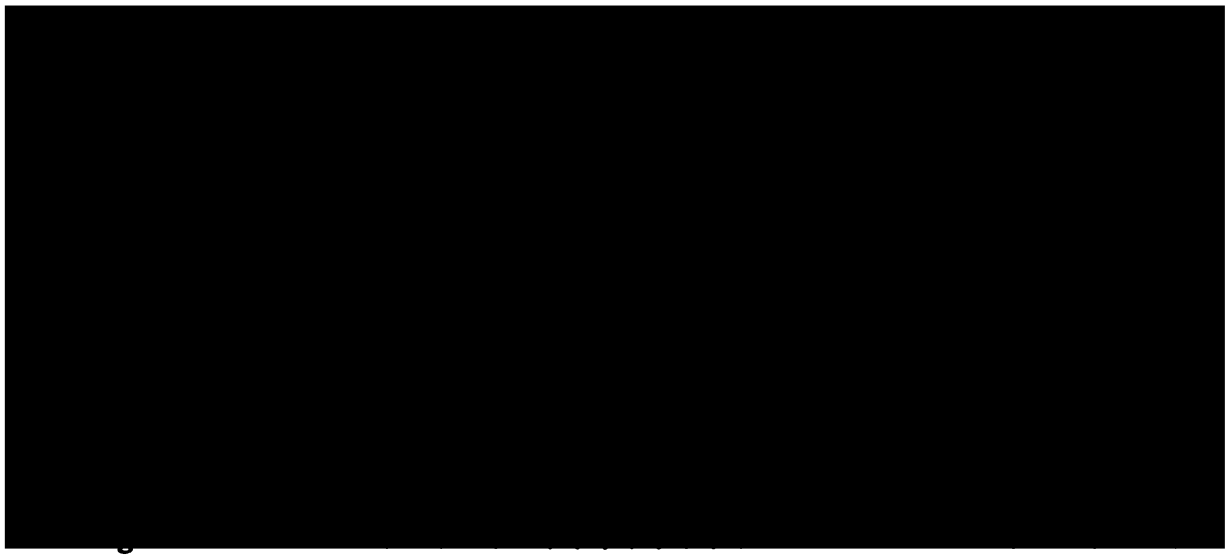
in the

GRADUATE DIVISION

(San Francisco)

of the

UNIVERSITY OF CALIFORNIA



Acknowledgements

Appreciation is extended to the following individuals for their assistance during my graduate training.

to Dr. C. H. Hine, research advisor and chairman of my thesis committee, for his guidance and encouragement throughout all phases of my graduate training.

to Dr. H. C. Hodge, for his constant patience and assistance over the past four years.

to Dr. R. E. Rasmussen, for his advice and cooperation as a member of my thesis committee.

to Dr. J. Nemenzo, for his technical expertise and assistance with the cytogenetic study.

to my fellow graduate students for their repeated assurances that I was not alone in the precarious process.

This work was supported in part by a National Institutes of Health Training Grant USPHS-GM-00475.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

Abstract

The mutagenicity of benzene was evaluated using several techniques. A host-mediated assay using Salmonella typhimurium TA 1950 as the indicator organism in mice was conducted following two subcutaneous injections of benzene (0.1 ml/kg) or saline (0.1 ml/kg). The results showed no differences between the two treatments in the assay. A dominant lethal assay was conducted following a single intraperitoneal injection of olive oil (0.50 ml/kg), benzene (0.50 ml/kg) or triethylenemelamine (0.25 mg/kg) using male rats. Necropsy data from pregnant females obtained by mating two female rats/week with each treated male over a 10-week period were analyzed. No dominant lethal effects were observed in the female rats after benzene treatment of the male rats whereas after triethylenemelamine treatment of the male rats, a significant increase was observed in dead implants/pregnant female during weeks 2, 3 and 4. Cytogenetic studies were conducted on bone marrow and blood specimens collected at 1, 8, 30 and 70 days following a single intraperitoneal injection of olive oil (0.50 ml/kg) or benzene (0.50 ml/kg) using male rats. Metaphase spreads were analyzed for chromosome aberrations. A significant increase in the number of cells containing achromatic lesions, chromatid deletions, and chromosome deletions was observed in bone marrow specimens from the benzene-treated animals at 1 day and in chromatid deletions at 8 days. Double minutes, restricted solely to the benzene-treated animals, were observed at 1, 8, 30 and 70 days in bone marrow chromosome preparations. Technique problems in culturing the blood specimens to obtain metaphase spreads were encountered with the 30

and 70 day blood specimens from both treatment groups resulting in the loss of the 30 day samples and reduced growth in the 70 day samples. Chromosome pycnosis was observed in blood specimens from the benzene-treated animals at 1, 8 and 70 days and very few metaphase spreads were located. Double minutes were detected in the 1, 8 and 70 day blood specimens obtained from the benzene-treated animals. No double minutes were observed in blood specimens obtained from control animals. Results from testing benzene in the bacterial plate assay with Salmonella typhimurium TA 100 and TA 98 as the indicator organisms showed no increase in reversion rates. Liver homogenates prepared from rats treated with 3-methylcholanthrene or phenobarbital were incorporated into the assay. No increase in the reversion rates was observed using benzene at several concentrations in the activated bacterial plate assay. Testing of benzene after the addition of 1,1,1,-trichloro-2,3-epoxypropane at several concentrations to the liver homogenate mixture in the activated bacterial plate assay produced data similar to the results observed with benzene alone. Bromobenzene and chlorobenzene were tested in the activated bacterial plate assay with Salmonella typhimurium TA 100 and TA 98 using a liver homogenate from 3-methylcholanthrene-treated animals; both compounds were negative. Use of bone marrow homogenates from rats treated with 3-methylcholanthrene or untreated in the activated plate assay in place of liver homogenates was unsuccessful. Using the micronucleus test in rats, three concentrations of benzene (0.05, 0.10 and 0.50 ml/kg) were evaluated

following intraperitoneal injection of one-half the dose 30 hours before sacrifice and one-half the dose 6 hours before sacrifice. Olive oil (1.0 ml/kg) and triethylenemelamine (0.25 mg/kg) injected similarly served as the negative and positive controls, respectively. A significant dose-related increase in polychromatic erythrocytes containing micronuclei was observed in the benzene-treated animals. The results of the various techniques are compared and discussed. The results are also discussed in relation to the mutagenic risk posed by benzene exposure.

HISTORICAL

Information concerning the metabolism and toxicity of benzene has been available from the literature for several years. My interest in benzene has been stimulated by the appearance in the literature in the last few years of several articles which increasingly point to benzene exposure as having an effect on human chromosomes. Most of these articles are based on clinical observations following exposure at one time or another to varying concentrations of benzene alone or in combination with other solvents. Preliminary studies have also demonstrated the chromosome effect experimentally. Since the biotransformation of benzene gives rise to several metabolites which may be involved, in addition to the benzene itself, a consideration of the metabolism must be included.

Background

Benzene has wide use in the present day industrial setting. It is used extensively as a solvent in the chemical and drug industries and as a starting material and intermediate in the synthesis of numerous chemicals. The lastest figures available indicated production in excess of one billion gallons in 1969 (NIOSH, 1974).

It is probably one of the most hazardous solvents in common use. Acute poisoning by benzene appears to be due to a narcotic action. The inhalation of high concentrations may cause exhilaration followed by drowsiness, fatigue, vertigo, nausea and headache. At higher concentrations or longer

exposure times, convulsions followed by paralysis and loss of consciousness may ensue. Death can result quickly from respiratory paralysis. The acute toxicity problem concerning benzene is of secondary importance in industrial situations to the chronic toxicity.

The effects of benzene inhalation over prolonged periods of time, measured in terms of years, are of paramount importance to the occupational use of this agent. The National Institute for Occupational Safety and Health (NIOSH) estimates that two million individuals in the work force have a potential for exposure (NIOSH, 1974). The Threshold Limit Value (TLV) has been set at 25 ppm but it is considered not good practice to exceed this even for brief periods without suitable respiratory protection (Fed. Reg., 1972). The NIOSH recommended standard for occupational exposure to benzene suggests the lowering to 10 ppm with a ceiling of 25 ppm.

Due to its lipid solubility, benzene tends to be accumulated in fat and the bone marrow. The chronic toxic effects of benzene exposure therefore are those of injury to the bone marrow, producing degenerative changes in the hematopoietic system, with the salient feature of a plastic anemia sometimes coexisting with areas of hyperplasia. The symptoms, signs and blood changes may not always present a typical picture of severe anemia and leukopenia. In addition, there appear to be host differences in susceptibility and in the capacities of individuals to reestablish normal hematopoietic function following chronic exposure.

The relation of benzene poisoning to leukemia has never definitely been resolved but there have been many reports which tend to substantiate such a relation. The pathogenesis of leukemia is obscure and its etiological relation to any particular exposure, possibly 15 years earlier, is difficult to establish. Vigliani and Saita (1964) published a study conducted between 1942 and 1963 in Italy. Of forty-seven patients observed with benzene hemopathy from industrial exposures, six developed leukemia. The patients were all documented benzene exposures although the exact concentrations were never determined. It was assumed that the concentrations were significantly above the maximum allowable values. Published in this report were similar results from a French study in which six cases of leukemia were observed out of 42 cases of benzene myelopathy. According to the Italian authors, the risk of death from leukemia in subjects exposed to benzene is 20-fold that of the general population in the area. More to the point was the observation that of those patients who died from chronic benzene poisoning, approximately 50% died from one form or another of leukemia. These authors suggested it would be interesting to undertake a systematic study of the chromosomes of bone marrow cells in chronic benzene poisoning and to follow clinically and hematologically the patients with altered patterns.

Benzene Metabolism

The biotransformation of benzene has been well documented. The classic studies were carried out in rabbits by Parke and Williams (1953) and are outlined by Williams (1959) in

Detoxification Mechanisms. Following the initial outline little was added until recently when several workers mainly at the National Institutes of Health (NIH) initiated studies on aromatic hydroxylation reactions in mammalian liver systems.

It is now thought that formation of an epoxide intermediate may be the primary metabolic step by which hepatic monoxygenase enzymes transform aromatic compounds. While this concept is not a new idea, Boyland and Wolf (1950) suggested that this was a likely method, most of the experimental evidence has been produced in the last few years. Vogel and Gunther (1967) pushed the work forward when they developed synthetic methods for the production of several of the postulated intermediates. These intermediates were called arene oxides and refer to the epoxidation of one double bond of the aromatic nucleus. The production of several synthetic arene oxides by the NIH group and in vitro testing published in a series of articles documented the biological existence of epoxide intermediates.

The enzymatic conversion of arene oxides was first shown to produce the metabolites found following the in vivo transformation of the parent molecule. Jerina et al. (1968a) demonstrated this using a benzene oxide-oxepin tautomer. Phenol, 1,2-dihydro-1,2-dihydroxybenzene, catechol and a glutathione conjugate were isolated from in vitro incubation of the benzene oxide in a rat liver system. All of the products formed are also seen when benzene is administered and metabolized in vivo. The isolation of an arene oxide in a

biological system following the administration of the parent compound had to be shown. Jerina et al. (1968a) attempted this in a liver system using benzene-C^{14} but no benzene-C^{14} oxide could be isolated. The authors reported that this may have been due to technique problems or the low rate of metabolism in vitro and instability of the benzene oxide. A direct demonstration of an arene oxide in the metabolism of benzene has yet to be reported (Jerina and Daily, 1974). Jerina et al. (1968b) did isolate 1,2-naphthalene oxide formed from naphthalene. Jerina et al. (1970) demonstrated that in the case of naphthalene, the oxide was the primary oxidation product and that its formation probably was the rate limiting step.

Data has been accumulating which indicates that the production of an epoxide on biotransformation of aromatic hydrocarbons can occur with a wide variety of compounds. Udenfriend (1971) suggested that from a chemical basis, all microsomal hydroxylations of aromatic compounds could involve similar mechanisms and that a pattern of metabolites such as phenols, dihydrodiols, catechols and mercapturic acid derivatives may be taken as presumptive evidence of an active arene oxide intermediate. Isolation of more complex ring system epoxide intermediates has been reported from the microsomal metabolism of phenanthrene, benzo(a)pyrene, benzo(a)anthracene, dibenzo(a,h)-anthracene (Sims et al., 1971; Wang et al., 1972).

The production of an active intermediate epoxide has been

suggested as one possible mechanism for the toxicity, mutagenicity and carcinogenicity of biological unreactive aromatic compounds such as the polycyclic aromatic hydrocarbons (PAH) and simpler monocyclic aromatic hydrocarbons. Following conversion of the compound to an epoxide, there appear to be several means to detoxify the intermediate. Boyland and Williams (1965) reported on a rat liver enzyme, glutatione S-epoxidetransferase, and suggested that this was probably the enzyme responsible for *in vivo* glutathione conjugation of epoxy derivatives. This conjugation has been suggested as a protective reaction for tissue nucleophilic sites from electrophilic attack thereby protecting the nucleophilic sulfhydryl groups of enzymes (Boyland and Chasseaud, 1969). Jerina *et al.* (1968a) demonstrated the conversion of arene oxides to phenolic compounds in a non-enzymatic but protein catalyzed rearrangement making possible the detoxification in the liver by conjugation with glucuronic and sulfuric acid then elimination. A third pathway appears to be the conversion of the epoxide to a dihydrodiol catalyzed by a microsomal enzyme, epoxide hydrase (Jerina *et al.*, 1968a; Oesch *et al.*, 1971).

Weibel *et al.* (1971) reported that there appear to be at least two forms of this enzyme system in rat tissues which can be differentially induced *in vivo* by phenobarbital or 3-methylcholanthrene. As a consequence, the profile of metabolites may also differ in various tissues producing differences in the levels and distribution of the active intermediates. The induction of the monoxygenase to produce the epoxide and of the

epoxide hydrase for further transformation does not occur in parallel and the combined effect of both enzyme systems may depend on the degree of association between the two in the microsomes (Jerina and Daily, 1974).

Most of the work concerning monocyclic aromatic hydrocarbon epoxide formation as a mechanism of toxicity has been with halogenated benzenes. Brodie et al. (1971) and Mitchell et al. (1971) reported on in vivo studies of bromobenzene-induced hepatonecrosis. Phenobarbital induction of microsomal enzymes increased the severity of necrosis from bromobenzene administration. Those animals which had the greatest hepatic damage had the lowest concentration of bromobenzene in the liver and plasma. The severity of necrosis was decreased by beta-diethylaminoethyl diphenylpropyl acetate (SKF 525-A), a microsomal enzyme system inhibitor, which increased plasma and liver levels of bromobenzene. These workers suggested that the active epoxide produced the necrosis by alkylation of various macromolecules in hepatocytes. Evidence of the binding in hepatonecrosis was provided by autoradiograms using C^{14}-bromobenzene (Krishna et al., 1971).

Experiments depleting liver glutathione using diethyl maleate pre-treatment or increasing the level of glutathione by administration of cysteine established a protective role for glutathione in halobenzene-induced liver necrosis (Reid and Krishna, 1973). Similar attempts to show the epoxide hydrase enzyme in a protective role against hepatonecrosis induced by halobenzenes were inconclusive (Oesch et al., 1973).

Benzene Exposure and Chromosome Studies.

Industrial workers. Tough and Court Brown (1965) published a preliminary investigation on chromosome aberrations and exposure to ambient benzene from 1 to 20 years without signs of toxicity. On-site personnel who were considered not to have been exposed and randomly selected males from the general population were used as controls. Cells containing aberrations were scored and the two control groups did not significantly differ. Tough and Court Brown did report a significant increase in the number of cells with aberrations from the exposed workers as compared to both control groups. The authors were confident that radiation exposure was not responsible. In a later paper, Tough et al. (1970) took two other groups of factory workers from different locations who had been exposed to benzene, ran cytogenetic studies and compared the data from these two factories with the previous study. In the two new groups there were no significant increases observed in cells with aberrations in the exposed workers when compared to their on-site controls. In one of the new groups, there was an increase when compared to the general population sample but in the other, exposed workers, on-site controls and general population controls were all about the same. Various reasons were suggested to account for the discrepancy in results including the possibilities, a) that the on-site controls could have had significant benzene exposure, b) that the age of the workers in the groups which tended to be somewhat different could account for the results and c) the suggestion that the benzene exposure

level of the group where no differences were seen could have been less than for the other two groups.

Forni et al. (1971a) reported on chromosome studies carried out on workers exposed to benzene, toluene or both chemicals. The benzene workers ran the gamut from no signs of toxicity to severe benzene hemopathy. Controls were matched for sex and age. The proportions of chromosome aberrations were significantly higher in the benzene group as compared to the toluene and control groups. There was a non-significant increase observed between the toluene and control groups. In another study, Forni et al. (1971b) observed chromosomes of lymphocytes from 25 subjects who had recovered from benzene hemopathy, 4 subjects with bone marrow toxicity from benzene and 3 subjects who had recovered from acute benzene poisoning. All had significantly increased rates of chromosome aberrations which were still present several years after cessation of exposure and clinical recovery from the poisoning. No correlation seemed to exist between the severity of benzene poisoning and the persistence of chromosomal changes.

Benzene-induced leukemia. Forni and Moreo (1967) reported on a patient who had benzene induced anemia which evolved into acute myeloblastic leukemia after a long latent period. At a time when the patient showed severe anemia, aplastic bone marrow and no inmature leucocytes, a high rate of chromosome aberrations were found. Later, still in the absence of inmature leucocytes, numerous cells with 47 chromosomes were detected in bone marrow. When myeloblasts appeared in the blood all cells with 47 chromosomes, prepared from bone marrow and blood,

showed the same karyotype with an extra C group chromosome. The many chromosome changes observed in bone marrow cells before the onset of leukemia changed when clinical leukemia was diagnosed to a single abnormal clone present in leukemic cells of bone marrow and blood. A hypothesis was suggested that benzene exposure may induce different chromosome changes and that leukemia may occur in those cases where a potentially leukemic clone with selective advantage was produced by the benzene. These two workers reported (1969) on another patient in whom cytogenetic studies were carried out who had a case of acute erythroleukemia which occurred after benzene exposure. In preparations from blood and bone marrow, a stemline of 46 chromosomes was present with a pseudodiploid karyotype due to the absence of 2 chromosomes of group E and the presence of 2 small markers. The abnormal chromosome complement seemed to characterize a stem-cell common to both series.

Hartwich et al. (1969) reported on chromosome studies from a patient who developed acute myeloid leukemia after four years of benzene exposure. Their studies showed an anomaly rate of 20% as compared to controls of 5%. All anomalies were simple break phenomena.

Experimental studies. While the clinical data that have been accumulating, mainly from the Italian studies, seem to indicate that benzene inhalation will produce chromosome aberrations, little experimental work has been carried out using controlled conditions. Dean (1969) published a study demonstrating the applicability of cytogenetic methods in rats to the evaluation of chemically induced damage and benzene was

one of four substances tested to demonstrate the technique. He injected benzene subcutaneously in single doses of 0.5, 1.0 and 2.0 ml/kg with male and 0.5 and 1.0 ml/kg with female rats. Aberrations were observed in bone marrow cells in rats of both sexes 24 hours after treatment with the 1.0 ml/kg dose. Only the male rat showed aberrations 24 hours after the 0.5 ml/kg injection and the male treated with 2.0 ml/kg showed less chromosome damage than the male at 1.0 ml/kg. In preparations made 8 days after treatment no damage was seen. Peripheral blood cultures which were made only with female rats showed damage 24 hours after treatment at both 0.5 and 1.0 levels and those prepared 8 days after treatment showed a much reduced level of damage. The study was extremely limited with one animals used for each treatment and can not be considered conclusive.

Philip and Jensen (1970) published a brief report on the induction of chromosome abnormalities in rat bone marrow cells using benzene. Five groups of 3 rats each were injected subcutaneously with benzene (2.0 ml/kg). Group I receive benzene 48 and 24 hours before sacrifice and group II, 72, 48 and 24 hours before sacrifice. Groups III, IV and V received a single injection 12, 24 or 36 hours, respectively, before sacrifice. All treatment groups except V showed structural aberrations which were exclusively chromatid deletions.

Dobrokhotov (1972) in one section of his study administered benzene (0.2 g/kg) subcutaneously to 5 rats daily for 12 days and analyzed bone marrow samples for chromosome aberrations. He reported a 3-fold increase in aberrations

INTRODUCTION

It has become increasingly recognized in recent years that many substances to which man is exposed present potential genetic hazards but to test the entire spectrum would exhaust the abilities of those workers in the field of chemical mutagenicity. The approach suggested by many groups is to consider first all available information such as previous mutagenicity testing, population exposure potential, type of usage and chemical structure among other criteria to determine where to place priorities for extensive testing. At the time of planning my thesis, application of this approach to benzene seemed warranted.

While there have been no definitive epidemiologic studies published, the available evidence collected from industrial situations (Tough & Court Brown, 1965; Tough et al., 1970; Forni et al., 1971a & 1971b; Forni & Moreo, 1967 & 1969; Hartwich et al., 1969) seems to indicate that chronic benzene exposure with or without progression to a leukemic state may produce chromosomal aberrations. The limited animal studies reported to date re-inforce these epidemiologic observations (Dean, 1969; Philip & Jensen, 1970; Dobrokhotov, 1972). In addition, there is strong circumstantial evidence that the mammalian biotransformation of benzene leads to a highly reactive intermediate epoxide (Jerina & Daly, 1974) which may be responsible for many of the toxic symptoms seen in chronic benzene intoxication and may also be mutagenic based on evidence available for other epoxides (Mrak Commission, 1969).

This information, when considered in conjunction with both the high production volume and the large occupational force exposed (NIOSH, 1974), points out the significant genetic threat posed by benzene exposure. The seriousness of the situation mandates additional, extensive study.

Discussions at a recent workshop on the evaluation of chemical mutagenicity (de Serres & Sheridan, 1973) emphasized the concern felt by many workers in the field regarding the reliability of individual test procedures employed in mutagenicity testing. While awaiting the development and trial of more reliable tests, the best approach seemed to be the judicious use of a battery of tests which might give more assurance in the estimation of risks. In 1969, the Advisory Panel on Mutagenicity of Pesticides (Mrak Commission, 1969) reviewed various methods for mutagenicity testing and recommended the testing of compounds in three mammalian systems, the dominant lethal, the host-mediated and *in vivo* cytogenetic, along with ancillary microbial systems. This basic approach has also been endorsed by others (Legator, 1969; Malling, 1970).

Because the available information increasingly pointed to the mutagenic threat from benzene exposure, this study was initiated to evaluate the problem more extensively. The first phase of the study was designed to evaluate the mutagenic potential of benzene using three mammalian test systems: a) the host-mediated assay in mice using *Salmonella typhimurium* as the indicator organism (Legator, 1973), b) the dominant lethal test in rats (Epstein, 1973) and c) *in vivo* cytogenetic

analyses of bone marrow and blood specimens from rats (Farrow et al., 1975). The development by Nemenzo et al. (1975) of techniques for obtaining bone marrow samples for cytogenetic analyses repeatedly from the same rat permitted the simultaneous assessment of cytogenetic and dominant lethal mutagenicity over a 10-week period. In the second phase, ancillary microbial testing of the mutagenicity of benzene using a liver-activated Salmonella typhimurium plate assay (Ames et al., in press, 1975) was incorporated into the study. Liver homogenates from rats treated with 3-methylcholanthrene or phenobarbital were employed as the activation system. Use of 1,1,1-trichloro-2, 3-epoxypropane in an attempt to block the in vitro biodegradation of the theoretical epoxide intermediate was included in several of the bacterial tests. Trial use of bone marrow homogenates from rats treated with 3-methylcholanthrene or untreated as the in vitro activation system in the bacterial plate assay was attempted for comparison. Further assessment with another mammalian system, the micronucleus test using rats (Schmid, 1975), was included for a dose-response evaluation. All testing with benzene, excluding the microbial procedures, was conducted following single, relatively large doses, parenterally injected. It is hoped that this work may serve as a starting point for further animal mutagenicity studies involving chronic inhalation of benzene. In the interim period, the data obtained should provide answers for application to the question of benzene mutagenicity.

METHODS

Solutions and Media

The complex mixtures used for the research procedures are given below along with the reference for their composition. Simple mixtures used in the work will be described when mentioned.

VB Salts (Vogel & Bonner, 1956)

warm distilled water (45° C.)	670 ml
magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$)	10 g
citric acid monohydrate	100 g
potassium phosphate, dibasic, anhydrous (K_2HPO_4)	500 g
sodium ammonium phosphate ($NaNH_4HPO_4 \cdot 4\ H_2O$)	175 g
	1000 ml

Add the salts in the order indicated. Allow each salt to dissolve completely before adding the next. When the salts are all dissolved, let the solution cool to room temperature. Add 5 ml chloroform per liter of solution and store in a capped bottle at room temperature.

Nutrient Broth

Bacto-nutrient broth (Difco)	0.8 g
sodium chloride	0.5 g
distilled water	100 ml

Dissolve the ingredients and dispense into capped closure tubes. Autoclave and store at room temperature.

Minimal Glucose Agar Plates (Ames, 1971)

A.	VB salts	20 ml
	distilled water	500 ml
B.	agar (Difco)	15 g
	distilled water	500 ml
C.	40% glucose solution	50 ml

Autoclave solutions A, B and C in separate containers. While still hot, mix together and pour plates (30 ml/plate).

Top Agar (Ames, 1971)

agar (Difco)	0.6 g
sodium chloride	0.5 g
distilled water	100 ml

Dissolve the ingredients and autoclave. Maintain at 45° C. to prevent hardening. Add a trace of histidine and an excess of biotin to the solution prior to adding the bacteria (10 ml/100ml-histidine-biotin solution).

Histidine-Biotin Solution (Ames, et al., in press, 1975)

L-histidine·HCl (0.5 mM) and biotin (0.5 mM) in distilled water. Do not autoclave! Sterilize by filtration. Use 10 ml/100 ml of Top Agar.

S-9 Mix (Ames, et al., in press, 1975)

S-9 fraction (3 ml sterile KCL solution (1.15%) per gram of liver)	1.0 ml
sodium phosphate buffer, 200 mM (pH 7.4)	5.0 ml
magnesium chloride solution, 8 mM	1.1 ml
glucose-6-phosphate (Sigma)	14 mg
NADP (Sigma)	35 mg
distilled water, sterile	2.9 ml

The magnesium chloride and sodium phosphate buffer are prepared in advance and stored. Before use, they are autoclaved. The NADP and G-6-P are not autoclaved but are dissolved in the sterile distilled water. Variations in the amount of S-9 fraction used require compensation by increased or decreased amounts of 1.15% KCl solution.

Blood Culture Media (Moorehead, et al., 1960)

Minimum Essential Medium (MEM) (Microbiological Associates)	100 ml
Fetal Calf Serum (Microbiological Associates)	25 ml
Phytohemaglutinin P (Difco) 1.5 ml dissolved in 4.7 ml MEM	6.2 ml
sodium heparin, 100 units/ml (Invenex)	2 ml
L-glutamine, 200 mM	2 ml
penicillin G, aqueous (Squibb)	200,000 units
streptomycin sulfate (Pfizer)	4 mg

Copper Sulfate-Sodium Potassium Tartrate Solution (Lowry, et al., 1951)

copper sulfate pentahydrate 1% in water	1 ml
sodium potassium tartrate 2% in water	1 ml
sodium carbonate 2% in 0.1 N sodium hydroxide solution	98 ml

Mix right before use.

Giemsa Stain

Giemsa dye, 1 gram, mixed in 66 ml of glycerin. Heat to 60° C. for 2 hours and cool. Add 66 ml of methanol, stir for 24 hours and filter.

May-Greenwald Stain

Mix 0.3 grams of May-Greenwald dye in 100 ml of methanol, warm to 50° C. then cool to room temperature. Let stand with occasional shaking for 24 hours and filter.

Host-Mediated Assay

A total of 16 male Swiss albino mice (23-28 g) were used with 5 in the control group, 5 in one benzene-treated group and 6 pre-treated for enzyme induction then treated with benzene. The 6 mice in the last group received phenobarbital sodium intraperitoneally (35 mg/kg) twice daily for 3 days prior to initiation of the experiment.

Nutrient broth was inoculated from a standard culture of TA 1950 strain of Salmonella typhimurium LT-2 and incubated overnight at 37° C. The grown culture was then diluted with sterile saline 1:4. On the day of the experiment, each mouse was injected intraperitoneally with 2 ml of this fresh bacteria cell suspension. Following this, all mice were injected subcutaneously, behind the head at the base of the neck, with either 0.1 ml of sterile saline solution (control) or 0.1 ml of benzene. This injection was repeated one hour later at a slightly different area at the base of the neck. The usual third injection an hour later was not given due to the deteriorated condition noted in the benzene-treated mice.

The mice were sacrificed by cervical dislocation $3\frac{1}{2}$ hours after injection of the bacteria cell suspension. The initial injections were made at 10 minute intervals and the mice sacrificed at 10 minute intervals to allow time for removal of the bacteria from the peritoneal cavity while keeping the time

sequence constant for all of the mice. To recover the bacteria, the abdominal region of the sacrificed mouse was swabbed with ethanol, 1.5 ml of sterile saline was injected into the peritoneum and the abdominal area gently massaged to mix the bacteria with the saline solution. The abdominal cavity was opened aseptically using sterile scissors and the exudate withdrawn using a sterile 1 ml disposable syringe. As much fluid as possible was removed from each mouse and placed in separate sterile test tubes. Dilution blanks, containing 1.8 ml of sterile saline for the initial dilution and 4.5 ml for the following dilutions, were prepared in advance for each specimen. Tenfold serial dilutions were made of each peritoneal sample (0.2 ml + 1.8 ml then 0.5 ml + 4.5 ml) yielding a concentration series from 10° (undiluted peritoneal exudate) through 10^{-7}.

To determine the total bacteria count and the number of revertants obtained from each mouse, 0.1 ml of each concentration in the series was plated on minimal glucose agar plates. A standard pour-plate technique was used: 0.1 ml of the bacteria sample and 2.2 ml of molten top agar containing histidine-biotin solution were mixed in a sterile tube and poured over the surface of the base plate. This was allowed to harden, the plate inverted and incubated at 37° C. for 48 hours. Enumeration for $\underline{his}^+$ prototrophic revertants was made using plates prepared from the undiluted peritoneal samples while total cell count was determined from plates prepared from the 10^{-5} dilutions.

Colony-forming units (CFU) per ml of the undiluted exudate

were calculated for each mouse from the total cell count X the dilution factor and a mutation frequency (MF) calculated.

$$MF = \frac{\underline{his}^{+} \text{ revertants/ml undiluted exudate}}{\text{CFU/ml undiluted exudate}}$$

The MF of the control group of animals was compared with that of the benzene-treated groups.

An _in vitro_ assay using the TA 1950 strain was conducted to parallel the host-mediated assay. Fifteen minimal glucose agar plates were overlayed with 2.2 ml of top agar containing histidine-biotin solution and 0.1 ml of undiluted TA 1950 culture. To prepare the mixture, 100 ml of top agar was autoclaved then held in a water bath at 45° C. Ten ml of histidine-biotin solution was mixed into the agar and 5 ml of the undiluted culture was added. From this mixture, 2.3 ml was pipetted directly onto each minimal glucose agar plate and the overlay allowed to harden. Five plates were left untreated for determining the spontaneous reversion rate, 5 had a crystal of nitrogen mustard placed in the center of the plate and 5 had one drop of benzene placed in the center. The plates were inverted and incubated in the dark at 37° C. for 48 hours. The number of _his_$^{+}$ revertants per plate was determined and comparison made between the untreated, benzene-treated and nitrogen mustard-treated plates.

The methods used are a modification of those reported by Legator (1973) for the host-mediated assay and Ames _et al_. (1973b) for the bacterial plate assay.

Liver-Activated Bacterial Plate Assay

Several activated bacterial plate assays were conducted using a modification of the methods outlined by Ames et al. (in press, 1975). TA 100 and TA 98 strains of Salmonella typhimurium LT-2 were employed as the indicator organisms. Separate tubes of nutrient broth (5 ml) were inoculated from frozen cultures and placed in an agitating water bath. The bacteria samples were cultured overnight at 37° C. with shaking.

The liver homogenate used in the activation system was prepared in advance and stored. Eight male, Long-Evans rats (180-200 g) were intraperitoneally injected to induce liver enzymes. Two induction procedures were used with 4 rats in each treatment group; 1) phenobarbital sodium (75 mg/kg) once daily for three days prior to sacrifice and 2) 3-methylcholanthrene (3-MC) in corn oil (75 mg/kg) 24 hours prior to sacrifice. On the day of preparation, all equipment, glassware and several containers of 1.15% KCl solution were sterilized then placed on ice. The animals were sacrificed by decapitation and the livers removed as quickly and aseptically as possible. The individual livers were rinsed in KCl solution to remove any adhering hairs and weighed in tared beakers containing KCl solution. The livers were removed and homogenized as rapidly as possible without excessive heating using 3 ml of KCl solution per gram of liver. A Potter-Elvehjem tissue grinder immersed in ice water was used for homogenization. All liver homogenates from the same induction treatment were pooled, mixed and centrifuged at 9000 X g for 20 minutes using a refrigerated centrifuge (4° C.). The supernatant

(S-9 fraction) was pipetted into sterile vials, quick frozen in dry ice-acetone and stored in a freezer at -80° C.

Protein was determined on the pooled liver samples of each treatment procedure by the method of Lowry et al. (1951). Four separate protein concentration measurements were made for each liver sample at dilutions with distilled water of 30, 90, 270 and 810. The four values were averaged to obtain the final value by comparison with prepared dilutions of Bovine Serum Albumin (BSA). One ml of all samples was mixed with 1 ml of copper sulfate-sodium potassium tartrate solution and allowed to sit for 10 minutes. Then 0.5 ml of Folin-Ciocalteu Reagent (0.1 N) was added to each sample and allowed to develop for 30 minutes. The samples were read at 660 nanometers using a Beckman DU and the protein concentrations calculated by comparison with the known BSA concentrations.

On the day of the experiment, a vial of S-9 fraction was removed from storage, thawed and kept on ice. The activation mixture (S-9 mix) was prepared using sterilized solutions and containers. The amount of S-9 fraction incorporated into the S-9 mix varied depending on the experiment. The final volume of the S-9 mix was adjusted using 1.15% KCl solution to maintain the desired concentrations of co-factors. After preparing, the S-9 mix was always kept on ice and the leftover portion discarded at the end of the day.

Individual, covered culture tubes were autoclaved and placed in a water bath at 45° C. A 250 ml flask containing 100 ml of top agar was autoclaved, placed in a water bath at 45° C. to prevent hardening and 5 ml of fresh, fully grown

culture was added. Ten ml of histidine-biotin solution was added to the mixture using a filter sterilization system attached to a syringe. The solution was agitated to mix, 2.3 ml pipetted into each culture tube and the tube returned to the water bath.

Test compounds were dissolved in sterile DMSO before addition to the tubes containing the prepared top agar and bacteria. Volumes of less than 100 µl of these solutions were added to the culture tubes using a Schwarz-Mann Biopitte delivery system. The tubes were agitated to mix the test compounds in the agar and 0.5 ml of S-9 mix was pipetted into the tube. The tube was immediately and rapidly rotated between the palms of the hands to thoroughly mix the contents which were then poured onto a minimal glucose agar plate. The plates were allowed to harden, inverted and incubated in the dark at 37° C. for 48 hours. The number of $\underline{his}^+$ revertants on each plate was recorded and compared to a negative and positive control.

Benzene at concentrations of 0.1, 0.5 and 1.0 µl per plate was evaluated using concentrations of S-9 fraction per plate of 0, 1, 10, 20 and 50 µl. Benzo(a)pyrene (BP) in DMSO at concentrations of 10 µg/plate and 1 µg/plate was used as the positive control and DMSO (25 µl/plate) served as the negative control to determine the spontaneous reversion rate. The compounds were examined with both TA 100 and TA 98 as the tester strain and using S-9 mix prepared from phenobarbital and 3-MC-induced animals. As a general screen, only one plate was used for each test condition.

A series of experiments using 3-MC-induced liver and TA 100 tester strain was conducted with the addition of 1,1,1-trichloro-2,3-epoxypropane (TCPO) in DMSO to the reaction mixture in an attempt to block one of the pathways of benzene metabolism. The amount of S-9 fraction in the activation mixture was maintained constant for all runs at 50 µl/plate. Concentrations of benzene from 0.1 to 2.0 µl/plate were evaluated with and without the addition of TCPO. TCPO at final concentrations of 0.05, 0.10 and 0.25 mM in the top agar-S-9 mix overlay was used. BP at concentrations of 0.5, 1.0 and 5.0 µg/plate was tested simultaneously as a positive control without TCPO. TCPO was added to an additional BP plate (0.5 µg/plate) overlay to test for any enhancement of reversion of the positive control.

One experiment was conducted in which the S-9 mix (50 µl/plate), bacteria and test solutions were mixed in sterile culture tubes and incubated in a water bath (37° C.) for 50 minutes with agitation. Following this procedure, top agar containing histidine-biotin solution was added to each tube (2.2 ml) and the tubes pour plated on minimal glucose agar plates. The plates were then handled as usual. Concentrations of benzene in the culture tubes was reduced to 0.05, 0.15 and 0.3 µl to prevent lethality to the bacteria. TCPO at a final concentration in the tube of 0.25 mM was added to a second series of benzene tubes at the same concentrations. A BP series (0.1, 0.4 and 0.8 µg) was used as positive control with TCPO included in an additional BP tube at the lowest concentration.

An experiment employing the usual method was conducted on the benzene analogs, bromobenzene and chlorobenzene. The concentrations of these agents was the same as for benzene, 0.1, 0.5 and 1.0 µl/plate. Liver activation was provided by phenobarbital induced S-9 at concentrations of 1, 10, 20 and 50 µl S-9 fraction per plate.

Bone Marrow Activated Bacterial Plate Assay

An attempt was made to use rat bone marrow as an activation system in conjunction with the bacterial plate assay. Two, large untreated male Long-Evans rats (397 & 424 g) were sacrificed by carbon dioxide inhalation in a chamber and both femurs from each rat were removed. An opening was made at each end for removing the marrow in the same manner as that used in the micronucleus test. The marrow was flushed out using 2 ml of ice-cold 1.15% KCl solution. The marrow from all four femurs was flushed into a small Potter-Elvehjem tissue grinder and homogenized. The fluid was centrifuged at 9000 X g for 20 minutes at 4° C. and the supernatant collected for use. Because of possible contamination problems the supernatant was then sterilized by filtration, pore size 0.35 micron. The sterile fluid was then used as the S-9 fraction in making S-9 mix. The concentration of all co-factors was identical to that used when liver served as the S-9 fraction source. Concentrations of the bone marrow S-9 fraction per plate of 10, 20, 50 and 100 µl were tested using benzene concentrations of 0.1, 0.5 and 1.0 µl. DMSO (25 µl) and benzo(a)pyrene (10 µg) were used as the negative and positive controls, respectively, with TA 100 serving

as the indicator organism. Pour-plating and handling of the minimal glucose agar plates was conducted in the usual manner.

The above experiment was repeated using 3 male Long Evans rats (400 g each). The animals were pre-treated with 3-methylcholanthrene (75 mg/kg) 24 hours prior to sacrifice. The animals were sacrificed and the femurs removed as before. A total of 5 ml of ice-cold 1.15% KCl was used to flush out all six femurs in an attempt to concentrate the marrow. The suspension was homogenized, centrifuged and filtered. Concentrations of bone marrow S-9 fraction per plate of 0, 10, 20, 50 and 90 µl were employed using benzene concentrations of 0.1, 0.5, 1.0 and 3.3 µl. DMSO (25 µl) and benzo(a)pyrene (10 µg) were used as the negative and positive controls, respectively, with TA 100. Handling was as before.

The protein concentration in the final filtered S-9 fraction was determined using a modification of the method of Lowry *et al*. (1951). The procedure employed was exactly that used for determination of liver samples except dilutions of 1, 3, 9 and 27 were employed.

Bacteria Strains

The bacteria strains used for mutagen testing were donated by Dr. B. N. Ames, University of California, Berkeley. The initial samples were cultured and stored at our laboratory at -80° C. The following table from Ames *et al*. (in press, 1975) indicates the genotype of the TA strains used.

histidine mutation		additional mutations		
hisG46	hisD3052	LPS	Repair	R factor
TA100	TA 98	rfa	ΔuvrB	+ R
TA 1950		+	ΔuvrB	- R

All strains were originally derived from Samonella typhimurium LT-2. The rfa (deep rough) mutation eliminates the polysaccharide side chain of the lipopolysaccharide (LPS) that coats the bacterial surface. Wild-type LPS gene is indicated by a +. The deletion (Δ) through the uvrB region of the chromosome eliminates the excision repair system for DNA. Two strains, TA 98 and TA 100, contain an R factor (plasmids carrying antibiotic resistance genes), pKM101, which increases mutagenesis with certain mutagens (McCann et al., 1972).

The TA 98 strain contains the histidine mutation hisD3052, a frameshift mutation, which is reverted by a variety of polycyclic aromatic hydrocarbons (Ames et al., 1973b). Strains TA 100 and TA 1950 contain the histidine mutation hisG46 which is a missense mutation. It is well reverted by a variety of mutagens that cause base-pair substitutions (Ames, 1971).

Dominant Lethal-Cytogenetic Study

A preliminary LD50 range test of a benzene-olive oil mixture (1:1) was made using mature Long-Evans male rats prior to initiation of the study. Twelve rats were injected intraperitoneally with the mixture, 6 at 1.0 ml/kg of benzene and 6 at 2.0 ml/kg of benzene to check the estimated LD50 obtained from the literature. From this work, a rough estimation of 2.0 ml/kg of benzene for the 24 hour LD50 of a 1:1 solution of

benzene-olive oil was established. Following recommendations outlined in other dominant lethal studies, approximately 0.2 X LD50 or 0.5 ml/kg of benzene was selected as the optimal dose for use in the study. The dose of aqueous triethylene melamine (TEM) was the same as that used in previous dominant lethal studies conducted by the author in the same laboratory (0.25 mg/kg).

A total of 43 male Long-Evans (320-400 g) rats was used in the combined dominant lethal-cytogenetic study. These animals were 12 weeks old, mature breeders required for mating in the dominant lethal portion. The animals were randomly divided into the three treatment groups and ear punched for identification. Fourteen animals received olive oil (0.5 ml/kg) as a negative control group, 10 received aqueous TEM (0.25 mg/kg) for use as a positive control in the dominant lethal study and 19 were injected with sufficient 1:1 benzene-olive oil solution so that they received 0.5 ml/kg of benzene. All treatments were single intraperitoneal injections.

Ten control, 10 TEM-and 15 benzene-treated males were immediately placed into individual cages containing 2 mature, virgin female Long-Evans rats. The remaining 4 control and 4 benzene-treated males were housed in two separate cages in the same area as the other animals. All animals received food and water ad libitum throughout the study. In the dominant lethal portion of the study, two virgin females were supplied each male every week. Twenty female rats were assayed for each male over the 10 week course of the experiment (2/week) for a total of 700 females. After caging with the male rat for one

week, the two females were placed in a separate cage for additional holding. The females were sacrificed by carbon dioxide inhalation in a chamber 17 days after first caging with the male. The pregnant females were necropsied for gross abnormalities: corpra lutea, total implants, early fetal deaths and late fetal deaths were scored. At the time of necropsy, the pregnancies were between 10 to 17 days into term.

The four additional males in the control and in the benzene-treated groups were used for cytogenetic analysis. These animals were treated at the same time as the ones used for the dominant lethal study and sampled during the course of the experiment. Bone marrow biopsies and blood samples were obtained from each of these animals at 24 hours, 8 days, 30 days and 70 days.

To obtain blood and bone marrow samples for cytogenetic analysis, the rats were anesthetized using methoxyflurane (Abbott) and wrapped with a towel, leaving the lower extremities uncovered. The initial puncture was made in the skin at the center of the patellar groove using a heparinized spinal needle containing a stylus. The needle was inserted through the boney structure into the distal end of the femoral canal, the stylus removed and a 5 ml dispossable sterile syringe containing 0.1 ml of heparin solution fitted tightly into the needle adapter. The plunger was retracted gradually until a brownish-red material entered the syringe and mixed with the heparin solution. Approximately 0.1 to 0.2 ml of bone marrow was collected from one femur of each animal. The bone marrow

samples were immediately flushed into 5 ml of pre-warmed (37° C.) Minimum Essential Medium containing 10 µg/ml of colcimed (Denecolcine-Ciba) and incubated for two hours at 37° C.

The blood samples were collected by making a small transverse incision just above the clavicle at the mid-clavicular line exposing the cubclavian vein as it emerges at the supraclavicular fossa. A 2 ml heparinized sterile plastic syringe with a 20 gauge needle was used to extract 1 to 1.5 ml of blood. The incisions were closed by surgical wound clips and the rats returned to their cages.

Two-tenths to 0.3 ml of the freshly drawn blood was added to 5 ml of pre-warmed (37° C.) blood culture media made the day of the experiment. The tube was sealed with a rubber stopper and the suspension incubated at 37° C. for 3 days. The suspension was agitated once daily during the incubation. At the end of the 3 day period, 1 µg of colcemid in aqueous solution was added to the suspension and the suspension was incubated for another 4 hours.

After incubation with the colcimed, both the bone marrow and the blood culture suspensions were centrifuged at 100 g for 5 minutes. The supernatant was removed and the cell buttons resuspended with 0.075 M KCl for 15 minutes at room temperature. The suspension was then recentrifuged as before and the supernatant removed. The cell buttons were resuspended in 5 ml of fixative (3:1, methanol: glacial acetic acid), then allowed to stand at room temperature for 20 minutes before being recentrifuged. The process of changing the fixative was repeated twice until a final suspension was made for smear

preparation. A chromosome spread was made by dropping a few drops of the preparation onto a pre-cleaned microscope slide previously dippled into distilled water. The slides were air dried then stained with Giemsa stain diluted 1:20 with distilled water for 10 minutes. All slides were coded with a diamond pencil and then covered with a glass cover slip to protect the preparation.

The slides were scanned under low power magnification (70X) and cells selected for inclusion on the basis of the quality of chromosome spreading. Once selected, the cell was microlocated using the stage calibrations so as to preclude the same cell being used more than once. Seven to 10 slides were made from each rat bone marrow or blood suspension and the cells used for analysis were always obtained from more than one slide. In the case of some of the blood preparations, all available slides were completely searched before it was recorded that no metaphases were present. All metaphases selected for use were photographed under oil immersion magnification (2900X) using a Zeiss photomicroscope, enlarged (2X), and printed for analysis. These prints were coded to prevent bias. Independent scoring was carried out by two investigators. In case of doubts or disagreements between the two, the original cell was re-checked with the microscope and a third opinion obtained.

The metaphases were scored for numerical and structural aberrations. Numerical aberrations consisted of polyploidy, aneuploidy and endoreduplication. Structural aberrations consisted of achromatic lesions, chromatid deletions, chromosome deletions, rearrangements, minutes and fragmentation. An arbitrary definition was used to distinguish between lesions and deletions: deletions- any distinct discontinuity in the chromatin which exceeds the width of a chromatid of that cell. Lesion- any discontinuity which is less than the width of a chromatid and in which the distal piece is aligned with the proximal piece, or which contains visible material connecting the proximal

and distal portions, regardless of the width (Nichols et al., 1972).

A maximum of 20 metaphases were analyzed at each time period for every rat using slides prepared from blood specimens. In some cases, abnormal cell-types were found containing several nuclear bodies of varying size. These were denoted as chromosome pycnosis (Koller, 1972) and the frequency of this type of cell per 1000 normal-appearing nucleated cells were scored. For bone marrow analysis, 50 metaphases were scored for each rat at the indicated time periods.

A modified version of the mouse dominant lethal technique reported by Epstein (1973) was used for testing the rats. The cytogenetic blood culture procedure employed was similar to that reported by Farrow et al. (1975). Biopsy technique and culturing methods used for serial sampling of bone marrow specimens were developed and refined by J. Nemenzo of this department (1975).

Micronucleus Assay

Twenty-five male, 5 week old, Long-Evans rats (110-135 g) were randomly divided into groups of five for use in a micronucleus assay. The procedure used was a modification of that reported by Schmid (1975). The negative control group received olive oil (1.0 ml/kg) and the positive control group was treated with aqueous TEM (0.25 mg/kg). The remaining three groups received injections of benzene-olive oil. The benzene doses used for the 3 groups were 0.05 ml/kg, 0.10 ml/kg and 0.50 ml/kg. All treatments were intraperitoneally injected, one-half of the total dose at 30 hours and $\frac{1}{2}$ at 6 hours prior to sacrifice. Thirty-six hours after the initial injection, the rats were sacrificed by carbon dioxide inhalation in a chamber and the right femur was dissected from each animal. The cartilagious cap was removed and a small piece of the apiphysis at the distil end of the femur was clipped off

opening the femoral canal. The ball joint was cut from the proximal end and a 19 gauge disposable needle tightly inserted into the remaining portion of the ball joint shaft. A syringe containing 5 ml of fetal calf serum was securely attached to the needle and the bone marrow flushed from the femur into a tube. The mixture was immediately agitated to separate and suspend the bone marrow cells. The tubes were centrifuged at 100 X g for 5 minutes and the supernatant drawn off. The cell button remaining was mixed by agitation and a small amount aspirated into a siliconized capillary. A drop was placed on a pre-cleaned glass slide and spread by pulling a cover slip over the slide. Ten slides were prepared from each animal and allowed to air dry overnight. All slides were coded using a diamond pencil.

The following day, the slides were stained in groups of 30 using horizontal staining jars and a staining rack. The slides were stained in undiluted May-Greunwald solution for 3 minutes, in May-Greunwald solution diluted with buffered (pH 7.4) distilled water 1:6 for 10 minutes. The rack of slides was rinsed in buffered distilled water, the individual slides removed from the rack and blotted dry on filter paper. The back of each slide was cleaned with methanol and the slides replaced in the rack. After all 30 slides were cleaned, they were cleared in xylene for 10 minutes and covered with glass slips using slide cement. The slides were air dried for 2 days to allow the cement to set and then scanned under oil immersion (2900X) magnification using the Zeiss photomicroscope.

For each animal, 2000 polychromatic erythrocytes were counted and the number of these cells containing micronuclei recorded. The number of mature erythrocytes containing micronuclei noted while scanning the 2000 polychromatic cells was recorded as an internal control for artifacts resembling micronuclei.

Statistical Procedures

Comparison of sample means by the t-test was used in several of the experiments. This consisted of comparing the magnitude of the observed difference between the two means $(\bar{X}_1-\bar{X}_2)$ with an estimate of its standard error, $S_{(\bar{X}_1-\bar{X}_2)}$, and referring to the t distribution as expressed below.

$$t = \frac{(\bar{X}_1-\bar{X}_2)}{S_{(\bar{X}_1-\bar{X}_2)}}$$

If the observed t exceeded the tabulated critical value for t at p = 0.01 or p = 0.05 with the calculated degrees-of-freedom (df) contributed by both samples, it was concluded that the means differed from each other significantly (Goldstein, 1964). The following formula was used to calculate the above values.

n = sample size

x = sample measurements

$\bar{X} = \Sigma x/n$ = mean

$df = n_1 + n_2 - 2$

$$S_{(\bar{X}_1-\bar{X}_2)} = \sqrt{\frac{\Sigma X_1^2 - \frac{(\Sigma X_1)^2}{n_1} + \Sigma X_2^2 - \frac{(\Sigma X_2)^2}{n_2}}{n_1 + n_2 - 2} \left[\frac{1}{n_1} + \frac{1}{n_2}\right]}$$

In the analysis of two measurements made in the dominant lethal experiment, dead implants/pregnant female and dead implants/total implants per pregnant female, an arcsin transformation of the basic data was applied before computation of the t value. This was used to stabilize the variances which were derived from basic observations that have a binomial distribution. These observations are proportions, where the variance and means are related and transformed with the following

formula (Winer, 1962):

$$X' = 2 \arcsin (X)^{\frac{1}{2}}$$

A Chi-Square test using a 2 × 2 contingency table was used for determining significance between enumeration data in the experiments.

	category 1	category 2
# observed	a	b
# without	c	d

An estimated expectation for each cell of the table from the pooled data was determined. The contribution of each cell to Chi-Square was calculated making the Yates correction for continuity using the method outlined by Goldstein (1964) with the following formula:

$$N = a + b + c + d$$

$$\text{Chi-Square} = \frac{(|ad-bc| - \frac{1}{2}N)^2 (N)}{(a+b)(c+d)(a+c)(b+d)}$$

The calculated values were compared to tabulated critical values of Chi-Square.

In extreme cases, where one or more of the cells in the 2 × 2 contingency table contained values smaller than 5, the above method was not valid. In these cases, it was necessary to compute the exact probability to determine significance. The following formula was used for this purpose (Goldstein, 1964):

$$p = \frac{(a+b)!\ (c+d)!\ (a+c)!\ (b+d)!}{N!\ a!\ b!\ c!\ d!}$$

An analysis of variance (AOV) was conducted on the control olive oil data in the dominant lethal experiment. Five one-way AOV were made of samples

drawn from the data and a variance estimate computed for each. The variance was partitioned for that arising between males for each test week and within the males for each week. An F-ratio of the variance estimate between treatment weeks and within treatment week was compared to critical table values for significance at p = 0.01. The F-ratio was calculated from the following formula (Goldstein, 1964):

$$SS = \text{sum of squares}$$

$$\Sigma N_m(\bar{X}_m-\bar{X})^2 = \text{between-sample SS}$$

$$\Sigma(X_m-\bar{X}_m)^2 = \text{within-sample SS}$$

where:

X_m = each measurement in a particular sample

$\bar{X}$ = overall mean

$\bar{X}_m$ = mean of particular sample

N_m = number of measurements in a particular sample

df(between males) = number of males - 1

df(within males) = number of females assigned to individual male that become pregnant - 1

$$\text{F-ratio} = \frac{\text{between-sample SS}}{\text{within-sample SS}}$$

RESULTS

Host-Mediated Assay

Results of the host-mediated assay conducted using benzene are presented in Table 1. No deaths occurred in the control mice and the mice treated only with benzene. The mice pre-treated with phenobarbital (Pb) appeared to be severely affected by the subsequent injections of benzene. These mice were sedated and lying on their backs following the first benzene injection. One mouse out of the six originally in this group died within the hour after this injection. The second benzene and saline injections were given to all the animals but the third injection was withheld as the Pb group appeared as if they would not survive.

During the procedure involving the injection of saline intraperitoneally to flush and remove the bacteria, the loss of another data point from the Pb group occurred due to contamination of the peritoneal sample. This left only four animals in this group.

The mean mutation frequency (MF) and standard deviation for each group along with the individual MF calculated for each mouse are shown in Table 1. There was extreme variability noted in these values. Within the control group differences approaching 10-fold were observed. Considering this variability, it was surprising that the mean MF for the groups were within 1 unit of each other. No significant differences can be demonstrated between the group means.

Results of the bacterial plate assay using TA 1950 as the indicator organism are presented below. This assay was conducted in conjunction with the host-mediated assay for an *in vitro* comparison.

treatment	number of plates	counts/plate his^+ revertants + standard deviation
control	5	13.4 ± 4.0
benzene	5	7.0 ± 4.2
nitrogen mustard	5	280.0 ± 164.9

The mean spontaneous reversion rate of 13.4/plate is similar to that observed using this strain in other plate assays. On the benzene-treated plates, a kill-area in the center of the plate of 5 to 10 mm was observed on all plates. In this area no revertants or background growth was observed. The mean reversion rate of 7.0/plate indicates some lethality to the organism by high concentrations of benzene encountered in the central area of application as noted above. A 20-fold increase in the reversion rate per plate was observed with the nitrogen mustard.

Liver-Activated Plate Assay

Tables 2 and 3 contain the data obtained testing the effect of the two differently induced liver fractions at several concentrations with benzene in the activated bacterial assay. TA 100 and TA 98 were used with several concentrations of benzene as shown in Tables 2 and 3, respectively. Benzo(a)-pyrene (BP) was used as the positive control in both systems. With both bacterial strains, the liver prepared from 3-methylcholanthrene (3-MC) induced animals produced more reversions using BP than the phenobarbital (Pb)-induced livers did. At 50 µl/plate of S-9 fraction, the highest activation was obtained with the 3-MC liver with both bacteria strains. Use of 1 µl/plate appeared to be below the concentration necessary for activation with the 3-MC liver while the 10 µl/plate series produced some activation with both strains and both liver fractions. The 10 µl/plate concentration appeared to be the optimal concentration tested for activation using Pb induced-liver; decreased

activation was noted when more-or-less S-9 fraction was used. The BP did not produce reversion without activation at the levels used as demonstrated on the plates which contained 0 µl/plate of S-9 fraction.

Control levels for spontaneous reversion using DMSO show little variation at each liver concentration with both strains. There appeared to be no increased reversion produced by benzene with any of the liver concentrations used. The differences observed fall within the observed range of individual plate variation.

The average of the four protein determinations for each of the pooled liver specimens were 25.7 mg/ml for the Pb-induced animals and 23.7 mg/ml for the 3-MC-induced animals. Considering the methodology, these values are identical.

The work conducted using the bacterial plate assay technique with the addition of 1,1,1-trichloro-2,3-epoxypropane (TCPO) is summarized in Table 4 and is a combination of several experiments. These data were obtained using a liver activation system prepared from rats treated with 3-methylcholanthrene. In the text table below are shown the data obtained without the addition of any activation system to check the response of the bacterial test system (TA 100) and to test benzene mutagenicity and lethality without metabolic activation.

test compounds	plate conc.	his^+ revertants/ plate	test compounds	plate conc.	his^+ revertants/ plate
spontaneous	-	82	benzene	1.0 µl	83
DMSO	25 µl	87	benzene	5.0 µl	62
MNNG	crystal	1000	benzene	10.0 µl	39

The spontaneous reversion rate (negative control) and the rate seen with the addition of DMSO are essentially the same, 82 and 87, respectively. One plate containing a crystal of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a

positive control used to check the response of the system, was reverted with a count exceeding 1000 for the plate. Benzene in DMSO at the three concentrations tested, 1.0 µl, 5.0 µl and 10.0 µl, produced no increase above the spontaneous reversion rate. At the highest benzene concentration (10.0 µl/plate) a definite lethality was observed with a reduction in revertants on that plate versus plate counts observed for the spontaneous control and the lowest benzene concentration (1.0 µl/plate), 39 versus 82 and 83, respectively.

The four series of test in Table 4 were conducted with the addition of S-9 mix prepared from rats induced with 3-MC and a concentration of 50 µl S-9 fraction/plate was used throughout the series. In the various sections of this work, different spontaneous reversion rates can be observed. This is a result of the tests being conducted at different times and accounts for the requirement that simultaneous controls be included each time. In the four experiments, an activation of the BP with allied increases in the reversion rates can be seen. The increased reversion rates followed a dose-response with the highest rate seen at the highest concentration of BP. No increase in the spontaneous reversion rate was produced by the addition of TCPO at the levels used (0.05-0.25 mM). Preliminary work had indicated that TCPO at a concentration of 0.5 mM and above produced increased reversion with the TA 100 strain therefore, requiring use of the lower concentrations. A dose range of benzene from 0.1 to 2.0 µl produced no increase in the reversion rate and the addition of TCPO to the system had no effect within the range used when compared to the simultaneous controls. The combination of TCPO at all concentrations and the lowest concentration of BP (0.5 µg) also produced values within range of those seen with the BP alone indicating no enhancement of the mutagenicity of BP by the addition of TCPO to the top agar. In the last series, incubated in the water bath, the levels of

revertants/plate are lower indicating lethality to the bacteria while incubating but the pattern of the results remained the same; namely, no enhancement of reversion with the addition of TCPO.

Data from the experiment performed using analogs of benzene in combination with Pb-induced liver S-9 mix are presented in Table 5. The DMSO and BP control plates are the same as those presented in Table 2 because the assays were simultaneously conduced. As can be observed, there was no increased reversion of the TA 100 strain noted with either bromobenzene or chlorobenzene at the concentrations used.

The results obtained from the two separate experiments attempting the use of homogenized bone marrow as the activation system in a bacterial plate assay are presented in Table 6. Protein determinations from the pooled S-9 fraction of bone marrow from the non-induced rats indicate the presence of 0.97 mg/ml. The femurs from rats used to obtain the marrow were each flushed with 2 ml of KCl solution. The other S-9 fraction used was obtained from 3-MC-induced rats and each of these femurs were flushed with 0.8 ml of KCl solution in an attempt to provide a more concentrated S-9 fraction. The concentration of protein from this fraction was 2.0 mg/ml.

The results from both S-9 fractions verus benzene indicated no increase in the reversion rate at any concentration combination used. The number of revertants counted was within the range of that obtained using DMSO as control. A concentration of benzene (3.3 µl) was used in the 3-MC-induced series and this concentration had a lethal effect on the indicator organism. The number of revertants per plate was reduced by 1/2 to 1/3. It was also noted that the background lawn of TA 100 was reduced. BP (10 µg) was not activated with either S-9 fraction at any concentration.

Dominant Lethal Study

Presented below in the text table is the preliminary range-finding study conducted to determine the optimal dose for use in the subsequent study. The animals were administered a single dose of benzene-olive oil mixture (1:1) intraperitoneally and observed for two weeks to determine the death ratio.

Dose	rat #	weight (grams) day 0	day 7	day 14	death ratio
benzene	1	347	382	400	
	2	371	375	397	
1 ml/kg	3	254	257	273	0/6
	4	241	262	301	
	5	230	251	286	
	6	260	264	325	
benzene	1	264	252	338	
	2	273	dead 10 hours		
2 ml/kg	3	239	dead 10 hours		4/6
	4	253	290	329	
	5	252	dead 10 hours		
	6	268	dead 10 hours		

The recommended dose for preliminary dominant lethal assays is 0.2 LD50 (Epstein, 1973). The observed 2 week mortality of 4/6 using a dose of 2 ml/kg was used to calculate a dose of 0.4 ml/kg which was rounded up to 0.5 ml/kg.

No deaths were noted in 19 rats injected with benzene (0.5 ml/kg) for use in the dominant lethal-cytogenetic study. Similarly, no deaths were observed in the olive oil control or triethylenemelamine (TEM) groups. The group weights (mean ± standard deviation) of the treated male rats used for breeding and cytogenetic samplings are presented below. The animals were weighed before injection and at the termination of the breeding sequence (70 days).

	olive oil	benzene	TEM
number of animals	10	19	10
day 0	362 ± 19 g	353 ± 22 g	367 ± 28 g
day 70	541 ± 21 g	500 ± 41 g	497 ± 53 g
weight gain (mean ± S.D.)	180 ± 17 g	147 ± 45 g	129 ± 35 g

Comparison of the mean weight gain between the olive oil groups and the TEM and benzene groups indicate significant depressions, $p < 0.01$ and $p < 0.05$, respectively.

A summary of the necropsy data from the 10 week dominant lethal study is presented in Table 7. The parameters measured are listed with the group value determined by week. Five one-way analyses-of-variance were performed on the control group data for the 10 week period. Parameters analyzed were number of pregnant females, number of implantations/pregnant female, number of dead implantations/pregnant female, number of dead implantations/total implantations for each pregnant female and the pre-implantation loss. No significant differences were detected in these categories in the control group over the course of the experiment. One male rat in the olive oil group proved to be sterile for the entire experiment and all data derived from this rat were discarded.

A comparison was made each week between the olive oil group and the benzene and TEM groups of the fertility index defined as the number pregnant/number mated. Using a Chi-Square analysis, no significant differences were detected at any week.

The total implantations/pregnant female for each group by week was analyzed using a t test. The olive oil and benzene data showed little variation by week. A decrease was observed

for the TEM group week 2, 3 and 4 with the minimum at week 4. This decrease was significant ($p < 0.01$) each of the three weeks when compared to the olive oil control values of the concurrent week.

The group ratios of dead implantations/pregnant female and dead implantations/total implantation are presented in the next two columns. No significant differences were detected at any week in either parameter between the olive oil and benzene groups. The TEM group showed a significant ($p < 0.01$) increase the first 5 weeks in both values when compared to the control. The highest increases were observed in weeks 2, 3 and 4 with the values returning to control levels by week 6. The pre-implantation loss/pregnant female, defined as the corpora lutea count minus total implantation count/pregnant female, is given by week for the groups. No significant differences were detected at any week between benzene and TEM data when compared to the controls.

Bone Marrow Cytogenetic Study

The data obtained from cytogenetic analyses of bone marrow samples taken at 1, 8, 30 and 70 days following a single administration of benzene or olive oil are presented in Tables 8 and 9 then summarized in Table 10. In Table 8 are shown the total number of each type of aberration for each individual animal detected from screening 50 metaphases per animal at each time period. Table 9 contains similar data presented on the basis of the number of cells from each individual animal which contained the aberration. From these two tables containing the data of each animal, it can be seen that the aberrations

observed were spread among the animals in that group and not concentrated entirely in one member. The data from Tables 8 and 9 were pooled for analysis and are presented in Table 10. Several observations can be made regarding the data in this table.

Achromatic lesions (gaps) are variable even in the olive oil group. This parameter was recorded and included for analysis even though Nichols et al. (1972) and Legator et al. (1973) report that gaps are the least conclusive measurement. A significant increase ($p < 0.01$) was noted in the benzene group at 24 hours. In contrast, at 8 days, the olive oil group was elevated above the benzene group but both values are much lower than that for benzene at 24 hours.

Chromatid deletions (breaks) were analyzed using a Chi-Square test. Based on the number of cells containing the aberration at each time period, a significant ($p < 0.01$) increase was noted at 1 and 8 days in the benzene group compared to the concurrent control. The data on this parameter at 30 and 70 days were not significantly increased but a trend can be seen. The increase observed in the number of cells with breaks in the benzene group gradually falls off approaching control level at 70 days. Control levels of breaks maintained a fairly constant level of 4 or less cells affected at each sampling period. Chromosome deletions present a striking example of this gradual fall with time. A significant increase ($p < 0.01$) was noted at 24 hours with diminishing numbers found until by 70 days no chromosome deletions were detected. In contrast, no chromosome

deletions were observed at anytime in the control group.

Rearrangements of different types were noted, always in the benzene group, but the number detected was too small to be significant statistically.

Double minutes present another type of aberration where none was found in the control group but they were detected in the benzene group. Again the number found was too small to be statistically significant but as can be seen, this type of aberration appears to persist. Per two hundred cells analyzed at each time period in the benzene group, 2 were found at 24 hours, 2 at 8 days, 1 at 30 days and 3 at 70 days.

One polyploidy was noted in a benzene treated animal at 8 days. No other polyploid cells were detected and no significance can be made of the one alone.

An analysis of the number of cells observed at each time period for each group that contained the normal count of chromosomes is presented below.

Percentage of cells containing the normal complement of 42 chromosomes (mean ± standard deviation)

	1 day	8 days	30 days	70 days
control	82.5 ± 8.5	88.0 ± 4.9	91.5 ± 5.3	93.5 ± 2.5
benzene	83.0 ± 2.0	89.5 ± 6.8	92.0 ± 3.7	88.8 ± 7.1

There are no significant differences between these values at each time period when compared with the simultaneously run control.

Blood Specimen Cytogenetic Study

In the same manner as the bone marrow data, the peripheral blood cytogenetic data are presented in Table 11 as the total number of aberrations found in the cells analyzed and in Table 12 as the number of cells containing the specific aberrations. Both are on the basis of the individual animal. The data were pooled and are summarized in Table 13.

Difficulty was encountered in the culturing of the lymphocytes from the blood samples at the last two sampling periods of 30 and 70 days. The blood samples were not stimulated by the phytohemaglutinin P used to a sufficient degree to obtain the minimum of 20 metaphases used for analysis. At 70 days, only one control animal produced enough metaphases for total analysis, two were somewhat responsive so that 13 and 14 metaphases were located using all ten slides available and one sample produced no metaphases. This makes it difficult to extract useful data from the 70 day data. The 30 day control data were entirely useless.

At 24 hours, only one benzene animal (#6) responded with the minimum 20 metaphases for analysis and two produced none while one metaphases was found for the other. All control animals were sufficiently stimulated to obtain 20 metaphases per animal. The 8 day sample from control animals again gave 20 metaphases per animal. In the benzene group, the same animal (#6) produced 20 again. Rat #5 appeared to be recovering from treatment and 18 metaphases were located. Rat #7 was still suppressed and only 5 were located while rat #8 was totally suppressed. Because a 100% response was noted in the control

animals at 1 and 8 days, it is felt that the failure to locate the minimum of 20 metaphases in the benzene-treated animals at 1 and 8 days is due to the benzene treatment and not to technique problems as encountered at 30 and 70 days.

A Chi-square analysis of the number of cells containing the specific aberrations was made for each time period between the pooled data of the olive oil and the benzene groups. Due to the very small numbers of cells analyzed and the corresponding number of aberrations found, no significant differences were detected. It should be noted that double minutes were again observed solely in the benzene group, 1/21 cells at 24 hours, 3/42 cells at 8 days and 1/53 cells at 70 days. The presence of this type of aberration was constant and persistent. No polyploid cells were observed and only 2 rearrangements in one cell of a benzene-treated animal at 8 days.

As in the bone marrow study, no significant differences were detected between the percentage of cells containing the normal complement of 42 chromosomes. The data are presented below.

	Percentage of cells containing the normal complement of 42 chromosomes (mean + std. dev.)		
	1 day	8 days	70 days
control	97.5 ± 2.9	80.0 ± 12.2	80.3 ± 4.2
benzene	97.5 ± 3.5	89.3 ± 9.3	86.3 ± 13.1

The slides prepared from the blood specimens of each rat were scanned for viable nucleated cells actively growing. In the benzene treated animals, cells were observed which contained chromosome pycnosis (fragmentation). No pycnosis was observed

in any of the olive oil control slides. The data from the benzene animals are summarized in Table 14. Two observations can be made from these data. The number of pycnotic cells observed/1000 viable nucleated cells falls off with time after treatment in three of the four animals. The animal (rat #6) in which the minimum 20 metaphases were found for analysis at 1 day was the lowest in pycnotic count. The same observation can be made at 8 days. The data are confused at 70 days by the failure of the PHA-P to stimulate fully but pycnosis was again observed in all animals.

Micronucleus Assay

The data from the micronucleus assay are presented in Table 15. The data obtained from all 5 animals in each group were pooled, the mean value calculated and comparisons made with the control olive oil values using a t test. The values observed with the lowest benzene dose of 0.05 ml/kg were not significantly different from the control value while the other two levels were significantly ($p < 0.01$) increased 2- to 3-fold above control levels. The number of micronuclei observed with TEM used as positive control was also significantly increased ($p < 0.01$), approximately 12-fold. While counting the number of polychromatic cells containing micronuclei, a running total of the number of normal erythrocytes containing micronuclei was kept for control purposes. In all rats, the number varied from 0 to 5 indicating little interference by artifacts in the procedure.

Table 1. Mouse Host-Mediated Assay with Benzene[a] using Salmonella typhimurium LT-2 TA 1950 as the Indicator Organism.

treatment	mouse no.	his^+ revertants/ ml undiluted exudate	colony-forming units/ ml undiluted exudate $\times 10^6$	mutation frequency (+ standard deviation)
control	1	160	68	2.35
	2	110	95	1.16
	3	180	14	12.86
	4	170	160	1.06
	5	180	29	6.31
				4.73 ± 5.0
benzene	6	160	91	1.76
	7	160	49	3.27
	8	110	47	2.34
	9	210	30	7.00
	10	200	32	6.25
				4.12 ± 2.4
benzene (Pb)[b]	11	200	93	2.15
	12	170	64	2.66
	13	110	72	1.53
	14	220	23	9.57
				3.98 ± 3.8

[a] Benzene (0.1 ml/mouse) subcutaneously administered two hours and one hour before sacrifice.

[b] Mice were pre-treated twice daily for three days with phenobarbital sodium (0.35 mg/kg).

Table 2. Liver-Activated Bacterial Plate Assay using *Salmonella typhimirium* TA 100

	his$^+$ revertants/plate [a]				
	µl/plate S-9 fraction (3-MC liver)				
compound	0	1	10	20	50
DMSO 25 µl	141	152	145	142	168
Benzene 0.1 µl	142	143	141	129	148
Benzene 0.5 µl	138	136	136	131	162
Benzene 1.0 µl	135	155	156	154	142
Benzo(a)pyrene 5 µg	123	155	252	714	1489
	µl/plate S-9 fraction (Pb liver)				
	0	1	10	20	50
DMSO 25 µl	-	159	164	134	157
Benzene 0.1 µl	-	146	164	152	147
Benzene 0.5 µl	-	159	170	173	168
Benzene 1.0 µl	-	158	169	153	136
Benzo(a)pyrene 10 µg	-	218	316	281	249

[a] One plate was used per compound with each liver concentration.

[b] 3-MC = 3-methylcholanthrene, Pb = sodium phenobarbital. See method section for procedure.

Table 3. Liver-Activated Bacterial Plate Assay using *Salmonella typhimirium* TA 98

	his$^+$ revertants/plate [a]				
	µl/plate S-9 fraction (3-MC liver)				
compound	0	1	10	20	50
DMSO 25 µl	17	18	18	19	32
Benzene 0.1 µl	23	22	24	35	33
Benzene 0.5 µl	26	17	23	30	23
Benzene 1.0 µl	15	20	17	24	34
Benzo(a)pyrene 5 µg	15	19	86	157	333
	µl/plate S-9 fraction (Pb livers)				
	0	1	10	20	50
DMSO 25 µl	-	18	23	23	24
Benzene 0.1 µl	-	24	33	27	23
Benzene 0.5 µl	-	17	19	24	22
Benzene 1.0 µl	-	16	21	31	30
Benzo(a)pyrene 1 µg	-	40	91	62	51

[a] One plate was used per compound with each liver fraction concentration.

[b] 3-MC = 3-methylcholanrene, Pb = sodium phenobarbital. See method section for procedure.

Table 4. Summary of Liver-Activated[a] Bacterial Plate Testing using Salmonella typhimurium LT-2 TA 100 with and without 1,1,1-trichloro-2,3-Epoxypropane (TCPO) added to Top Agar.

test compounds	plate conc.	counts/ plate	test compounds	plate conc.	counts/ plate	test compounds	plate conc.	counts/ plate
						TCPO (0.05 mM) added to top agar		
spontaneous	-	89				spontaneous	-	67
benzo(a)pyrene	0.5 µg	281				benzo(a)pyrene	0.5 µg	258
benzo(a)pyrene	1.0 µg	372	benzene	0.1 µl	76	benzene	0.1 µl	75
benzo(a)pyrene	5.0 µg	766	benzene	0.2 µl	75	benzene	0.2 µl	68
DMSO	25 µl	86	benzene	0.5 µl	88	benzene	0.5 µl	90
			benzene	0.8 µl	68	benzene	0.8 µl	105
			benzene	1.0 µl	67	benzene	1.0 µl	63
			benzene	2.0 µl	72	benzene	2.0 µl	86
						TCPO (0.10 mM) added to top agar		
spontaneous	-	51				spontaneous	-	33
benzo(a)pyrene	0.5 µg	291				benzo(a)pyrene	0.5 µg	258
benzo(a)pyrene	1.0 µg	399				benzene	0.7 µl	29
benzo(a)pyrene	5.0 µg	790				benzene	1.0 µl	27
DMSO	25 µl	40						
						TCPO (0.25 mM) added to top agar		
spontaneous	-	37				spontaneous	-	33
benzo(a)pyrene	0.5 g	66				benzo(a)pyrene	0.5 µg	78
benzo(a)pyrene	1.0 g	89				benzene	0.8 µl	56
benzo(a)pyrene	5.0 g	173						
						TCPO (0.25 mM) in incubation mix[b]		
spontaneous[b]	-	20				spontaneous	-	25
benzo(a)pyrene	0.1 µg	23	benzene[b]	0.05 µl	10	benzo(a)pyrene	0.1 µg	32
benzo(a)pyrene	0.4 µg	85	benzene	0.15 µl	13	benzene	0.15 µl	15
benzo(a)pyrene	0.8 µg	106	benzene	0.30 µl	12	benzene	0.30 µl	18
DMSO	25 µl	18						

[a] Liver homogenate prepared from rats induced with 3-methylcholanthrene (50 µl S-9 fraction/plate).

[b] This series was incubated for 50 mins. at 37° C. in a water bath before pour plating.

Table 5. Liver-Activated Bacterial Plate Assay of Benzene Analogs using Salmonella typhimurium LT-2 TA 100.

		his$^+$ revertants/plate[a]			
		µl/plate S-9 fraction (Pb livers)			
compound		1	10	20	50
DMSO 25 µl		159	164	134	157
Bromobenzene	0.1 µl	136	185	167	156
Bromobenzene	0.5 µl	141	180	178	159
Bromobenzene	1.0 µl	146	169	135	185
Chlorobenzene	0.1 µl	164	128	176	174
Chlorobenzene	0.5 µl	193	149	170	161
Chlorobenzene	1.0 µl	169	162	162	175
Benzo(a)pyrene	10 µg	218	316	281	249

[a] One plate was used with each compound at each liver concentration. Pb = phenobarbital.

Table 6. Activated Bacterial Plate Assay of Benzene using Salmonella typhimurium LT-2 TA 100 with Rat Bone Marrow Homogenate as the S-9 Fraction.[b]

	his$^+$ revertants/plate[a]				
	µl/plate S-9 fraction				
compound	0	10	20	50	100
DMSO 25 µl	141	153	145	154	144
Benzene 0.1 µl	142	144	139	119	156
Benzene 0.5 µl	138	138	174	140	125
Benzene 1.0 µl	135	135	135	139	131
Benzo(a)pyrene 10 µg	123	145	147	157	147
	µl/plate S-9 fraction (3-MC-induced)				
	0	10	20	50	90
DMSO 25 1	152	162	127	143	124
Benzene 0.1 µl	158	163	174	165	145
Benzene 0.5 µl	134	157	172	145	161
Benzene 1.0 µl	122	173	140	153	134
Benzene 3.3 µl	51	58	56	70	69
Benzo(a)pyrene 10 µg	158	156	155	156	162

[a] One plate was used with each compound at each S-9 fraction concentration.

[b] Protein determinations on the S-9 fractions indicated 0.97 mg/ml for the bone marrow from non-induced rats and 2.0 mg/ml for the 3-MC induced rats.

Table 7. Summary of Dominant Lethal Assay in Long-Evans Rats following a Single Intraperitoneal Administration of Olive Oil, Benzene or Triethylenemelamine.

treatment	week	no. mated	no. preg.	implants live	implants dead	tot. impl. preg. fem.	dead impl. preg. fem.	dead impl. tot. impl.	average pre-impl. loss[a]
Olive Oil	1	18	12	138	3	11.75	0.25	0.02	0.33
	2	18	15	153	17	11.33	1.13	0.10	0.60
0.5 ml/kg	3	18	17	187	8	11.47	0.47	0.04	0.59
	4	18	18	168	15	10.17	0.83	0.08	0.61
	5	18	17	181	8	11.12	0.47	0.04	1.00
	6	18	16	162	10	10.75	0.63	0.06	0.38
	7	18	15	172	11	12.20	0.73	0.06	0.20
	8	18	17	168	12	10.59	0.71	0.07	0.59
	9	18	15	143	11	10.27	0.73	0.07	0.87
	10	18	17	149	19	9.88	1.12	0.11	0.53
Benzene	1	30	24	227	17	10.17	0.71	0.07	1.00
	2	30	24	245	14	10.79	0.58	0.05	0.83
0.5 ml/kg	3	30	26	287	20	11.81	0.77	0.07	0.27
	4	30	25	241	32	10.92	1.28	0.12	0.56
	5	30	29	292	35	11.28	1.21	0.11	0.38
	6	30	27	277	15	10.81	0.56	0.05	0.52
	7	30	25	230	28	10.32	1.12	0.11	0.76
	8	30	26	280	29	11.88	1.12	0.09	0.19
	9	30	27	274	29	11.22	1.07	0.10	0.48
	10	30	28	276	16	10.43	0.57	0.05	0.54
Triethyl-	1	20	16	144	28	10.75	1.75 *	0.16 *	0.88
enemelamine	2	20	19	23	138	8.47 *	7.26 *	0.86 *	1.11
	3	20	16	18	118	8.50 *	7.38 *	0.87 *	1.38
0.25 mg/kg	4	20	18	4	102	5.89 *	5.67 *	0.96 *	1.11
	5	20	16	117	60	11.06	3.75 *	0.34 *	0.81
	6	20	17	167	22	11.12	1.29	0.12	0.35
	7	20	18	180	11	10.61	0.61	0.06	0.94
	8	20	17	167	14	10.65	0.82	0.08	0.71
	9	20	20	183	23	10.30	1.15	0.11	0.55
	10	20	17	176	8	10.82	0.47	0.04	0.47

[a] Calculated as the mean corpora lutea count minus the mean total implantation count.
* Values significantly differ from control values at $p < 0.01$.

Table 8. Cytogenetic Data from Serial Bone Marrow Specimens of Rats[a] following a Single Intraperitoneal Administration of Olive Oil (0.05 ml/kg) or Benzene (0.5 ml/kg).

			total number of aberrations/50 metaphases					
rat #	treatment	time after treatment	achromatic lesions	chromatid deletions	chromosome deletions	rearrangements	double minutes	polyploidy
1	olive oil	1 day	3	2	0	0	0	0
2	olive oil	1 day	0	2	0	0	0	0
3	olive oil	1 day	4	0	0	0	0	0
4	olive oil	1 day	7	0	0	0	0	0
5	benzene	1 day	13	8	5	0	0	0
6	benzene	1 day	8	9	2	0	0	0
7	benzene	1 day	23	23	3	1 exchange	3	0
8	benzene	1 day	10	9	3	1 exchange	0	0
1	olive oil	8 days	3	0	0	0	0	0
2	olive oil	8 days	5	2	0	0	0	0
3	olive oil	8 days	11	C	0	0	0	0
4	olive oil	8 days	4	0	0	0	0	0
5	benzene	8 days	7	5	0	1 dicentric	0	0
6	benzene	8 days	4	3	0	0	0	1
7	benzene	8 days	1	5	2	1 dicentric	0	0
8	benzene	8 days	2	2	1	0	2	0
1	olive oil	30 days	0	1	0	0	0	0
2	olive oil	30 days	0	0	0	0	0	0
3	olive oil	30 days	4	1	0	0	0	0
4	olive oil	30 days	2	3	0	0	0	0
5	benzene	30 days	4	5	2	0	0	0
6	benzene	30 days	5	6	0	0	1	0
7	benzene	30 days	3	0	0	0	0	0
8	benzene	30 days	4	2	1	0	0	0
1	olive oil	70 days	2	0	0	0	0	0
2	olive oil	70 days	0	0	0	0	0	0
3	olive oil	70 days	0	0	0	0	0	0
4	olive oil	70 days	3	1	0	0	0	0
5	benzene	70 days	3	2	0	0	2	0
6	benzene	70 days	5	1	0	0	0	0
7	benzene	70 days	2	5	2	0	1	0
8	benzene	70 days	3	0	0	1 ring	0	0

[a] Four animals were used in each treatment group and the same four sampled at each time period.

Table 9. Cytogenetic Data from Serial Bone Marrow Specimens of Rats[a] following a Single Intraperitoneal Administration of Olive Oil (0.05 ml/kg) or Benzene (0.05 ml/kg).

			number of cells with aberration/50 analyzed					
rat #	treatment	time after treatment	achromatic lesions	chromatid deletions	chromosome deletions	rearrangements	double minutes	polyploidy
1	olive oil	1 day	3	2	0	0	0	0
2	olive oil	1 day	0	2	0	0	0	0
3	olive oil	1 day	4	0	0	0	0	0
4	olive oil	1 day	6	0	0	0	0	0
5	benzene	1 day	10	8	5	0	0	0
6	benzene	1 day	5	8	2	0	0	0
7	benzene	1 day	17	15	3	1 exchange	2	0
8	benzene	1 day	7	7	2	1 exchange	0	0
1	olive oil	8 days	2	0	0	0	0	0
2	olive oil	8 days	4	2	0	0	0	0
3	olive oil	8 days	10	0	0	0	0	0
4	olive oil	8 days	4	0	0	0	0	0
5	benzene	8 days	5	4	0	1 dicentric	0	0
6	benzene	8 days	4	3	0	0	0	1
7	benzene	8 days	1	5	2	1 dicentric	0	0
8	benzene	8 days	2	2	1	0	2	0
1	olive oil	30 days	0	1	0	0	0	0
2	olive oil	30 days	0	0	0	0	0	0
3	olive oil	30 days	4	1	0	0	0	0
4	olive oil	30 days	2	2	0	0	0	0
5	benzene	30 days	4	4	2	0	0	0
6	benzene	30 days	5	5	0	0	1	0
7	benzene	30 days	3	0	0	0	0	0
8	benzene	30 days	4	2	1	0	0	0
1	olive oil	70 days	2	0	0	0	0	0
2	olive oil	70 days	0	0	0	0	0	0
3	olive oil	70 days	0	0	0	0	0	0
4	olive oil	70 days	3	1	0	0	0	0
5	benzene	70 days	3	2	0	0	1	0
6	benzene	70 days	3	1	0	0	0	0
7	benzene	70 days	2	5	2	0	1	0
8	benzene	70 days	2	0	0	1 ring	0	0

[a] Four animals were used in each treatment group and the same four sampled at each time period.

Table 10. Summary of Cytogenetic Analysis for Serial Bone Marrow Specimens from Rats[a] following a Single Intraperitoneal Administration of Olive Oil or Benzene.

		Aberrations					
treatment	criteria	achromatic lesions	chromatid deletions	chromosome deletions	rearrangements	double minutes	polyploidy
olive oil - 1 day	no. animals affected	3	2	0	0	0	0
	total no. found	14	4	0	0	0	0
	no. cells affected[b]	13	4	0	0	0	0
benzene - 1 day	no. animals affected	4	4	4	2	1	0
	total no. found	54	49	13	2 exchanges	3	0
	no. cells affected	39 *	38 *	12 *	2	2	0
olive oil - 8 days	no. animals affected	4	1	0	0	0	0
	total no. found	23	2	0	0	0	0
	no. cells affected	20	2	0	0	0	0
benzene - 8 days	no. animals affected	4	4	2	2	1	1
	total no. found	14	15	3	2 dicentrics	2	1
	no. cells affected	12	14 *	3	2	2	1
olive oil -30 days	no. animals affected	2	3	0	0	0	0
	total no. found	6	5	0	0	0	0
	no. cells affected	6	4	0	0	0	0
benzene -30 days	no. animals affected	4	3	2	0	1	0
	total no. found	16	13	3	0	1	0
	no. cells affected	16	11	3	0	1	0
olive oil -70 days	no. animals affected	2	1	0	0	0	0
	total no. found	5	1	0	0	0	0
	no. cells affected	5	1	0	0	0	0
benzene -70 days	no. animals affected	4	3	0	1	2	0
	total no. found	13	8	0	1 ring	3	0
	no. cells affected	10	8	0	1	2	0

[a] Four animals were used in each treatment group and the same four sampled at each time period.

[b] Two hundred cells were analyzed for each group at each time period, fifty metaphases per animal.

* Values significantly differ from control values at $p < 0.01$.

Table 11. Cytogenetic Data from Serial Blood Specimens of Rats[a] following a Single Intraperitoneal Administration of Olive Oil (0.05 ml/kg) or Benzene (0.5 ml/kg).

rat #	treatment	time after treatment	no. scored[b] metaphases	total number of aberrations				
				achromatic lesions	chromatid deletions	chromosome deletions	rearrangements	double minutes
1	olive oil	1 day	20	1	1	0	0	0
2	olive oil	1 day	20	3	0	0	0	0
3	olive oil	1 day	20	2	1	0	0	0
4	olive oil	1 day	20	0	0	0	0	0
5	benzene	1 day	0	0	0	0	0	0
6	benzene	1 day	20	2	2	0	0	1
7	benzene	1 day	1	0	0	0	0	0
8	benzene	1 day	0	0	0	0	0	0
1	olive oil	8 days	20	1	0	0	0	0
2	olive oil	8 days	20	3	1	0	0	0
3	olive oil	8 days	20	4	0	0	0	0
4	olive oil	8 days	20	2	0	0	0	0
5	benzene	8 days	18	7	1	0	2 exchanges	0
6	benzene	8 days	20	3	1	0	0	3
7	benzene	8 days	5	0	0	0	0	0
8	benzene	8 days	0	0	0	0	0	0
1	olive oil	70 days	20	0	1	0	0	0
2	olive oil	70 days	14	3	1	0	0	0
3	olive oil	70 days	0	0	0	0	0	0
4	olive oil	70 days	13	1	0	0	0	0
5	benzene	70 days	1	0	0	0	0	0
6	benzene	70 days	20	3	1	1	0	1
7	benzene	70 days	20	3	0	0	0	0
8	benzene	70 days	12	0	0	0	0	0

[a] Four animals were used in each treatment group and the same four sampled at each time period.

[b] A maximum of 20 metaphases were scored for each rat at each treatment time. In those cases where less are scored, all available slides were searched to obtain the maximum number possible.

Table 12. Cytogenetic Data from Serial Blood Specimens of Rats[a] following a Single Intraperitoneal Administration of Olive Oil (0.5 ml/kg) or Benzene (0.5 ml/kg).

rat #	treatment	time after treatment	no. scored[b] metaphases	number of cells with aberration				
				achromatic lesions	chromatid deletions	chromosome deletions	rearrangements	double minutes
1	olive oil	1 day	20	1	1	0	0	0
2	olive oil	1 day	20	1	0	0	0	0
3	olive oil	1 day	20	2	1	0	0	0
4	olive oil	1 day	20	0	0	0	0	0
5	benzene	1 day	0	0	0	0	0	0
6	benzene	1 day	20	1	2	0	0	1
7	benzene	1 day	1	0	0	0	0	0
8	benzene	1 day	0	0	0	0	0	0
1	olive oil	8 days	20	1	0	0	0	0
2	olive oil	8 days	20	3	1	0	0	0
3	olive oil	8 days	20	3	0	0	0	0
4	olive oil	8 days	20	1	0	0	0	0
5	benzene	8 days	18	5	1	0	2	0
6	benzene	8 days	20	3	1	0	0	2
7	benzene	8 days	5	0	0	0	0	0
8	benzene	8 days	0	0	0	0	0	0
1	olive oil	70 days	20	0	1	0	0	0
2	olive oil	70 days	14	1	1	0	0	0
3	olive oil	70 days	0	0	0	0	0	0
4	olive oil	70 days	13	1	0	0	0	0
5	benzene	70 days	1	0	0	0	0	0
6	benzene	70 days	20	2	1	1	0	1
7	benzene	70 days	20	3	0	0	0	0
8	benzene	70 days	12	0	0	0	0	0

[a] Four animals were used in each treatment group and the same four sampled at each time period.

[b] A maximum of 20 metaphases were scored for each rat at each treatment time. In those cases where less are scored, all available slides were searched to obtain the maximum number possible.

Table 13. Summary of Cytogenetic Analysis for Serial Blood Specimens from Rats[a] following a Single Intraperitoneal Administration of Olive Oil or Benzene

		aberrations				
treatment	criteria	achromatic lesions	chromatid deletions	chromosome deletions	rearrangements	double minutes
olive oil - 1 day	no. cells scored[b]	80	80	80	80	80
	no. animals affected	3	2	0	0	0
	total no. found	6	2	0	0	0
	no. cells affected	4	2	0	0	0
benzene - 1 day	no. cells scored	21	21	21	21	21
	no. animals affected	1	1	0	0	1
	total no. found	2	2	0	0	1
	no. cells affected	1	2	0	0	1
olive oil - 8 days	no. cells scored	80	80	80	80	80
	no. animals affected	4	1	0	0	0
	total no. found	10	1	0	0	0
	no. cells affected	8	1	0	0	0
benzene - 8 days	no. cells scored	42	42	42	42	42
	no. animals affected	2	2	0	1	1
	total no. found	10	2	0	2 exchanges	3
	no. cells affected	8	2	0	1	2
olive oil -70 days	no. cells scored	47	47	47	47	47
	no. animals affected	2	2	0	0	0
	total no. found	4	2	0	0	0
	no. cells affected	2	2	0	0	0
benzene -70 days	no. cells scored	53	53	53	53	53
	no. animals affected	2	1	1	0	1
	total no. found	6	1	1	0	1
	no. cells affected	5	1	1	0	1

[a] Four animals were used in each treatment group and the same four sampled at each time period.

[b] A maximum of 80 cells were analyzed for each group at each time period, 20 metaphases per animal. In those cases where less are scored, all available slides were searched to obtain the maximum number possible.

Table 14. Chromosome Pycnosis Data for Serial Blood Specimens from Rats[a] following a Single Intraperitoneal Administration of Benzene (0.5 ml/kg).

	pycnotic cells/1000 viable nucleated cells		
rat no.	1 day	8 days	70 days
5	21	12	10
6	3	8	2
7	17	19	8
8	31	14	5
	18 ± 1.2[b]	13 ± 4.6	6.3 ± 3.5

[a] The same four animals were sampled at each time period.

[b] Mean ± the standard deviation.

Table 15. Results of the Micronucleus Assay in Rats following Intraperitoneal[a] Administration of Olive Oil, Benzene or Triethylenemelamine (TEM).

	Percent Cells containing Micronuclei/2000 Polychromatic Erythrocytes Scored				
rat #	olive oil (1.0 ml/kg)	benzene (0.05 ml/kg)	benzene (0.10 ml/kg)	benzene (0.50 ml/kg)	TEM (0.25 mg/kg)
1	0.30	0.25	0.55	0.65	2.30
2	0.15	0.25	0.40	0.40	2.80
3	0.15	0.40	0.30	0.65	2.65
4	0.05	0.05	0.50	0.75	2.00
5	0.30	0.15	0.75	0.95	2.90
mean ± SD	0.19 ± 0.11	0.22 ± 0.13	0.50 ± 0.17**	0.68 ± 0.20**	2.53 ± 0.37**

[a] Each animal received one-half the dose 30 hours and one-half 6 hours before sacrifice.

** Significantly different from control value, $p < 0.01$.

DISCUSSION

Any discussion concerning the mutagenic potential of a chemical should be preceeded by a short review of the problem. A mutation can be defined as any hereditary alteration in the informational content or in the distribution of the hereditary material in an organism or cell not due to polyploidy or recombination (Freese, 1971). The mutation may be the result of a chemical transformation of an individual gene (point mutation) or it may involve a gain, loss or rearrangement of a chromosome or a part of a chromosome (chromosomal mutation). Mutations can occur in germ cells which are used for propagation of the organism and in somatic cells which do not contribute to the progeny of the organism but die with it. Mutations may lead to morphological or biochemical changes with the consequences depending on the developmental state of the organism. Most mutations producing effects large enough to be observed are deleterious, although they may produce effects of little or no consequence and in certain rare cases may be advantageous (Committee 17, 1975). It is generally accepted that most mutations are detrimental to the organism.

Assays for detecting mutagens have as their endpoint mutation and many organisms and systems have been developed to ascertain this effect. Basic recommended programs suggest the testing of compounds in three mammalian systems, the dominant lethal, host-mediated and *in vivo* cytogenetics (Mrak Commission, 1969) with any ancillary systems that may be useful. The recommendations indicate a degree of confidence in the precision

and interpretation of results from these methods but there is also uncertainty because of a lack of experience with the problems of methodology and interpretation (Friedman, 1973).

The research reported in this dissertation had as its endpoint an evaluation of the mutagenicity of benzene. In addition, a direct comparison of two methods was made possible by the simultaneous use of more than one procedure. The results from the various tests employed will be discussed in relation with each other as well as to the problem of benzene mutagenicity itself.

Mammals can convert non-mutagenic compounds to highly mutagenic metabolites. In order to combine the metabolism of mammals with the sensitive microbial systems for measuring point mutations, the host-mediated assay and *in vitro* activation systems were developed. In the host-mediated assay (Gabridge and Legator, 1969), a host animal is injected with an indicator microorganism in which mutation frequencies can be measured and the animals are then treated with a potential mutagen *via* a different route. After a time period, the microorganisms are withdrawn from the animal and the induction of mutants determined. A comparison is made between the ability of the test compound to mutate the indicator microorganism in the host and on the organism directly. The results can be used to indicate if the host can detoxify the compound or if mutagenic products can be formed as a result of host metabolism. Using this system, streptozotocin, dimethylmitrosamine (DMNA) and cycasin have been demonstrated to be sources of active mutagens as compared to *in vitro* assays where the parent molecules were inactive (Legator and Malling, 1971).

In my host-mediated study, a histidine auxtroph of Salmonella typhimurium LT-2 was used. The parent organism has a base substitution which alters one codon in the mRNA from the gene coding for the first enzyme of histidine biosynthesis and requires histidine for growth (Ames, 1971). The specific strain employed (TA 1950) has a deletion through the uvrB gene which also includes the nitrate reductase and biotin genes. This particular strain was used in place of the standard TA 1530 strain which has an additional mutation for partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria because in previous work at this laboratory, viability of the TA 1530 strain proved to be a problem in the mouse peritoneum. The TA 1950 was considered to be advantageous over the parent hisG46 due to the additional mutation, uvrB, which produces a defective excision repair system that greatly increases the sensitivity to mutagens.

From the results, benzene was not mutagenic in the host-mediated assay nor in the in vitro plate assay conducted without any activation. The proximity of the indicator organism to the site of metabolism is probably related to mutagenic activity in terms of orders of magnitude. Malling and Frantz (1973) presented data that indicated that the peritoneal cavity was a very ineffective place to measure labile mutagenic metabolites formed in the liver. Highly reactive metabolites may be short-lived and not easily survive transport from the liver to the peritoneum. Their work also demonstrated that an in vitro activated system was a few orders of magnitude more sensitive than the intraperitoneal host-mediated system. In

the in vitro activated assay, the indicator organism can more easily come in contact with an active metabolite. Another problem with the host-mediated assay is the survival of the host which determines the maximum dose that can be used. The toxicity noted after two injections of 0.1 ml of benzene precluded further injections or increased dose. The benzene may be too toxic to allow an adequate dose to be given the host animal so that a mutagenic concentration can be distributed to the peritoneal cavity and the indicator organisms.

Considering the methodological and possible theoretical problems associated with the host-mediated assay, emphasis was shifted to use of an in vitro plate assay in conjunction with an in vitro metabolism system. Ames et al. (1973) published a procedure using an in vitro activation system with his Samonella typhimurium strains. Use of in vitro activation circumvented the problems a) of toxicity of benzene to the host but not to the organism, b) of the viability of the organism in the host and c) of the potential transport of a labile metabolite to the organism. It also enabled the use of more sensitive indicator organisms.

Ames's research group improved the tester strains for mutagenic assays introducing two new strains, TA 100 and TA 98. The TA 100 strain has the same histidine mutation, a base substitution, and uvrB deletion as the previous used TA 1950. In addition, the TA 100 strain contains two refinements. A rfa (deep rough) mutation which removes the lipopolysaccharide coat down to the ketodeoxyoctanoate-lipid core that facilitates penetration of large mutagens to the cell membrane (Ames et al., 1973) and the addition of an R factor plasmid, pKM101, which

increases sensitivity to some compounds (McCann et al., 1975). The TA 98 strain contained a histidine frameshift mutation, hisD3052, as well as the previously discussed rfa, uvrB deletions and R factor. At present, these two strains are the most sensitive available from the Salmonella typhimirium series. TA 100 reverts well with methylmethanesulfonate, N-methyl-N'-nitro-N-nitrosoguanidine, furylfuramide, niridazole, and TA 98 with 4-nitroquinoline-1-oxide, 2-nitrosofluorene, ICR-191. Both strains with activation are reverted by aflatoxin B_1, benzo(a)pyrene, 7,12-dimethylbenzo(a)anthracene and 2-amino-anthracene (McCann et al., 1975).

The plate assay involving activation is not a simple enzyme assay. Various complicating factors influence the yield of revertants such as the amount of S-9 used on the plate, the activation then further inactivation of compounds, and the loss of activity of the system on incubation (Ames et al., 1973a). Over the range of S-9 fraction (0-50 µl/plate) and benzene (0.05-1.0 µl/plate) used, no mutagenic response was noted with either strain or either induction system for the animals.

The lack of reversion seen using the in vitro test system with an induced liver homogenate may be a function of several factors. Foremost would be the assumption that benzene itself or its metabolites are not mutagenic in the system. Alternatives would again be with the system itself. Although benzo(a)-pyrene was activated, in vitro metabolism of benzene may not be as efficient due to lack of the right cofactors or other conditions which are required for its biotransformation. Snyder et al. (1967) reported only 1-3% of the added benzene was metabolized using rat liver homogenates. Jerina and Daily (1974)

suggested that a low rate of metabolism of benzene and instability of the suspected epoxide intermediate in liver homogenates were responsible for their inability to detect the metabolite. Incorporation of 1,1,1-trichloro-2,3-epoxypropane (TCPO) into the system was an attempt to circumvent the problem of further metabolism of the intermediate. Oesch et al. (1971) reported that TCPO was a potent uncompetitive inhibitor of epoxide hydrase. The problem encountered in this attempt was that TCPO was mutagenic in the assay system by itself. The concentration had to be kept below the threshold level of mutagenicity which may be also below the level necessary for adequate inhibition of the epoxide hydrase. At any rate, if the data reported by Williams (1953) using rabbits are any indication, phenol and its conjugates account for the major end products of benzene metabolism. This step is thought to be the result of spontaneous isomerization of the epoxide (Jerina et al., 1968a), therefore blockage of enzymatic hydration by the addition of TCPO would have minor consequences on the concentration of the epoxide intermediate.

Testing of the two benzene analogs, bromobenzene and chlorobenzene, was conducted using the liver activation system because these agents have as their prime toxicity, hepatonecrosis. This action is believed to be the result of an epoxide intermediate produced in the liver (Brodie et al., 1971). It was felt that there was an increased possibility that these compounds would be metabolized in the in vitro liver system. Phenobarbital-induced livers were used as this type of induction was reported to enhance hepatonecrosis using in vivo experiments.

Neither agent was positive.

Use of bone marrow homogenate as the S-9 fraction was an attempt to try another activation system for use with benzene. It seemed possible that bone marrow may provide activation since this organ system is the major site of action of benzene. The first experiment using non-induced rats indicated no activation with either benzene or the benzo(a)pyrene used for control. After making protein determinations, it was found that the concentration was 25-fold less than that of the liver fractions. In attempting to increase the concentration a doubling was obtained but because of a fairly large volume of fluid must be used to completely flush the femur with the technique used no higher concentration could be achieved. Induction of the animals with 3-MC before removal of the marrow was tried because this treatment gave the best activation with liver S-9 fraction. No activation of benzene or the control benzo(a)pyrene was observed; several possibilities could account for this failure. The cofactor system used in the S-9 mix was adapted directly from the liver system and may not be optimal for bone marrow. The concentration of protein from the bone marrow samples, even from the 3-MC-induced animals, was still much below that used for best activation using liver. Maximum activation of benzo(a)pyrene using 3-MC-induced liver was achieved with 1.25 mg of protein per plate with no activation at 0.025 mg per plate and little activation at 0.25 mg/plate. The highest protein concentration per plate used with bone marrow was 0.20 mg and this is at the lower range for activation of the benzo(a)pyrene even for 3-MC-induced liver. As a

beginning, I would suspect the metabolic activation system in the bone marrow plate assay as being inactive.

Finally, some consideration should be given to the detection system employed in the preceeding work. The most recent tester set, TA 98 and TA 100, are the most sensitive available of the Salmonella typhimirium series for detecting point mutations. This system is based on a reverse mutation at a specific site within the histidine gene and the original mutants were produced by a known type of DNA damage, base-pair substitution or frameshift mutation. It is possible that point mutations using benzene may occur in other base sequences not represented in the reverse system of the two strains. Ames (1973) reported that carcinogens and mutagens have specificity as to the DNA sequence in which they produce mutations. Different results may be obtained in experiments with forward mutation systems which detect genetic damage throughout the gene rather than a specific sequence for reversion (de Serres, 1973).

The dominant lethal assay was used to evaluate potential mutagenic damage to germ cell lines. Other methods are available such as cytogenetic evaluation of chemicals on mouse germ cells but the dominant assay is the most widely used and thus has the advantage of much background data. A dominant lethal mutation is a genetic event that kills the individual carrying it in a heterozygous state. The genetic basis of the test is reported to be the induction of structural and numerical chromosomal anomalies which sequentially may induce pre-implantation losses of non-viable zygotes and early embryonic death (Bateman

and Epstein, 1971). This type of damage would be self-limiting but it is felt that agents that produce dominant lethality would also cause point mutations. With rats, use of a 10 week serial mating sequence provides a differential evaluation of all the spermatogenic stages exposed to the chemical. Effects manifested in week 1 and 2 following administration of the chemical represent damage to sperm in the epididymis, weeks 3, 4 and 5 damage to spermatids, weeks 6 and 7 to spermatocytes and weeks 8, 9 and 10 to spermatogonia (Grice, 1974).

The assay conducted using benzene with triethylenemelamine (TEM) as the positive control demonstrated no dominant lethal effects associated with the benzene treatment. Neither compound produced any antifertility effect. The TEM showed a definite dominant lethality at weeks 1-5 representing damage at the postmeiotic stage of spermatogenesis. The lack of effect by TEM on pre-implantation loss as measured by corpora lutea count was not without literature precedent. Green and Springer (1971) reported pre-implantation loss variability which may be due to corpora lutea count problems and Epstein et al. (1972) reported the same. The lack of effect seen with in this test may not completely rule out a germinal effect for this compound.

Dominant lethal effects induced in meiotic and premeiotic stages of male germ cells tend to be selectively eliminated prior to fertilization (Bateman, 1973). It is possible that benzene treatment may induce effects in the early stages and the damaged cells are selectively removed. The dominant

lethality would not be recognized if it killed or sterilized the gametes carrying it. In the assay, the test chemical is assumed to gain access to the spermatogenic cells in the seminiferous tubules, tubuli recti, rete testes, efferent ducts, epididymis and vas defferens (Dixon and Lee, 1973). The blood-testicular barrier, a permeability barrier surrounding the seminiferous tubules of mammalian testis, may exclude from the lumen of these tubules many substances present in testicular blood (Setchell et al., 1969: Fawcett et al., 1970: Dyn and Fawcett, 1970). This suggests the possibility of negative results due to distribution. Based on the ready passage through the blood-brain barrier, benzene would probably not be hindered but a labile metabolite such as an epoxide intermediate could be excluded. Benzo(a)pyrene has been reported to be positive in dominant lethal testing in mice but further testing is indicated because of a lack of consistency between experiments (Epstein et al., 1972). If the genetic effects of this compound are mediated through an epoxide intermediate as suggested (Ames et al., 1973a), then the relative stability and reactivity of the intermediates may account for the different dominant lethal results observed for it and for benzene. To be effective, a compound must achieve an adequate concentration at the site of action, a function of exposure, absorption, distribution, binding, metabolism and excretion. The end determinant of the action of an individual chemical on chromosomes at the cellular level could vary according to the metabolic pathways open to it at that site. The absence of localized benzene metabolism

within the testis could be responsible for the lack of dominant lethality observed. Finally, the dominant lethal assay measures a very narrow spectrum of germinal genetic effects and the many other possible effects would pass undetected in the assay.

Cytogenetic analysis of bone marrow and blood specimens stands as one of the most widely used methods of mutagenicity evaluation. One of the goals of genetic toxicology is to develop tests that detect genetic damage and mutations with great sensitivity and to have these test relevant to man. No test completely fulfills this requirement but the cytogenetic technique is an excellent method for detecting chromosomal abnormalities with sensitivity and the only one directly applicable to man.

It has been stated that any discussion of the genetic implications of induced chromosomal aberrations involves more speculation than fact (Grice, 1974). Changes in chromosomes can be heritable and are significant to the human population (Carter, 1969). The question is whether clastogenic events can serve as an indicator for point mutations. There is a high correlation between the ability of an agent to produce point mutations and chromosome breaks (Nichols, 1973). We would like to be able to make quantitative estimates of the relation between a given dose of an injurious agent, the number and type of chromosome aberrations produced and the ultimate genetic changes or disease state which would result but this is beyond the present day state of the art (Nowell, 1969). Many chromosome aberrations will result in cell death or at least the inability to complete mitosis, but some changes which functionally alter

the cell still leave it capable of proliferation.

The results derived from the cytogenetic portion of the study clearly indicate a clastogenic response with benzene treatment. The work reported in this dissertation is unique in that the same animals were followed serially to ascertain the fate of the lesions. The results indicate a gradual falling off of the number of cells containing chromatid deletions, chromosome deletions and rearrangements but not double minutes (DM). This last named type of aberration, restricted exclusively to the benzene-treated animals, maintained a low but persistent presence even at 70 days post treatment. The blood specimen study did not prove to be as concise as that of the bone marrow. The fragmentation as shown by chromosome pycnosis and suppression of metaphases in the initial portion of the study eliminated the possibility of detecting any significant number of aberrations. In contrast to bone marrow cells, which represent a heterogenous cell population, actively undergoing proliferation, blood specimens consist of circulating lymphocytes which do not divide unless stimulated by phytoheamaglutinin (PHA). In the later portion of the study at 30 and 70 days, the variation in the response of the lymphocytes to PHA further complicated the problem. Lack of stimulation of rat lymphocytes using commercially derived batches of PHA has been reported previously (Farrow et al., 1975). The presence of DM exclusively in metaphases from the benzene-treated animals' blood specimens reinforces the findings in bone marrow. As before, the number seen was extremely small but some were

present at all time periods and the number was relatively consistent regardless of length of time post-exposure.

DM are extremely rare aberrations in rat preparations from the author's experience. Sometimes called interstitial deletions (Evans and O'Riordan, 1975), DM have been associated with neoplastic conditions. Mark (1966) reported the finding of DM during chromosome work with Rous sarcomas in mice. Cytogenetic analyses of human neuroblastoma tissue (Levan et al., 1969), of human medulloblastoma and neuroblastomas tissue (Lubs and Salmon, 1965), and of human meningiomas (Zankl and Zang, 1971) also indicate the presence of DM-like aberrations. In all of these cases, metaphases were located containing from one to several hundred DM. In contrast, with the benzene treatment reported on here, DM were usually singly located with the exception of two cells each of which contained two DM.

Chromosomal aberrations induced either by radiation or chemicals are selected against in proliferating systems but in some cases have been found to persist for long times in these systems (Puck, 1958; Bender and Booch, 1963). In cells containing an aberration several possible fates have been postulated for the aberration at cell division (Carrano and Heddle, 1973). Lacking a centromere, the aberration could be lost to both daughter nuclei or it could split with incorporation into one or both daughter nuclei. The third case would be incorporation of the aberration into one daughter nucleus. Data of Sasaki and Norman (1967) indicates that the second case rarely occurs. This means that fragment transmission appears to be an all-or-

none process with either both pieces going to a single daughter nucleus or the fragment is lost possibly to become a micronuclei. It appears from the data with benzene treatment that aberrations affecting large portions of a chromosome such as the chromatid and chromosome deletions produce cell death but the DM follows the third procedure and are incorporated into one of the daughter nuclei upon cell division. The possibility also exists that cells missing the DM chromatin fragments may be present. Those cells if they survived would be scored normal but the loss in itself may be deleterious.

The micronucleus test was developed for screening chemicals for clastogenic effects in vivo (Schmid, 1975). This method detects the presence of fragments which resemble a nucleus but are much smaller. These satellite nuclei result from particles of chromatin material which do not migrate to the poles during anaphase and are not incorporated into the nuclei of the daughter cells. Use is made of polychromatic erythrocytes of the bone marrow to ease the analysis. Shortly after completion of the last mitosis, erythroblasts expel their nucleus but not the satellite nuclei. For several hours following the last division, the cytoplasm of these young erythrocytes retain their basophilic staining capacity (polychromatic) allowing the differential scoring of those cells in mitosis during a restricted period. By scoring these cells from bone marrow 24 hours after chemical treatment of the animal, a selected cell population which was undergoing mitosis at the time of treatment is obtained. The method is relatively

rapid and the procedure permits the analysis of a large number of cells as opposed to metaphase cytogenetic tests. Relatively little background data are available for this method. Several strong alkylating agents, trenimon, mitomycin C and TEM, have been evaluated and the results were in line with those expected from cytogenetic metaphase work. In contrast, recently published work with this method using TEM in a dose-response study was not as concise (Jellema and Schardein, 1975) probably a result of technique problems, such as artifacts.

The benzene study reported here demonstrated a dose-related increase in micronuclei using 3 levels of benzene treatment. These results give added impact to the concern that benzene in vivo may be mutagenetic. The results did indicate a no effect level at the lowest level of benzene treatment (0.05 ml/kg). One regret regarding the combined dominant lethal-cytogenetic study is that I was not set-up to do micronuclei testing upon completion of the 70 day breeding sequence. Analysis of those erythrocytes that underwent mitosis the last 24 hours of the study could have provided interesting data for comparison with the chromosomal analysis.

The lack of effect in the dominant lethal study as opposed to the very strong response observed in the cytogenetic and micronuclei portions may be explained as outlined in the dominant lethal discussion. All three systems assay chromosomal mutations but the findings with one test substance in one test system are not necessarily to be expected with the same compound in another system (Schleiermacher, 1971). Results

from tests conducted by this worker with cytoxan were interpreted to indicate that damaged spermatogonia seem to be much more sensitive to the agent than bone marrow cells. They appeared to undergo cell death while bone marrow cells survived and reached the following mitosis. This same argument could be applied to the dual benzene study.

The use of chronic benzene treatment preferably by inhalation exposure as opposed to the single injection used in these studies may provide different results in the dominant lethal assay. It would be interesting to obtain data from such a dominant lethal assay using the maximum tolerated level at 5 days/week for 6 weeks to assist in the evaluation of occupational exposures.

The frequency of genetic effects measured in various assay systems can be affected by the usual pharmacologic factors such as distribution, absorption, metabolism and excretion of the mutagen. Additional factors in the detection of mutagenic effects would be the extent and effectiveness of repair processes and immunological response. An apparent threshold of mutation induction can be expected to occur and was detected using the micronucleus technique. But even though a compound may not be mutagenic at the concentration used in a specific test, the total number of deleterious mutations induced with this concentration in the whole population over a prolonged period of time could be substantial (Malling, 1970). It has been suggested that mutagenic effects are more insidious than toxic effects because a single mutated cell that multiplies

can produce a massive effect on an organism. In comparison, the death of a single cell is relatively harmless except when it occurs during embryogenesis (Freese, 1973). Bloom (1972) feels the evidence is convincing that populations with increased levels of induced aberrations are at increased risk in terms of development of malignant diseases. A no effect level in any study is a function of the test system employed and any alteration in the artificial test system can affect this level. To make recommendations based on these no effect levels in the case of mutagens and carcinogens may be extremely hazardous.

SUMMARY

Several conclusions may be drawn from this work. Acute benzene treatment appears to produce a strong clastogenic effect as demonstrated in both the metaphase cytogenetic study and the micronuclei test. The cytogenetic results indicate that one of these effects, the double minutes, are persistent. The possibility of other, more subtle and undetected effects, which persist can not be excluded. A dominant lethal effect and point mutations as measured in the bacterial plate assay with and without activation could not be demonstrated but are still not excluded. Based on the micronuclei test, an apparent no effect level can be ascertained for a mutagenic effect but extreme care should be exercised in its use. Further evaluation using chronic inhalation studies should be considered.

REFERENCES

Ames, B. N. (1971). The Detection of Chemical Mutagens with Bacteria. In: Chemical Mutagens. Principles and Methods for their Detection. (A. Hollaender, ed.) Plenum Press, New York. Vol. 1, pp. 267-282.

Ames, B. N. (1973). Carcinogens are Mutagens: Their Detection and Classification. In: The Evaluation of Chemical Mutagenicity Data in Relation to Population Risk. Environmental Health Perspectives, experimental issue No. 6, DHEW Publ. No. (NIH) 74-218, pp. 115-117.

Ames, B. N., W. E. Durston, E. Yamasaki and F. D. Lee. (1973a). Carcinogens are Mutagens: A Simple Test System Combining Liver Homogenates for Activation and Bacteria for Detection. Proc. Natl. Acad. Sci. USA 70, 2281-2285.

Ames, B. N., F. D. Lee and W. E. Durston. (1973b). An Improved Bacterial Test System for the Detection and Classification of Mutagens and Carcinogens. Proc. Natl. Acad. Sci. USA 70, 782-786.

Ames, B. N., J. McCann and E. Yamasaki. (in press, 1975). Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test. Mutation Research.

Bateman, A. J. (1973). The Dominant Lethal Assay in the Mouse. Agents and Actions 3, 73-76.

Bateman, A. J. and S. S. Epstein. (1971). Dominant Lethal Mutations in Mammals. In: Chemical Mutagens. Principles and Methods for their Detection. (A. Hollaender, ed.) Plenum Press, New York. Vol. 2, pp. 541-568.

Bender, M. A. and P. C. Gooch. (1963). Persistent Chromosome Aberrations in Irradiated Human Subjects. Radiation Research 18, 389-396.

Bloom, A. D. (1972). Induced Chromosomal Aberrations: Biological and Clinical Significance. J. Pediatrics 81, 1-8.

Boyland, E. and L. F. Chasseaud. (1969). The Role of Glutathione and Glutathione S-Transferases in Mercapturic Acid Biosynthesis. Adv. in Enzymol. 32, 173-219.

Boyland, E. and K. Williams. (1965). An Enzyme Catalyzing the Conjugation of Epoxides with Glutathione. Biochemical J. 94, 190-197.

Boyland, E. and G. Wolf. (1950). Metabolism of Polycyclic Compounds. 6. Conversion of Phenanthrene into Dihydroxy Dihydrophenanthrenes. Biochemical J. 47, 64-69.

Brodie, B. B., W. D. Reid, A. K. Cho, G. Sipes, G. Krishna and J. R. Gillette. (1971). Possible Mechanism of Liver Necrosis caused by Aromatic Organic Compounds. Proc. Natl. Acad. Sci. USA 68, 160-164.

Carrano, A. V. and J. A. Heddle. (1973). The Fate of Chromosome Aberrations. J. Theor. Biol. 38, 289-304.

Carter, C. O. (1969). Chromosomes and Chromosome Mutations. The Lancet 1, 1041-1045.

Committee 17. (1975). Environmental Mutagenic Hazards. Committee of the Environmental Mutagen Society. (J. W. Drake, chairman). Science 187, 503-514.

Dean, B. J. (1969). Chemical-Induced Chromosome Damage. Lab. Animals 3, 157-174.

de Serres, F. J. (1973). Development of Mutagen-Sensitive Strains of Neurospora to Detect Specific Locus Mutations. In: The Evaluation of Chemical Mutagenicity Data in Relation to Population Risk. Environmental Health Perspectives, experimental issue No. 6, DHEW Publ. No. (NIH) 74-218, pp. 97-99.

de Serres, F. J. and W. Sheridan. (1973). The Evaluation of Chemical Mutagenicity Data in Relation to Population Risk. (F. J. de Serres and W. Sheridan, eds.). Environmental Health Perspectives, experimental issue No. 6, DHEW Publ. No. (NIH) 74-218.

Dixon, R. L. and I. P. Lee. (1973). Possible Role of the Blood-Testicular Barrier in Dominant-Lethal Testing. In: The Evaluation of Chemical Mutagenicity Data in Relation to Population Risk. Environmental Health Perspectives, experimental issue No. 6, DHEW Publ. No. (NIH) 74-218, pp. 59-63.

Dobrokhotov, V. B. (1972). The Mutagenic Influence of Benzene and Toluene under Experimental Conditions. (Trans.). Gig i San. (USSR) 37, 36-39.

Dyn, M. and D. W. Fawcett. (1970). The Blood-Testis Barrier in the Rat and the Physiological Compartmentation of the Seminiferous Epithelium. Biol. Reprod. 3, 308-326.

Epstein, S. S. (1973). Use of the Dominant-Lethal Test to Detect Genetic Activity of Environmental Chemicals. In: The Evaluation of Chemical Mutagenicity Data in Relation to Population Risk. Environmental Health Perspectives, experimental issue No. 6, DHEW Publ. No. (NIH) 74-218, pp. 23-26.

Epstein, S. S., E. Arnold, J. Andrea, W. Bass and Y. Bishop. (1972). Detection of Chemical Mutagens by the Dominant Lethal Assay in the Mouse. Toxicol. Appl. Pharmacol. 23,

Evans, H. J. and M. L. O'Riordan. (1975). Human Peripheral Blood Lymphocytes for the Analysis of Chromosome Aberrations in Mutagen Tests. Mutation Res. 31, 135-148.

Farrow, M. C., A. M. Hawk, L. T. Wetzel and G. C. Boxill. (1975). A Method for Culturing Peripheral Blood Leukocytes for Chromosome Analysis in the Dog, Guinea Pig, Hamster, Monkey, Mouse and Rat. Mutation Res. 28, 145-146.

Fawcett, D. W., L. V. Leak and P. M. Heidger. (1970). Electron Microscopic Observations on the Structural Components of the Blood-Testis Barrier. J. Reprod. Fertil. Suppl. 10, 105-122.

Federal Register. (1972). 29 CFR Part 1910.93 published in the Federal Register, Vol. 37, October 18, 172, p. 22139.

Forni, A. and L. Moreo. (1967). Cytogenetic Studies in a Case of Benzene Leukemia. Europ. J. Cancer 3, 251-255.

Forni, A. and L. Moreo. (1969). Chromosome Studies in a Case of Benzene-Induced Erytholeukaemia. Europ. J. Cancer 5, 459-463.

Forni, A., E. Pacifico and A. Limonta. (1971a). Chromosome Studies in Workers Exposed to Benzene or Toluene or Both. Arch. Environ. Health 22, 373-378.

Forni, A., A. Cappellini, E. Pacifico and E. Vigliani. (1971b). Chromosome Changes and their Evolution in Subjects with Past Exposure to Benzene. Arch. Environ. Health 23, 385-391.

Freese, E. (1971). Molecular Mechanisms of Mutations. In: Chemical Mutagens. Principles and Methods for their Detection. (A. Hollaender, ed.) Plenum Press, New York. Vol. 1, pp. 1-56.

Freese, E. (1973). Thresholds in Toxic, Teratogenic, Mutagenic and Carcinogenic Effects. In: The Evaluation of Chemical Mutagenicity Data in Relation to Population Risk. Environmental Health Perspectives, experimental issue No. 6, DHEW Publ. No. (NIH) 74-218, pp. 171-178.

Friedman, L. (1973). The Need for Better Methods of Extrapolation. In: The Evaluation of Chemical Mutagenicity Data in Relation to Population Risk. Environmental Health Perspectives, experimental issue No. 6, DHEW Publ. No. (NIH) 74-218, 7-11.

Gabridge, M. G. and M. S. Legator. (1969). A Host-Mediated Microbial Assay for the Detection of Mutagenic Compounds. Proc. Soc. Expl. Biol. Med. 130, 831-834.

Goldstein, A. (1964). Biostatistics: An Introductory Text. The MacMillan Company, New York. pp. 34-128.

Grice, H. C. (1973). Mutagenicity. In: The Testing of Chemicals for Carcinogenicity, Mutagenicity and Teratogenicity. (H. C. Grice, ed.). Health and Welfare, Canada, Ottawa. pp. 69-134.

Green, S. and J. A. Springer. (1973). The Dominant-Lethal Test: Potential Limitations and Statistical Considerations for Safety Evaluation. In: The Evaluation of Chemical Mutagenicity Data in Relation to Population Risk. Environmental Health Perspectives, experimental issue No. 6, DHEW Publ. No. (NIH) 74-218, pp. 37-46.

Hartwich, D., G. Schwanitz and J. Becker. (1969). Chromosome Anomalies in a Case of Benzene Leukaemia. Germ. Med. Mth. 14, 449-450.

Jellema, M. M. and J. L. Schardein. (1975). Comparison of Two Methods for Determining the Cytogenetic Effects Induced by Triethylenemelamine. Toxicol. Appl. Pharmacol. 31, 107-113.

Jerina, D., J. Daly, B. Witkop, P. Zaltzman-Nirenberg and S. Udenfriend. (1968a). Role of the Arene Oxide-Oxepin System in the Metabolism of Aromatic Substrates. 1. In Vitro Conversion of Benzene Oxide to a Premercapturic Acid and a Dihydrodiol. Arch. Biochem. Biophys. 128, 176-183.

Jerina, D., J. Daly, B. Witkop, P. Zaltzman-Nirenberg and S. Udenfriend. (1968b). The Role of the Arene Oxide-Oxepin System in the Metabolism of Aromatic Substrates. III. Formation of 1,2-Naphthalene Oxide from Naphthalene by Liver Microsomes. J. Amer. Chem. Soc. 90, 1056-1061.

Jerina, D, J. Daly, B. Witkop, P. Zaltzman-Nirenberg and S. Udenfriend. (1970). 1,2-Naphthalene Oxide as an Intermediate in the Microsomal Hydroxylation of Naphthalene. Biochemistry 9, 147-155.

Jerina, D. and J. W. Daly. (1974). Arene Oxides: A New Aspect of Drug Metabolism. Science 185, 573-582.

Koller, P. (1972). The Role of Chromosomes in Cancer Biology. In: Recent Results in Cancer Research. (P. Rentchnick, ed.) Springer-Verlag, New York. No. 38, p. 107.

Krishna, G., M. Eichelbaum and W. D. Reid. (1971). Isolation and Characterization of Liver Proteins Containing Covalently Bound C^{14}-Bromobenzene Metabolites. The Pharmacologist, fall, p. 196 (abstr.).

Legator, M. S. (1969). Deficiencies in our Present Protocol for Chemical Evaluation and Possible Remedies. Ann. New York Acad. Sci. 179, 508-513.

Legator, M. S. (1973). Procedure for Conducting the Host-Mediated Assay Utilizing Bacteria ('Salmonella typhimurium'). Agents and Actions 3, 111-117.

Legator, M. S. and H. V. Malling. (1971). The Host-Mediated Assay, a Practical Procedure for Evaluating Potential Mutagenic Agents in Mammals. In: Chemical Mutagens. Principles and Methods for their Detection. (A. Hollaender, ed.) Plenum Press, New York. Vol. 2, pp. 569-589.

Legator, M. S., K. A. Palmer and I. Adler. (1973). A Collaborative Study of In Vivo Cytogenetic Analysis. Toxicol. Appl. Pharmacol. 24, 337-350.

Levan, A., G. Manolov and P. Clifford. (1968). Chromosomes of a Human Neuroblastoma: A New Case with Accessory Minute Chromosomes. J. Natl. Cancer Inst. 41, 1377-1387.

Lowry, H. O., W. J. Rosebrough, A. L. Farr and R. J. Randall. (1951). Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193, 265-275.

Lubs, H. A. and J. H. Salmon. (1965). The Chromosomal Complement of Human Solid Tumors. J. Neurosurgery 22, 160-168.

Malling, H. V. (1970). Chemical Mutagens as a Possible Genetic Hazard in Human Populations. Am. Ind. Hyg. Assoc. J. 31, 657-666.

Malling, H. V. and C. N. Frantz. (1973). In Vitro Verus In Vivo Metabolic Activation of Mutagens. In: The Evaluation of Chemical Mutagenicity Data in Relation to Population Risk. Environmental Health Perspectives, experimental issue No. 6, DHEW Publ. No. (NIH) 74-218, pp. 71-82.

Mark, J. (1967). Double-Minutes - A Chromosomal Aberration in Rous Sarcomas in Mice. Hereditas 57, 1-22.

McCann, J., N. E. Spingarn, J. Kobori and B. N. Ames. (1975). Detection of Carcinogens as Mutagens: Bacterial Tester Strains with R Factor Plasmids. Proc. Natl. Acad. Sci. USA 72, 979-983.

Mitchell, J. R., W. D. Reid, B. Christie, J. Moskowitz, G. Krishna and B. B. Brodie. (1971). Bromobenzene-Induced Hepatic Necrosis: Species Differences and Protection by SKF 525-A. Res. Comm. in Chem. Pathol. Pharmacol. 2, 877-887.

Moorehead, P. S., P. C. Nowell, W. J. Mellman, D. M. Battips and D. A. Hungerford. (1960). Chromosome Preparations of Leukocytes Cultured from Human Peripheral Blood. Exptl. Cell Res. 20, 613-616.

Mrak Commission. (1969). Mutagenicity of Pesticides. In: Report of the Secretary's Commission on Pesticides and their Relationship to Environmental Health. (E. M. Mrak, chairman). DHEW, Washington, D. C. pp. 565-653.

NIOSH. (1974). Criteria for a Recommended Standard... Occupational Exposure to Benzene. DHEW, Office of Research & Standards Development, Natl. Inst. Occupational Safety and Health, HEW Publ. No. (NIOSH) 74-137, Washington, D. C.

Nemenzo, J. N., A. Pasi and C. H. Hine. (1975). A New Technique; Serial Bone Marrow Biopsies for Monitoring Chromosomal Changes. The Society of Toxicology, 14th Ann. Meeting, Spring, pp. 89-90 (abstr.).

Nichols, W. W. (1973). Significance of Various Type Chromosome Aberrations for Man. In: The Evaluation of Chemical Mutagenicity Data in Relation to Population Risk. Environmental Health Perspectives, experimental issue No. 6, DHEW Publ. No. (NIH) 74-218, pp. 179-183.

Nichols, W. W., P. Moorehead and B. Brewen. (1972). Chromosome Methodologies in Mutation Testing. Toxicol. Appl. Pharmacol. 22, 269-275.

Nowell, P. C. (1969). Biological Significance of Induced Human Chromosome Aberrations. Fed. Proc. 28, 1797-1803.

Oesch, F., D. M. Jerina, J. W. Daly and J. M. Rice. (1973). Induction, Activation and Inhibition of Epoxide Hydrase: An Anomalous Prevention of Chlorobenzene-Induced Hepatotoxicity by an Inhibitor of Epoxide Hydrase. Chem.-Biol. Interactions 6, 189-202.

Oesch, F., N. Kaubisch, D. M. Jerina and J. W. Daly. (1971). Hepatic Epoxide Hydrase. Structure-Activity Relationships for Substrates and Inhibitors. Biochemistry 10, 4858-4866.

Parke, D. and R. Williams. (1953). Studies in Detoxification. 49. The Metabolism of Benzene Containing (C^{14}) Benzene. Biochemical J. 54, 231-238.

Philip, P. and M. Jensen. (1970). Benzene Induced Chromosome Abnormalities in Rat Bone Marrow Cells. Acta Path. Microl. Scan. A. 78, 489-490.

Puck, T. T. (1958). Action of Radiation on Mammalian Cells. III. Relationship between Reproductive Death and Induction of Chromosome Anomalies by X-Irradiation of Euploid Human Cells. Proc. Natl. Acad. Sci. USA 44, 772-780.

Reid, W. D. and G. Krishna. (1973). Centrolobular Hepatic Necrosis Related to Covalent Binding of Metabolites of Halogenated Aromatic Hydrocarbons. Exp. Mol. Pathol. 18, 80-99.

Sasaki, M. S. and A. Norman. (1967). Selection against Chromosome Aberrations in Human Lymphocytes. Nature 214, 502-503.

Schleiermacher, E. (1971). Chromosome Aberrations in Mitoses and Meioses In Vivo. Arch. Toxikol. 28, 105-114.

Schmid, W. (1975). The Micronucleus Test. Mutation Res. 31, 9-15.

Setchell, B. P., J. K. Voglmayr and G. M. H. Waites. (1969). A Blood Testies Barrier Restricting Passage from Blood into Rete Testis Fluid but not into Lymph. J. Physiol. 200, 73-85.

Sims, P., A. Hewer and P. Grover. (1971). Formation of Epoxides as Microsomal Metabolites of Polycyclic Hydrocarbons. Biochemical J. 125, 28p.

Snyder, R., F. Uzuki, I. Gonasun, E. Bromfeld and A. Wells. (1967). The Metabolism of Benzene In Vitro. Toxicol. Appl. Pharmacol. 11, 346-360.

Tough, I. and W. Court Brown. (1965). Chromosome Aberrations and Exposure to Ambient Benzene. The Lancet 1, 684.

Tough, I., P. Smith, W. Court Brown and D. Harnden. (1970). Chromosome Studies on Workers Exposed to Atmospheric Benzene. The Possible Influence of Age. Europ. J. Cancer 6, 49-55.

Udenfriend, S. (1971). Arene Oxide Intermediates in Enzymatic Hydroxylation and their Significance with Respect to Drug Toxicity. Ann. New York Acad. Sci. 179, 529-547.

Vigliani, E. and G. Saita. (1964). Benzene and Leukemia. The New England J. Med. 271, 872-876.

Vogel, J. J. and D. M. Bonner. (1956). Acetylornithinase of Escherichia coli: Partial Purification and some Properties. J. Biol. Chem. 218, 97-106.

Vogel, E. and H. Gunther. (1967). Benzene Oxide-Oxepin Valence Tautomerism. Angew. Chem. Intern. Ed. Engl. 6, 385-401.

Wang, I. Y., R. E. Rasmussen and T. T. Crocker. (1972). Isolation and Characterization of an Active DNA-Binding Metabolite of Benzo(a)pyrene from Hamster Liver Microsomal Incubation Systems. Biochem. Biophys. Res. Commun. 49, 1142-1149.

Wiebel, R., J. Leutz, L. Diamond and H. Gelboin. (1971). Aryl Hydrocarbon (Benzo(a)pyrene) Hydroxylase in Microsomes from Rat Tissue: Differential Inhibition and Stimulation by Benzoflavones and Organic Solvents. Arch. Biochem. Biophys. 144, 78-86.

Williams, R. (1959). The Metabolism of Aromatic Hydrocarbons - Benzene. In: Detoxification Mechanisms. 2nd. edition, Chapman and Hall, London. pp. 188-194.

Winer, B. J. (1962). Statistical Principles in Experimental Design. McGraw-Hill, New York. p. 221.

Zankl, H. and K. D. Zang. (1971). Cytological and Cytogenetical Studies on Brain Tumors. Humangenetik 12, 42-49.