January 1980

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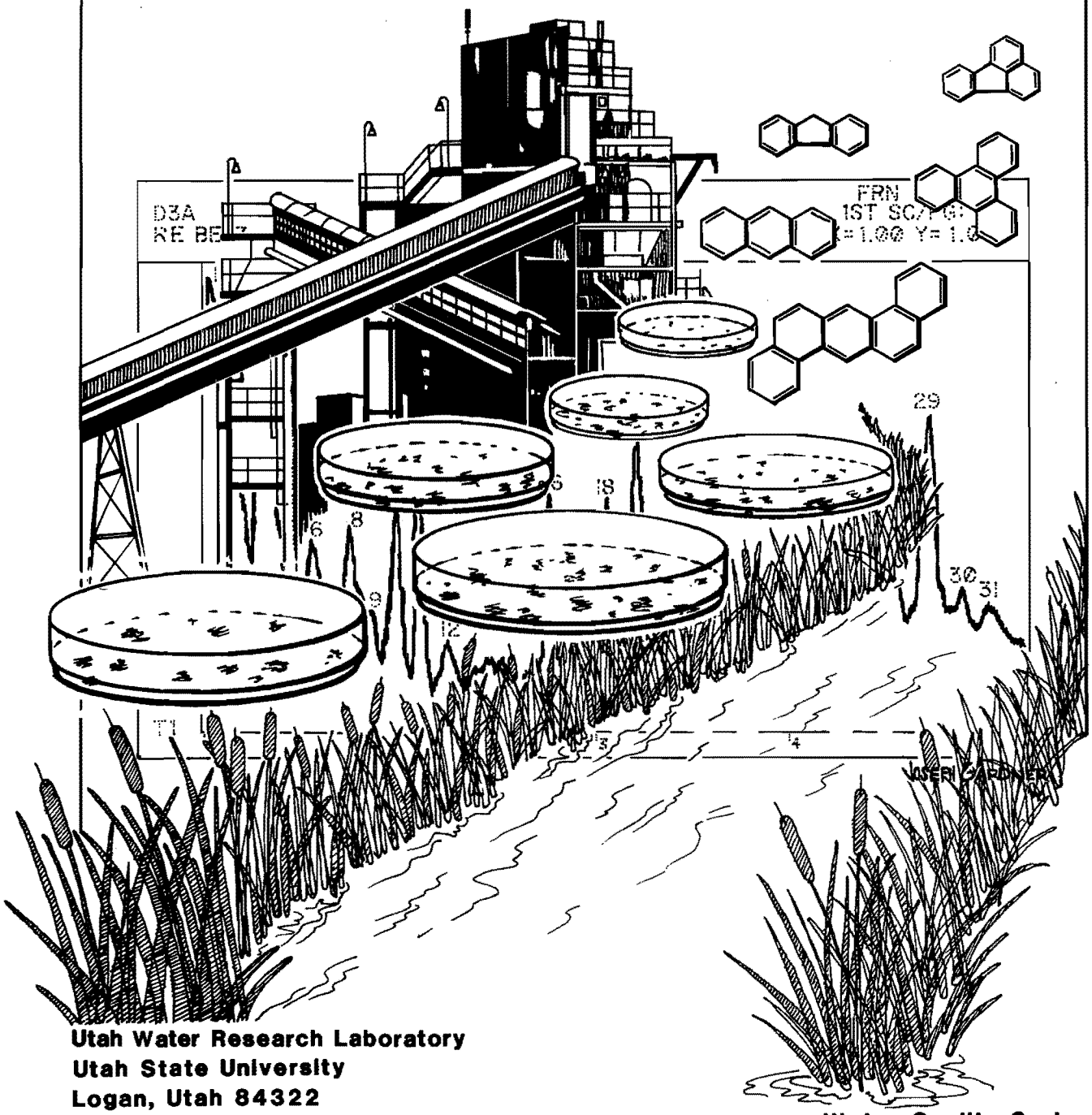

Utah Water Research Laboratory
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May 1980

Water Quality Series
UWRL/Q-80/01

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ABSTRACT

The Ames/*Salmonella* mutagenicity assay was used to screen processed oil shale extracts for potentially carcinogenic chemicals. Positive mutagenic activity was detected in organic solvent extracts of all four spent shales tested. This result combined with the fact that most carcinogens (90 percent) have been detected as mutagens in the Ames test, indicates that these extracts contain carcinogens. Aqueous extracts of spent shale exhibited marginal or no mutagenic response, presumably because the techniques for extracting and concentrating trace quantities of these nearly water-insoluble compounds are inadequate.

Several problems which might limit application of the Ames assay for environmental screening were also explored. The results of assays of one-to-one mixtures of two mutagens which exhibited different dose response curves when assayed separately indicated the response to the mixture was non-additive. Furthermore, the response to the mixture was determined to be statistically indistinguishable (chi square analysis) from the dose response curve of one of the mutagens in the majority of cases. This masking effect was found to persist for one strong mutagen (benzo(a)pyrene) even when it composed only 10 percent of the mixture. The effect of various non-toxic solvents on the mutagenic response of certain mutagens was also determined. The present study showed that the choice of solvent (ethanol, methanol, dimethylsulfoxide, p-dioxane) can significantly alter the magnitude of the mutagenic response. Certain solvents may also confer a toxicity to compounds which are not otherwise toxic when assayed at the same concentration in another solvent.

ACKNOWLEDGMENTS

We would like to acknowledge the Office of Water Research and Technology (Project No. B-154-UTAH, Contract No. 14-34-0001-8123), United States Department of the Interior, Washington, D.C., which provided funds for research and publication (WG 215), as authorized by the Water Research and Development Act of 1978, and the State of Utah (WR215) for additional funding. In addition, we would like to thank John Manwaring for technical assistance, Annette Brunson, Barbara South, and Betty Hansen for typing the manuscript, and Joseph Gardner for preparing the figures. We would also like to express our appreciation for Dr. Frederick J. Post, Dr. Mary L. Cleave, and Dr. L. Douglas James for reviewing the preliminary draft and for offering many valuable suggestions for its improvement.

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INTRODUCTION

Environmental Carcinogens

The United States is currently assessing the need to regulate technological and industrial development in order to protect the health of its citizens. Cancer, in particular, is of major concern. Through institutions such as the National Cancer Institute (NCI) and the National Institute of Health (NIH), the government is directing a national effort to eliminate this disease. Research funds are being used to identify the causes of cancer, so that it may be prevented and to elaborate the mechanisms of tumor formation and growth, so that the disease can be cured.

Environmental carcinogenesis, which is reported to account for up to 90 percent of the cancer mortality in the United States (Higginson 1972; Wynder and Mabuchi 1972) is receiving a large portion of the research attention because the opportunities for its prevention are great. By limiting man's exposure to environmental factors contributing to this disease (natural and man-made chemicals, ionizing and nonionizing radiation, and certain agents related to an individual's life style, e.g. smoking), a significant portion of this type of cancer could be eliminated.

One of the more important concerns in the area of environmental carcinogenesis has been to determine the extent to which drinking water contributes to cancer. The presence of organic chemical carcinogens in industrial effluents discharged to rivers which are subsequently used for public water supplies has been known for sometime (Middleton and Rosen 1956; Hueper and Conway 1964), but control efforts have been hampered by technical difficulties in detecting the very small amounts of these chemicals which can be hazardous. Only recently have instrumentation and analytical techniques become sophisticated enough to detect minute quantities of contaminants in drinking water. The use of gas chromatography and mass spectroscopy (GC/MS) and high pressure liquid chromatography (HPLC) have allowed the identification and quantification of microgram per liter levels of carcinogenic contaminants in river water (Harrison et al. 1975; Hites and Biemann 1972; Svec et al. 1973; Sheldon and Hites 1978) and in treated municipal water supplies (Hueper and Payne 1963; Andelman and Suess 1970; EPA 1972; Kleoper and Fairless 1972; Scheiman et al. 1974; Junk and Stanley 1975; Kraybill 1975; Glatz et al. 1978).

Because these carcinogenic contaminants are present in such low concentrations, their effect is not immediate, and people are unknowingly exposed to them over a long period of time. The effect of a low-dose, long-term exposure to these chemicals is only beginning to be known. Epidemiologic studies are currently used to determine high cancer risk areas of the United States. The potential environmental sources of carcinogens in these areas, including the drinking water source, are surveyed to determine the extent to which each source may contribute to cancer mortality. Surveys showing a high incidence of cancer in populations located along the Ohio, Missouri, and Mississippi Rivers point to drinking water as a source of carcinogens and a potential cause of cancer. One example is the statistically significant association (using multivariate regression techniques) between cancer mortality in Louisiana and the use of the Mississippi River as a source of drinking water (Harris 1974; Page et al. 1976). Similar studies conducted along the Ohio and Missouri Rivers have supported this correlation (Cook and Watson 1966; Buncher 1975).

Waterborne Carcinogens

Organic chemical carcinogens reach the aquatic environment from several sources and in a variety of ways. Surface waters may receive these pollutants in effluents from manufacturing and processing plants and sewage treatment plants (Wedgwood and Cooper 1955), in runoff and erosion from forests and agricultural lands (Kraybill 1977), and through fallout and rainout of air pollution particulates (Andelman and Suess 1970). Carcinogens of natural or industrial origin adsorbed onto vegetation or soil may leach into the groundwater as well (Hueper 1960).

The fate of the polycyclic aromatic hydrocarbons (PAH) in aquatic systems has received a great deal of attention due to the fact that many are known to be carcinogenic to animals and probably to man (Badger 1962; Hueper and Conway 1964). Although several researchers have suggested the endogenic formation of PAH in plants and microorganisms (Borneff 1963; Knoor and Schenk 1968; Tornabene 1977), the PAH resulting from human activities are quantitatively, by far the most significant and the sources are relatively easy to trace.

The major source of PAH pollution in freshwater is petroleum and its combustion by-products (Blummage 1975; MacKenzie and Hunter 1977) which enter the surface water systems through dustfall and stormwater runoff (Hites and Bieman 1972; Wakeham 1977). The presence of PAH in freshwater in quantities above background levels therefore suggests organic pollution from fuel development and use. As a preventive measure, the carcinogenic hazard from synthetic fuel development, including the oil shale industry proposed for the western United States should be assessed by determining its potential as a source of PAH and other carcinogens.

There are at least two ways to accomplish this objective. One way is to chemically analyze spent shale leachate and attempt to identify compounds which could potentially be carcinogenic. The primary indicator of carcinogenic PAH in the environment has historically been benzo(a)pyrene (BaP). The concentration of BaP in a substance or complex mixture has also been used to predict its carcinogenic potential. The reasons for this practice are clear: BaP was early known to be a potent animal carcinogen, it was easily identified by its fluorescence spectrum, and it was detected in the first substances identified as human carcinogens (soot, paraffin, oil). Therefore, inventories of PAH in the environment emphasize the presence of BaP (see Andelmann and Suess 1970; Suess 1967, 1972).

The more recent advent of sophisticated techniques and instrumentation (GC/MS, HPLC) has allowed researchers to determine directly the composition of PAH in a substance and lead to the findings that the concentration of BaP is not always related to that of other carcinogenic PAH, nor can it account for the carcinogenic potency of that material. Despite this problem, the chemical approach offers information regarding the variety of compounds present and has been pursued with relatively good success by colleagues at UWRL (Maase and Adams 1980).

An alternate method to assess the carcinogenic potential of spent shale extracts is by the use of bioassays. A sensitive organism can be found to give an integrated response of the hazard from spent shale. This is the approach taken by the present study, the objectives of which were to: 1) obtain an appropriate bioassay to assess the carcinogenic potential of spent shale leachates, 2) perform the bioassays, and 3) speculate as to the carcinogenic hazard of spent shale in light of the validity of the chosen bioassay.

Water Pollution Potential of Spent Oil Shale

Much attention has been directed toward the problem of minimizing the environmental impact of processed (spent) shale disposal. Trace quantities of heavy metals, salts, and residual organics adsorbed onto the shale particles become a considerable problem when put into the perspective of the quantity of processed shale that will be produced (over 1 ton of spent shale per barrel of shale oil, Schmidt-Collerus et al. 1976). This, in addition to the fact that the shale expands after processing and is generally disposed of above ground where it will be subjected to physical and chemical weathering thus allowing the contaminants to leach out, explains the necessity for determining the potential impact on the biological system. The present study was initiated to assess the carcinogenic potential of spent shale leachate.

The evaluation of the carcinogenic potential of materials implicated in epidemiological studies customarily involves two steps: 1) chemical analysis to determine the presence of carcinogenic materials (usually only BaP as an indicator of PAH, though the presence of other carcinogens is determined with less frequency), and 2) bioassays, involving extended contact of test animals (mice, rats, and hamsters) with suspected carcinogenic materials (Atwood and Coomes 1974). Chemical analyses are usually performed prior to bioassays as a screening procedure because bioassays are expensive and time consuming; however, the bioassays serve as the ultimate test for animal carcinogenesis.

Since the BaP content of spent shale is low (approximately three orders of magnitude lower than that of raw shale oil (Schmidt-Collerus 1974)), it was judged (Atwood and Coomes 1974) to be an insignificant source of environmental carcinogens and therefore to pose no threat to health. Other reasons for this judgment included: the lack of certain known accelerators of carcinogenic potency (hematite and asbestos) in spent shale; the similarity in composition of organic material in processed shale and that in raw shale which is thought to be non-hazardous; and the physical and chemical similarity of processed shale to carbon black, a material found to be a non-carcinogen by animal testing but shown to contain carcinogens when chemically analyzed (Atwood and Coomes 1974).

Despite these speculations about the carcinogenic nature of shale, a series of animal bioassays were performed (Schmidt-Collerus 1974) to test the tumorigenicity of spent shale. The bioassay procedure in use at this time involved the repeated application of the suspected compound, dissolved in an appropriate solvent, to the shaved skin of the laboratory rodent. This procedure had to be modified to simulate the exposure that humans might have with the solid shale material (breathing and ingesting aerosols). The spent shale was mixed in the laboratory animals' bedding material so that intimate contact could be maintained with the skin and respiratory and digestive tracts of the experimental animal (hairless rats). The results indicated that life-long contact with spent shale did not significantly increase the frequency of tumor formation (Schmidt-Collerus 1974).

The conclusions of this bioassay were that spent shale is non-hazardous. The results of this experiment also contributed to the accumulating evidence against the validity of using BaP concentration as an indicator of carcinogenicity. For many substances neither the composition of carcinogenic PAH, nor the carcinogenic potency, can be predicted by knowing the BaP content. For example, in the aquatic environment BaP accounts for between 1 and 20 percent of the total carcinogenic PAH (Andelman and Suess 1970). The carcinogenic activity of certain petroleum products can be greater or less than that which could be accounted for by the BaP content. The carcinogenic potential of various crude shale oils was found to be only partly attributable to the BaP content (Hueper and Cahnman 1958). Wallcave and coworkers (1971) came to a similar conclusion regarding the skin tumorigenesis of asphalts and coal-tar pitches. The presence of non-carcinogenic PAH promoters enhanced the carcinogenicity of coal-tar pitch. Finally, the discovery that BaP is present in substances considered to be non-hazardous, such as plant material (oak leaves, coconut and peanut oils) and soil (Coomes 1976), presumably as a result of biosynthesis, has made the prediction of carcinogenic potential based on BaP content even more tenuous.

How does one explain the presence of carcinogenic compounds yet the lack of carcinogenic hazard in substances such as spent shale? It has been suggested that either tumor promoter compounds are lacking in that substance or that the carcinogens are physically unavailable for metabolism (a prerequisite for tumor initiation) (Coomes 1976). The evidence tends to refute the first hypothesis. The carcinogens, BaP and dimethlybenz(a)anthracene (DMBA), present in benzene extracts of shale, have been determined to be "complete carcinogens"; once metabolized, these compounds can affect tumor initiation and promotion (Berenblum 1941; Mottram 1944). In addition, certain of the PAH contained in the benzene extract of spent shale (e.g. fluoranthene, pyrene, and benzo-(e)pyrene) while not known to initiate tumors, have been found to promote tumor growth (Hoffmann and Wynder 1962; Van Duuren et al. 1973; Hoffmann et al. 1976, 1978).

The majority of the evidence seems to support the hypothesis of physical unavailability. Benzo(a)pyrene, and probably other carcinogens, are bound up in a structure and/or chemical form which renders them inactive. The strong adsorbent property of finely divided carbon black and spent shale has previously been used to explain their lack of carcinogenic potential when assayed in solid form despite the fact that benzene extracts of these substances contain PAH and exhibit carcinogenicity in experimental animals (Schmidt-Collerus et al. 1976; Nau et al. 1962). The extraction of carcinogens in these substances requires intense physical and chemical treatment (refluxing in an organic solvent) and may indicate the presence of strong physical or chemical bonds that complex the PAH to the spent shale. A process which weakens these interactions (soxhlet extraction in the laboratory, or physical and chemical weathering in the field) could release these compounds into the environment where they would be available for biological uptake and accumulation, metabolism. or elimination.

The research conducted by Schmidt-Collerus and colleagues (1976) indicates that PAH compounds can leach from processed oil shale and can migrate with the saline water. Depending upon shale age and retorting process, between 20 percent and 40 percent of the PAH in benzene extracts of spent shale can enter the percolating water. The PAH content of this percolate could be as much as 3 to 4 orders of magnitude higher than that of the pristine ground or surface water in the area. The water pollution potential from commercial oil shale development becomes very real in view of the resulting large quantities of spent shale. The probability that compounds having blastomogenic or mutagenic properties will enter into the environment from this source warrants an intensive monitoring program and an in-depth study of the consequences of these compounds in the environment and on biological organisms in the environment.

Fate and Effect of PAH in the Aquatic Environment

The fate of PAH in the environmental waters is affected by its physical and chemical characteristics and the physical, chemical, and biological factors of the environment. In pure water, 4 to 5 ring PAH are nearly insoluble (1 to 10 ng/l (Borneff and Knerr 1960)), however, there is sufficient evidence to suggest that the solubility of PAH can be increased by a wide variety of organic compounds (lactic acid, acetone, ethanol) that may be found in certain waters (Ekwall and Sjöblöm 1952; Suess 1967). The majority of the PAH which exist in excess of its solubility is sorbed to surfaces. The transport and settling characteristics of the particles play an important role in the dispersion and persistence of the PAH carried.

There are two primary modes by which polycyclic aromatic hydrocarbons are degraded. These are physical oxidation and biological degradation. The most important process, quantitatively, is photo-oxidation. The photodecomposition of BaP both in solution and while adsorbed onto calcium carbonate particles in a suspension has been investigated (Suess 1967). While pH and ionic strength of the water had no effect on BaP degradation, oxygen concentration, temperature, illumination, and exposure time increased the decomposition rate. This study led to the conclusion that BaP (and presumably other PAH) degradation will fluctuate on a daily and seasonal basis and will occur at

a faster rate in the upper layers of the water and decrease with depth. Also, it is expected that PAH in river, lake, and sea sediments will degrade physically very slowly, if at all, because of the lack of penetration of oxygen and radiation (Suess 1967).

The decomposition of the PAH fraction that escapes physical oxidation is dependent upon the processes of biodegradation. A diversity of organisms (bacteria, fungi, and algae) are capable of PAH degradation (Walker and Colwell 1974; Walker et al. 1975a, 1975b) in laboratory and field studies, both in terrestrial (Lijinsky and Quastel 1956; Jobson et al. 1972, 1974) and marine (Barnsley 1975; Atlas and Bartha 1972) environments.

There have been few studies to determine the response of freshwater organisms to PAH in their environment. Metcalf (1975) used a 3-day model aquatic system to evaluate the ecological effects of BaP. Using the radiolabeled compound, it was demonstrated that BaP was accumulated and stored in the tissues of organisms from each trophic level. Bioaccumulation factors (the ratio of the BaP concentration in tissue to the BaP concentration in water) were for fish, 930; algae, 5258; mosquito, 11,536; snail, 82,231; and Daphnia, 134,248. Some degradation of this compound to unknown polar compounds was observed.

Marine organisms have also been shown to accumulate BaP and other PAH (Lee et al. 1972a, 1972b, 1976; Neff et al. 1976). Using radiolabeled compounds, the pathway of uptake, metabolism, and storage of PAH (^{14}C-Napthalene and ^{3}H-BaP) in fish has been demonstrated (Lee et al. 1972a). The pollutants are taken up through the gills within minutes of exposure, they are metabolized in the liver, then the hydrocarbons are transferred to the bile and their metabolites are excreted in the urine. The storage site of labeled hydrocarbons is the gall bladder. The major metabolic product of BaP was 7,8-dihydro-7,8-dihydrobenzopyrene and of napthalene was 1,2-dihydro-1,2-dihydroxynapthalene after 24 hours of exposure. Some organisms may not metabolize these compounds but rather are able to detoxify and excrete them when exposed for a relatively short period of time (Corner et al. 1973). The rate of release of the accumulated compound when the organism is placed in compound-free water also varies for different organisms; it may take as long as 58 days for clams exposed to BaP (Neff et al. 1976).

Effect of Water and Wastewater Treatment on PAH

The majority of the research to determine the effect of some water treatment processes on the removal of PAH has been performed by European investigators (see Andelman and Suess (1970) for a complete review of the subject). Simple clarification was found inefficient in PAH removal, presumably because the colloidal materials which adsorb the PAH are too small to settle (Borneff and Fischer 1963). However, when flocculation precedes sedimentation, the removal efficiency of PAH from river water increases (Lawerenz 1967). The work of Borneff and Kunte (1964; 1965) indicated that the combination of rapid sand-filtration and chlorination or ozonation were not particularly effective in reducing the concentration of the three compound groups studied: BaP, carcinogenic PAH (BaP, 3,4-benzfluoranthene, benz(a)anthracene, 10,11-benzfluoranthene and indeno(1,2,3-cd)pyrene) or total PAH (the five compounds above plus 11,12-benzfluoranthene, 1,12-benzperylene, fluoranthene, and pyrene) in lake water (percent reductions for these three groups were: 0 percent, 42 percent, and 19 percent, respectively). The addition of activated carbon to the filter, however, gave greater reduction in concentration of these three compound groups (99 percent, 69 percent, and 65 percent reductions in BaP, carcinogenic PAH, and total PAH, respectively).

Numerous studies have reported the effectiveness of oxidizing agents such as chlorine and ozone, on the removal of total PAH from water. Chlorination (using hypochlorite) does not significantly increase the removal of PAH using the typical disinfection time (one half hour) and dosage (0.3 - 0.5 mg Cl_2/l) (Gräf and Nothhafft 1963). However, disinfection with gaseous chlorine in dose and contact periods commonly practiced in water treatment was found to yield a high removal of BaP (Reichert 1968a, 1968b, 1968c). Using ultraviolet and infrared fluorescence spectra and mass spectroscopy, Reichert (1968a, 1968b, 1968c) has identified eight isolated derivatives of the reaction of BaP with chlorine dioxide. The majority of these (90 percent) are non-carcinogens (3,4-benzopyrene-1,5-dione; 3,4-benzopyrene-5,8-dione, and 3,4-benzopyrene-5,10-dione). The residual derivatives have a lower carcinogenic effect than BaP (5-chloro-3,4-benzopyrene, 5,8,10-trichloro-3,4-benzopyrenes). Two isomeric dichloro-3,4-benzopyrenes have also been identified but their carcinogencity is unknown. Disinfection by chlorine dioxide may decrease the carcinogenic hazard of polluted wastewaters, however, for the trace quantities (ng/l) of PAH normally present in groundwaters, chlorine dioxide is not an effective treatment because PAH cannot be removed in practically useful periods (Reichert 1968a). Experimental studies with ozone have shown it can be effective in removing up to 99 percent of BaP in aqueous solution after 30 minutes contact time (Borneff 1969); BaP can be resistant to degradation by ozone if it is adsorbed onto soil particles, which is generally the case.

Filtration through activated carbon is the most effective conventional process for

removal of PAH, though no known method has been found to reduce the concentration of carcinogenic PAH below 10 ng/l, a typical value for uncontaminated groundwater (Reichert 1968a; Borneff 1969).

Human Exposure to PAH and Health Hazard Assessment

As a result of biosynthesis and anthropogenic sources, BaP and other PAH are widely distributed on this planet. Humans are exposed directly to PAH compounds in food, water, and air. It is postulated that concentrations of BaP exist on the order of 10 to 20 µg/kg of dry organic substance (Gräf and Diehl 1966). The concentration of carcinogenic PAH in freshwater ranges from 1 ng/l in unpolluted groundwater to 25 ng/l for treated surface water to greater than 100 ng/l for very contaminated surface water (Borneff and Kunte 1964). Numerous reports have identified carcinogenic PAH in urban air and in cigarette smoke (Hoffmann and Wynder 1962) adsorbed to particulates which can be inhaled by man. The organisms of the lower trophic levels, zoo- and phytoplankton and worms have all been shown to contain carcinogenic PAH. These organisms serve as the food source and also a source of PAH for edible fish and shellfish from which PAH can reach man. The existence of carcinogenic PAH in cooked meats has also been demonstrated (Bailey and Dungal 1958; Dungal 1961; Kraybill 1969).

Obviously, humans are exposed to carcinogenic PAH from many sources. The effect on human health of this exposure is very difficult to determine because the presence of so many uncontrollable factors complicates analysis. The general consensus of researchers working with experimental animals is that repeated exposure to carcinogenic chemicals is more effective than an equivalent single dose, and that there is a potential danger of continuous exposure to carcinogenic PAH introduced into the gastrointestinal tract by water and food, even at low concentrations (Payne and Hueper 1960; Poel 1963). Many investigators believe that there is no safe level of PAH to which humans can be exposed without adverse effects (Ilnitsky and Varshavskaya 1964), and therefore some suggest that its threshold be set practically at zero (Gerarde 1960). This recommendation is impractical because of the natural background concentrations of PAH in the environment from biosynthesis. It is agreed that the presence of carcinogens in the air, food, and water are undesirable, however, a low level of exposure appears to be inevitable. Therefore, consideration should be given to avoid increasing the level of carcinogenic PAH to which humans are exposed, where possible.

Monitoring Carcinogens in the Environment

The early detection of carcinogens in the environment is necessary in order to be timely in organizing public and private support for their removal. The Early Organic Detection System (EODS) under the authority of the Ohio River Valley Water Sanitation Commission is an example of such a monitoring program (Hadeed 1978). In response to studies which linked cancer mortality to chemicals in the Ohio River drinking water, this system was established to develop stream quality criteria for certain compounds that may affect public health or the environment and to instigate regulatory programs to control such compounds in point source discharges. The degradation of water quality in several rivers used as drinking water sources in the United States warrants this type of program. In order to avoid this happening in relatively unpolluted river systems, the magnitude of impact of a potential pollution source must be estimated prior to its existence.

Mutagen/Carcinogen Screening Systems

The water pollution potential of spent oil shale residues is a major concern regarding the environmental impact of oil shale development (Ward 1971; Dassler 1976; Schmidt-Collerus et al. 1976; Slawson 1979; Cleave 1979). Salts, trace elements and organics including carcinogenic PAH, that may leach out of spent shale as a result of weathering and into surface and groundwater may pose a serious problem to public health and the environment. Despite the relatively low concentraton of the benzene-soluble organics in spent shale (200-2000 ppm (Schmidt-Collerus 1974)), the large quantities of spent shale that will be produced during a day of full production (estimated at one facility, the White River Shale Project, to be 86,000 metric ton (Slawson 1979)), will contain 17-170 metric ton of benzene-soluble materials per day. Schmidt-Collerus (1974) found that 20-40 percent of the total benzene-soluble organic matter in shale can be leached by water and that most of the carcinogens in the shale were dissolved and concentrated in the salt residue. Therefore, it would be appropriate to perform additional bioassays to assess the carcinogenic hazard of spent shale.

The long-term, expensive animal bioassays that were at one time the rule, are presently being preceded with a series of short-term, in vitro, bioassays several of which are listed in Table 1. The advantages of using these new bioassays for initial screening are their relatively low cost, simple procedure, reproducibility and accuracy in detecting environmental carcinogens (Table 2).

Recent scientific advancements in chemical carcinogenesis and in molecular biology have suggested that carcinogenic chemicals activated by either chemical or biochemical means are frequently mutagenic (Miller and Miller 1971; Vogel and Röhrborn 1970; Strong 1976; Mueller et al. 1978).

These two fields had previously remained distinctly separate because the indicator organisms used to detect mutagens were not capable of transforming a procarcinogen to its active form. Provided suitable activation can be achieved in an in vitro or in a host-mediated assay, mutagenesis appears to be a useful indicator of carcinogenicity (Legator 1972; Garner et al. 1972; Ames et al. 1973; McCann et al. 1975). The results of comparative studies on the accuracy and sensitivity of these short-term tests are presently being compiled with the ultimate goal of developing a standard screening procedure for existing chemicals of unknown hazard and for the multitude of chemicals continually being created.

The preliminary results of these comparative studies have revealed that a few short term submamallian mutagenicity tests are sufficiently accurate to use prior to mammalian testing. Bridges (1976) has outlined a three tier system for chemical carcinogen screening (Figure 1). Most chemicals and environmental samples would be tested by one or more of the short term, submammalian assays in the first tier. These assays are used to assess a wide variety of genetic damage, including DNA mutation and chromosomal aberration. Damage to DNA leading to heritable changes may be important to man not only because of the correlation with carcinogenicity, but because it may also cause hereditary disease (Legator 1972; Drake et al. 1975). Both the outcome and the priority of the substance would determine the next phase of testing. A negative outcome would allow that compound (sample) to pass if it is of low priority (i.e. few humans will be exposed to it and precautions can be made to protect them). A negative outcome would be further tested in tier 2 if the compound is of high priority (i.e. a large number of humans will be exposed to this compound and may even consume it) to ensure against the occurrence of a false prediction. Those samples which gave a positive response would either be rejected, if a non-mutagenic substitute were available or further tested in tier 3, if not. The assays of tier 2 would include both long- and short-term tests involving in vitro and in vivo systems. Again the sample would be subjected to a battery of tests. A repeated negative response in tier 2 would allow the chemical to pass. A positive response would result in rejection if a non-mutagenic substitute is available or further testing in tier 3 if it is a potentially valuable substance. In the third tier further testing using whole animal carcinogenesis assays could be performed, if necessary, to corroborate the mutagenic potential of the substance, however the primary objective of this stage is to evaluate, as quantitatively as possible, the hazards to humans from substances shown to be potentially carcinogenic in the first two tiers. Only compounds whose use or presence seems inevitable would be subject to this evaluation. A risk-benefit

Table 1. Types of genetic damage detected by currently employed mutagen screening systems.[a]

Screening System		Type of Damage Detected						
		Chromosome Aberrations				Gene Mutations		Induced recombination
Category	Organism	Dominant lethality	Translocations	Deletions and duplications	Nondisjunction	Forward or reverse or both	Multiple specific locus	
Bacterial	Salmonella typhimurium					+		
	Escherichia coli					+		
Fungal	Neurospora crassa			+	+	+	+	
	Aspergillus nidulans				+	+	+	+
	Yeasts	+			+	+	+	+
Plant	Vicia faba		+	+	+			
	Tradescantia paludosa		+	+	+	+		
Insect	Drosophila melanogaster	+	+	+	+	+	+	+
	Habrobracon juglandis	+	+			+	+	+
	Bombyx mori	+				+	+	
Mammalian cell culture	Chinese hamster		+	+	+	+		
	Mouse lymphoma		+	+	+	+		
Intact mammal	Mouse	+	+	+	+		+	
	Rat	+	+	+	+			
	Man		+	+	+			

[a]Taken from Drake et al. (1975), copyright 1975 by American Association for the Advancement of Science, and presented here with their and the authors permission.

assessment could then be used to help determine the regulatory steps needed to reduce human exposure.

The first tier screening tests play an essential role in this system, because the scheme for further testing is based on their predictive value. The most successful assays currently under scrutiny are those that detect DNA damage by the induction of mutations in bacteria. Reversion to prototrophy is generally believed to be the most sensitive type of assay, and there are currently two tests of this type in general use. These two screening assays utilize: the histidine-requiring mutants of the Salmonella typhimurium/mammalian microsome system (Ames test) and the DNA polymerase-deficient mutants of the Escherichia coli system. Comparative tests have shown that both tests are good predictors of environmental carcinogens (Purchase et al. 1976) but each test has its individual strengths and weaknesses. For example, the E. coli mutants are more sensitive than the S. typhimurium mutants are in detecting mutagens that cause base-pair substitutions, but much less sensitive in detecting frameshift mutagens. The Ames test, on the other hand, can detect a wider range of chemical mutagens, because it provides strains which respond to both base-pair substitution and frameshift mutagens (Bridges 1976). Since the purpose of this investigation was to provide an initial survey of the carcinogenic/mutagenic hazard of spent shale leachates and because the Ames test has been successful in detecting known PAH (McCann et al. 1975) and mutagens in petroleum, petroleum products, and effluents (Epler et al. 1978a, 1978b; Rubin et al. 1976), it was the preferred assay.

The Ames Test

The data compiled for validation of the Ames test by McCann and coworkers (1975) are shown in Table 3. Overall, the test correctly identified 90 percent (158/175) of the carcinogens as mutagens and 79 percent (49/62) of the non-carcinogens as non-mutagens. For the classes of compounds found in petroleum, its products and by-products (including spent oil shale), the predictability was 96 percent accurate (85/88) for carcinogens detected as mutagens and 72 percent (18/25) for non-carcinogens not mutagenic to bacteria. An independent

Table 2. Operational characteristics of mutagen screening systems.[a]

Test System	Time to Run Test	Operating Costs[b]	Initial Investment Costs	Relative East of Detection[c]	
				Gene Mutations	Chromosome Aberrations
Microorganisms with metabolic activation:					
Salmonella typhimurium	2 to 3 days	Very low	Low	Excellent	
Escherichia coli	2 to 3 days	Very low	Low	Excellent	
Yeasts	3 to 5 days	Very low	Low	Good	Unknown
Neurospora crassa	1 to 3 weeks	Moderate	Moderate	Very good	Good
Cultured mammalian cells with metabolic activation	2 to 5 weeks	Moderate to high	Moderate	Excellent to fair	Unknown
Host-mediated assay with:					
Microorganisms	2 to 7 days	Low to moderate	Low to moderate	Good	
Mammalian cells	2 to 5 weeks	Moderate to high	Moderate	Unknown	Good
Body fluid analysis	Variable	Variable	Low to moderate	Variable	
Plants:					
Vicia faba	3 to 8 days	Low	Low		Relevance unclear
Tradescantia paludosa	2 to 5 weeks	Low to moderate	Moderate	Potentially excellent	
Insects:					
Drosophila melanogaster:					
Gene mutations	2 to 7 weeks	Moderate	Moderate	Good to excellent	
Chromosome aberrations	2 to 7 weeks	Moderate	Moderate		Good to excellent
Mammals:					
Dominant lethal mutations	2 to 4 months	Moderate to high	Moderate		Unknown
Translocations	5 to 7 months	Moderate to high	Moderate		Potentially very good
Blood or bone marrow cytogenetics	1 to 5 weeks	Moderate	Moderate		Potentially good
Specific locus mutations	2 to 3 months	High to very high	High to very high	Unknown	

[a] Taken from Drake et al. (1975), copyright 1975 by American Association for the Advancement of Science, and presented here with their and the authors permission.

[b] Operating costa vary widely depending upon the protocol specified and upon the number of substances tested simultaneously. Very approximately, very low is $1,000; low is $1,000 to $5,000; moderate is $3,000 to $10,000; high is $10,000 to $20,000; and very high is $25,000 upward (based on 1975 economics).

[c] Since most of these test systems do not detect all classes of gene mutations or chromosome aberrations (see Table 1), these columns refer only to the detectable mutations.

examination of the Ames test conducted by Purchase and coworkers (1976) gave similar results. Ninety-one percent (53/58) of the carcinogens were mutagenic and 93 percent (58/62) of the non-carcinogens were not mutagenic. For the PAH compounds tested, in particular, 95 percent were correctly identified. Other researchers (Bartsch 1976; Commoner 1976a, 1976b) have attested to the predictive value of the Ames test. Additional advantages of this assay are the tentative indication that a quantitative relationship exists between carcinogenic and mutagenic potency (Meselson and Russel 1977) which is especially shown for a number of PAH (Teranishi et al. 1975), and the apparent suitability of the test to analyze complex mixtures (food, human body fluid, hair dyes).

Although additional data are still being accumulated on the accuracy of the Ames test, the current estimate is that the test can correctly predict the carcinogenicity or non-carcinogenicity of 90 percent of the compounds tested. That means that there are some chemicals that are carcinogenic but will not be detected by the assay (false negatives), and some chemicals that will be incorrectly identified as carcinogens (false positives). This is part of the compromise that is to be expected when using a short term screening test. The short term assays are to be used only as a preliminary step (the first tier) of the complete testing procedure. There is some question, however, about the certainty of the number of false positives implicated by this test. McCann and Ames (1976) believe that many of the

Three Tiers for Carcinogen Screening

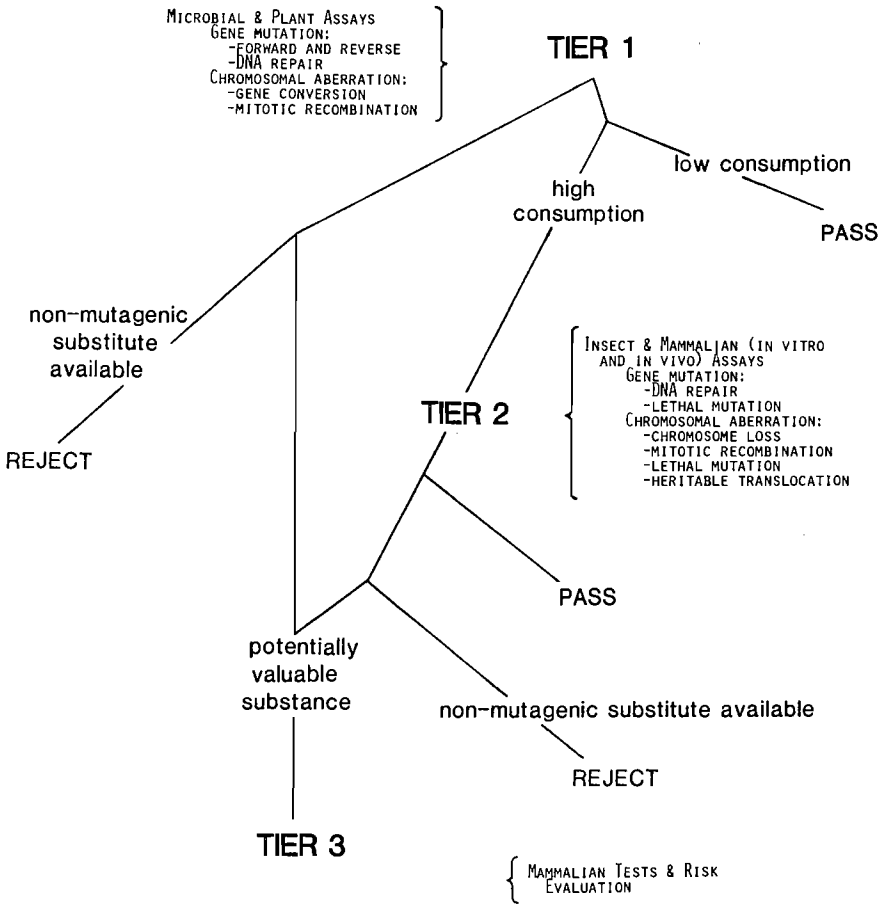

Figure 1. Hierarchical testing scheme for identifying mutagens and carcinogens. Three-tier approach adapted from Bridges (1974).

so-called "false" positives detected by the Ames test will eventually be shown to be carcinogens. This has already been shown with the Ames test mutagen (and now proven animal carcinogen), furylfuramide (a food additive previously used in Japan), that had given negative results in two carcinogenicity trials (Miyaji 1971a, 1971b).

Ames Test Applied to Environmental Carcinogens

The bacterial test system developed by Ames has been used primarily for testing pure compounds. In its extended application of screening for potential environmental carcinogens, various substances of complex chemical

Table 3. Summary of Ames test results indicating prediction accuracy for various chemical groups.[a]

Class of Compounds	Percent Correctly Identified[b]	
Aromatic Amines	25[c]	11
Alkyl Halides	20	3
Polycyclic Aromatics	26	8
Esters, Epoxides and Carbamates	17	8
Nitro Aromatics and Heterocycles	28	4
Miscellaneous Aliphatics and Aromatics	5	12
Nitrosamines	21	NT[d]
Fungal Toxins and Antibiotics	8	2
Mixtures	1	NT
Miscellaneous Heterocycles	4	7
Miscellaneous Nitrogen Compounds	9	4
Azo Dyes and Diazo Dyes	11	3
Total	175	62

[a]Data taken from McCann et al. (1975).

[b]There are two bars for each class of compounds; the stippled one refers to the known carcinogens of that class and the blank bar refers to the non-carcinogens.

[c]The number appearing at the right of each bar indicates the number of different compounds assayed.

[d]NT means no chemicals were assayed from that group.

composition have been fractionated and assayed. Mutagens have been detected in many types of environmental mixtures: tobacco smoke condensates (Kier et al. 1974; Hutton and Hackney 1975); urban air (Pitts et al. 1978); hair dyes (Ames et al. 1975); petroleum products, by-products, and effluents (Rubin et al. 1976; Voll et al. 1977; Rao et al. 1977; Commoner 1977; Chrisp et al. 1978; Epler 1979; Epler et al. 1978a, 1978b); insecticides (Grant et al. 1976); pesticides (Shirasu et al. 1976); flame retardent additives (Blum and Ames 1977); urine (Durston and Ames 1974; Yamasaki and Ames 1977); surface and potable water (Simmon and Tardiff 1976; Pelon et al. 1977; Glatz et al. 1978; Loper et al. 1978a, 1978b); and aqueous waste material (Commoner 1977; Ander et al. 1977; Epler et al. 1978a, 1978b; Epler 1979). The Ames test has also been applied to extracts of organisms from industrially polluted terrestrial and marine environments (Barnes and Klekowski 1978; Parry et al. 1976).

It is usually necessary to apply chemical techniques to fractionate these mixtures prior to biological testing. Many researchers have relied on the procedures originally provided by Swain and colleagues (1969) for fractionating cigarette smoke condensate. When this technique is applied to petroleum-related products and by-products, it is found that the biologically active chemicals are found with the basic and neutral fractions (Epler et al. 1978a; Rubin et al. 1976; Rao et al. 1977; Kier et al. 1974). Epler and coworkers (1978a) suggest that the mutagens (and carcinogens) responsible for this activity in the basic fraction could be quinoline, substituted quinolines, alkyl pyridines, acridine, napthyl-amines and/or aromatic amines. In the neutral fraction, the compounds implicated are benzanthracenes, dibenzanthracenes, substituted anthracenes, benzopyrenes, benzofluorenes, pyrene, substituted pyrenes and/or chrysenes.

Limitations of the Ames Test

The validation process for determining the applicability of the Ames test for environmental screening of mutagens has encountered several problems. Several researchers have noted that their results could be biased as a function of sample collection and storage, or that artifacts could be generated by the extraction and fractionation procedures (Epler et al. 1978a). A number of biological variables also may affect the outcome of the assay (Epler et al. 1978a). For example, a mutagen present in one sample may go undetected due to the interfering toxicity of another compound (Ames et al. 1975), or the choice of inducer for liver enzymes could be inappropriate to activate certain compounds (Ames et al. 1975; Epler et al. 1978a; Bartsch 1976). Finally, the nature of the interactions (i.e. co-mutagenesis) of two or more mutagens in solution has received limited attention, and its effect on the response of the Ames tester strains is only beginning to be known.

MATERIALS AND METHODS

Sample Extraction and Preparation

Extracts of spent oil shale were obtained from Maase and Adams (1980) who studied two extraction procedures. The first method employed soxhlet extraction using organic solvent and separation techniques for easier identification and bioassay of compounds in the complex mixture; the second was actually a series of methods for obtaining and concentrating identifiable compounds in aqueous leachates of spent shale. A general outline of both methods follows.

The purpose of the soxhlet extraction was to indicate the total quantity of mutagens which could be obtained from the shale matrix. A diversity of samples were obtained (in effect eluted from the sorbent shale) by varying the sequence of solvents, the composition of solvents in mixtures, and the extraction time. Most of the samples for mutagenicity testing were obtained in methanol after a period of extraction with benzene, although a few samples were developed in pentane, or cyclohexane. The use of solvents other than methanol frequently elicited a toxic response during mutagenicity testing (benzene) or were incompatible with the bioassay procedure due to volatilization (pentane) or hydrophobicity (cyclohexane). Attempts were made to evaporate the original solvent and redissolve the sample in dimethylsulfoxide (DMSO), however, this procedure met with little success; a majority of samples still showed toxicity or failed to become resolubilized.

The potential for water to leach mutagens from shale was evaluated by providing extended contact of water with shale in both an upflow column and in a large teflon-lined drum equipped with a motor-driven mixer. The organic compounds suspended in the water were then either concentrated on an exchange resin (XAD-2 and XAD-7, Rohm-Haas Co.) and eluted with solvents of varying polarity, or extracted directly with organic solvent (liquid-liquid extraction). As with the organic solvent extracts obtained from the soxhlet, these samples were concentrated by either roto-evaporation or Kuderna-Danish concentration. Thin-layer chromatography was used to further separate the components in these mixtures, if necessary. In the presentation of the results, the procedures used for obtaining the individual samples are outlined.

Mutagenicity Tests

In applying the Salmonella/mammalian microsome test or Ames test (Ames et al. 1973), the Salmonella typhimuriam mutant strains employed for the screening (provided by Professor B. N. Ames, U. C. Berkeley) were TA 98, 1537, 1538, and TA 100. Strain TA 1535 was also used initially but later excluded from general testing because it responded to so few of the known mutagens (see Results Section).

The microsomal enzyme fraction (S-9) was obtained from rat liver homogenate after induction with a polychlorinated biphenyl mixture, Aroclor 1254 (Ames et al. 1973). After initial attempts to obtain an active S-9 mixture failed due to high temperature denaturation, it was found that low enough temperatures could be maintained by the addition of a glass jacket to the tissue grinding tube (see Figure 2) through which ice-cold water could circulate and by allowing sufficient time to elapse (15 seconds) between successive passes with the pestle.

The Salmonella strains employed were histidine-dependent due to a specific mutation in the genes coding for this biosynthetic pathway. They could have been reverted back to the ability to grow without histidine by exposure to various mutagenic agents. Strains TA 100 and 1535 are reverted by substances causing base-pair substitutions in their DNA and strains TA 98, 1537 and 1538 are reverted by frameshift mutagens. Positive, or mutagenic, activity is defined by a response showing at least twice the number of revertant colonies as that appearing in solvent controls. It has been suggested that the numbers of revertants is correlated to carcinogenic activity (McCann and Ames 1976), and therefore the results are presented in terms of strength of mutagenicity. In order to compare the sensitivity of strains to mutagens, the response was normalized to the spontaneous revertant response. A ratio was calculated by dividing the number of revertant colonies resulting from assays of the test compound by the number of revertant colonies resulting spontaneously in the solvent controls. For each experiment conducted, the ratio was calculated and a mean value and standard error was determined for each chemical, strain, and concentration.

The samples resulting from spent shale extraction were assayed using the standard plate incorporation assay over a wide concentration range (representing from 0.1 g to 10 g of spent shale per plate), with and without the addition of the S-9 fraction. For comparison, the dose-response of several known PAH standards, some of which had previously been identified in spent shale extracts, were determined.

A few minor modifications of the protocal described by Ames and coworkers (1973) became standard procedure in this laboratory because they improved the interpretation of the assay: 1) the percentage of agar in the top agar was increased from 0.5 percent to 0.75 percent, thereby speeding the hardening process and minimizing top agar slippage; 2) the bacteria used were always fresh overnight cultures because the overall sensitivity and response were improved slightly; 3) strain TA 1535 was excluded rather than TA 1538 in screening unknowns because of the sensitivity of TA 1538 in detecting nitrogen-containing PAH (Hutton and Hackney 1975; Kier et al. 1974), and 4) the concentration of S-9 used per volume of S-9 mix was increased over the recommended proportion (Ames et al. 1973) to 100-150 µl/plate.

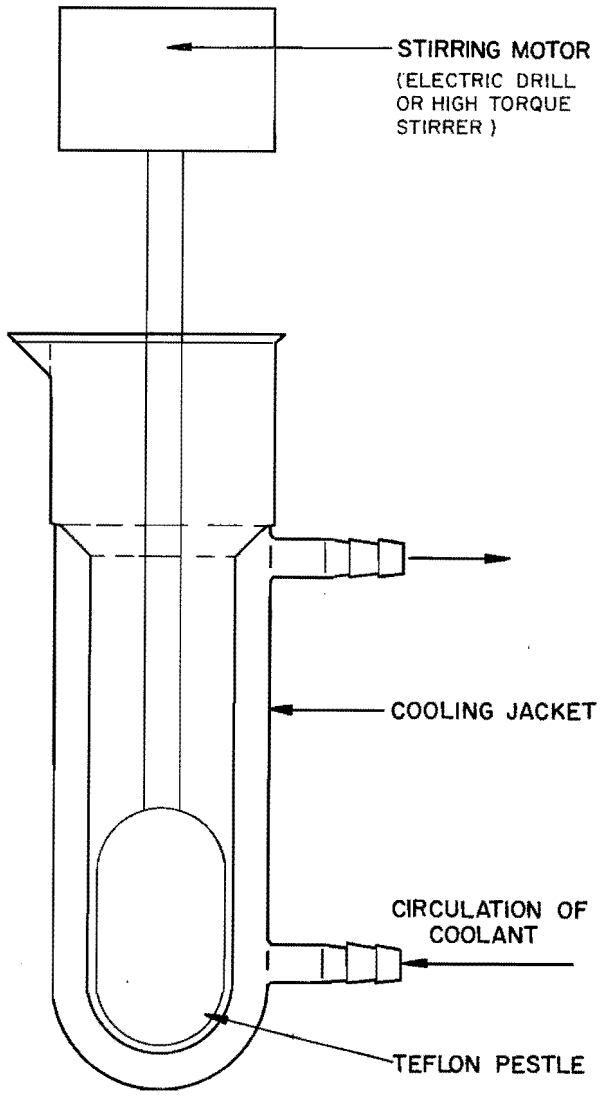

Figure 2. Schematic diagram of jacketed tissue homogenizer.

In addition to the plate incorporation assay two other techniques were attempted. In order to conserve a limited amount of sample the "well" test, introduced by Turner and coworkers (1978) was used. This methodology combines the advantage of the spot test (small quantity of chemical required per test) and that of the plate incorporation assay (allows incorporation of hydrophobic substances, i.e. PAH, into the agar, thereby increasing the exposure of the bacteria to the mutagens). The method differs from the plate incorporation method in that histidine and biotin are added to the molten minimal agar prior to plate pouring, thus eliminating the need for top agar. Then a small aliquot (0.1 ml) of tester strain culture (2 x 10^8 cells) is spread evenly over the surface of the agar. Two 13 mm plugs are cut out on opposing sides of a single petri dish and plugged with 0.1 ml of molten well agar (same as top agar minus histidine and biotin). In one well only the test chemical solution (in various concentrations) is added; in the other well, an identical quantity of chemical is added along with 50 µl of S-9. The two wells are filled with well agar to the same level as the agar surface. A response is taken as positive if a distinguishable ring of revertant colonies appears surrounding the well but does not appear in the control plates (prepared as described above except that solvent alone is used rather than the chemical solution).

For assaying the chemical mixture in aqueous extracts of spent shale, a technique described by Saxena and Schwartz (1979) was employed. The aqueous sample is filter-sterilized and used to make up the Vogel-Bonner E media for the minimal glucose plates (see the original methods paper by Ames et al. 1975). The test water composes 90 percent of the total media volume and 20 ml of media is distributed to each petri dish (representing $\sim$ 0.15 g of spent shale per plate). The bacteria and S-9 are added to the dish in the top agar as usual. The technique exposes the bacteria to all chemicals present in the water. A positive response could be determined quantitatively as with the plate incorporation procedure.

As mentioned, the soxhlet extracts of spent shale were taken in benzene and in methanol, whereas the known PAH compounds

were dissolved in DMSO prior to mutagenesis assay. In order to assess whether the choice of solvent has an effect on mutagenic response, the known compounds (those which were soluble) were assayed in solutions of methanol. The use of benzene in the Ames assay is not recommended, but a bacterial tolerance limit to the toxic effect of the solvent was also determined in order to assess whether small quantities of benzene, which may contaminate the methanol during the second phase of soxhlet extraction, could be responsible for any observed toxic response.

The potential complexity of determining mutagenic response to chemical mixtures was investigated by assaying two known PAH compounds at various concentrations (equivalent to 5, 10, 50 µg each per plate) in a one-to-one solution (by weight) and in two other weight ratios: 10 percent compound A with 90 percent compound B and vice versa. The response was compared to that of the single compound at the same concentration to which an appropriate amount of pure solvent blank was added.

General procedure maintained for all tests included positive controls to test for S-9 activity, and blanks to test for solvent effect and for sterile technique. Four replicate plates were used initially for each compound or sample tested (coefficient of variance (C.V.) = 13.4 percent, n = 27), but two replicates were later used with little loss of precision (C.V. = 14.0 percent, n = 22).

Experiments on the known PAH compounds were conducted at least twice. Due to the limited amount of available sample, many of the unknown mixtures (90 percent) were assayed only once.

RESULTS

Mutagenicity Testing of Known Compounds

Eighteen known polycyclic aromatic hydrocarbons (PAH) were assayed for mutagenicity using the plate incorporation procedure of the Ames test. Eleven of these compounds have been identified in spent shale extracts (indicated by the * in Table 4). The others are examples of nitrogen- and sulfur-containing PAH which could possibly be formed during fossil fuel combustion. The response of the Ames test mutant strains to the diversity of compounds represented by this group was used to evaluate the applicability of this test for mutagenicity screening of spent shale extracts. To be suitable for this use, a test must consistently distinguish between mutagens and non-mutagens with a low rate of false positives (non-mutagens detected as mutagens) and false negatives (mutagens not detected). The mutant strains must be sensitive to small concentrations of test compound and consistently demonstrate the same dose-response curve.

A unique pattern of mutagenic response, in terms of a particular strain sensitive to the mutagen and the shape and magnitude of the dose response curve, was demonstrated for each chemical mutagen. Examples of these characteristic patterns are shown in Figures 3 and 4 for a strong carcinogen, benzo(a)-pyrene (BaP) and a moderate carcinogen, benz(a)anthracene (BA), respectively. The data presented in these figures indicate that strain TA 100 is the most responsive of the four strains to these two chemicals and that responsiveness also varies among the other three strains. Since the spontaneous reversion rate peculiar to a strain enhances its ultimate response, the average response at each chemical concentration for a given strain was normalized to the average spontaneous response (solvent control) determined for each experiment (no. revertants per plate for each treatment/no. spontaneous revertants); thus, a comparison of the relative response of each strain to mutagens could be made. This ratio, henceforth referred to as the revertant ratio or R.R., proved to be a simple way to determine a positive mutagenic response, which according to co-developers of the Ames test (McCann et al. 1975) is defined by R.R. $\geq$ 2.0.

The advantage of expressing the data in terms of the revertant ratio is demonstrated

Table 4. Polycyclic aromatic hydrocarbons identified in spent oil shale extracts.

Compound Name	Symbol	Investigator			Carcinogenic Potential[d]
		DRI[a]	DRI[b]	Colony[c]	
*Acridine		X			-
Anthanthrene (dibenz(c,d,j,k)pyrene)		X	X	X	-
*Benz(a)anthracene	BA	X	X	X	+
*Benzo(g,h,i)perylene	BP		X		+
*Benzo(a)pyrene	BaP	X	X	X	+++
*Carbazole		X			-
Dibenz(a,j)acridine		X			+
*Dibenz(a,h)anthracene	DBA		X		++
*7,12 Dimethylbenz(a)anthracene	DMBA	X	X	X	++
*Fluoranthene	F	X	X	X	-
3-Methylcholanthrene			X	X	++
*Perylene	P	X	X	X	-
Phenanthrene		X	X	X	-
*Phenanthridine		X	X	X	?
*Pyrene		X	X	X	-

[a] DRI (1974) Source: Schmidt-Collerus (1974).
[b] DRI (1976) Source: Schmidt-Collerus et al. (1976).
[c] Colony (1974) Source: Atwood and Coomes (1974).
[d] Relative carcinogenic activity on mouse skin, +++ = high, ++ = moderate, + = weak, - = inactive, ? = unknown, source: Hoffman and Wynder, in Searle (1976).

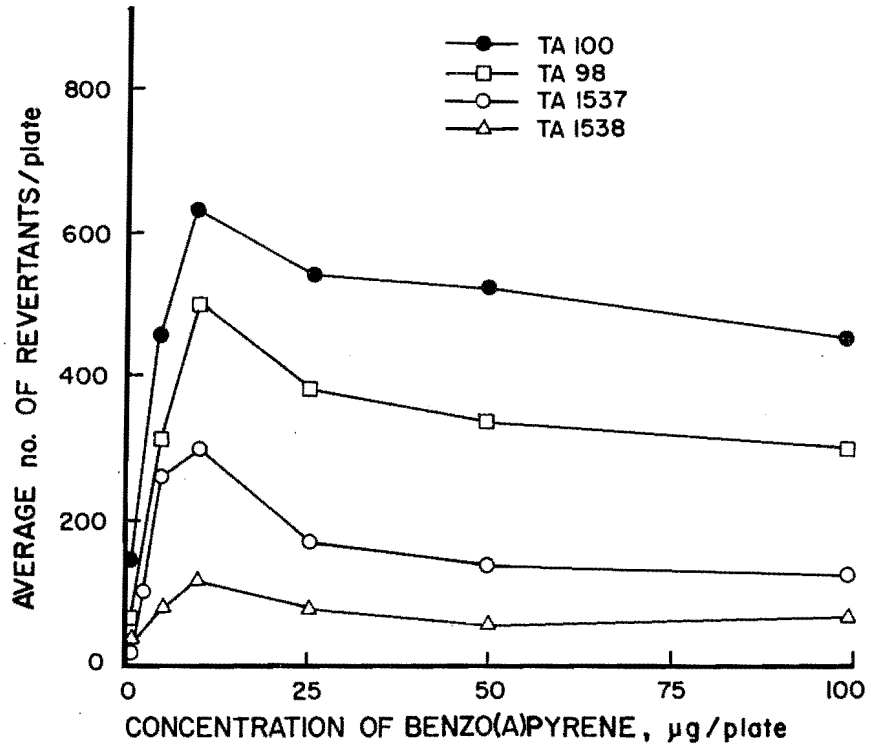

Figure 3. Dose response curves using mutant Salmonella strains TA 100, 98, 1537 and 1538 for benzo(a)pyrene. Each point represents the average value obtained from at least two replicates in at least two experiments.

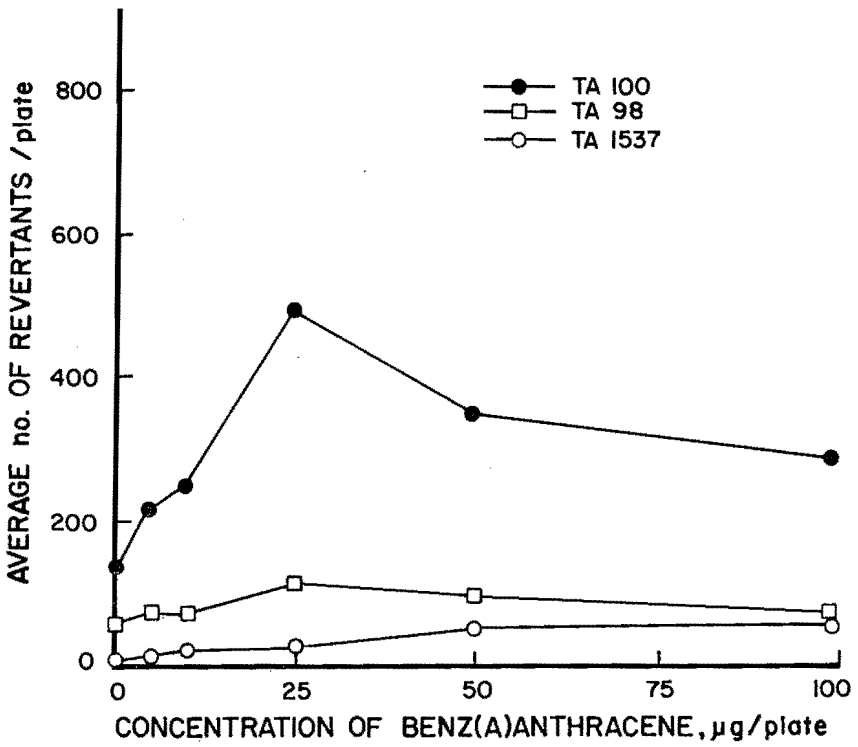

Figure 4. Dose response curves using mutant Salmonella strains TA 100, 98 and 1537 for benz(a)-anthracene. Each point represents the average value obtained from at least two replicates in at least two experiments

in Figures 5 and 6 which are transformations of Figures 3 and 4, respectively. The transformed dose-response curves indicate the relative sensitivity of each strain and whether that strain can be used to clearly identify the compound as a chemical mutagen (R.R. $\geq$ 2.0). In the figures plotted from the data transformation, TA 1537 is shown to be the most sensitive of all those strains which responded to either BaP or BA. The other strains show a lesser response and are therefore less reliable for clearly detecting these compounds as mutagens.

The results of the Ames test assay of the standard PAH compounds (including anthracene, a non-carcinogen frequently formed during combustion of fossil fuels) are compiled in Table 5. The values presented in this table are the average revertant ratio values per plate determined for the concentration at which that strain responded maximally. The R.R. value given in parentheses is accompanied by the symbol(s), - or + which indicate the relative magnitude of the R.R. value (thus relative mutagenic strength) according to the scheme in Table 6.

The data compiled in Table 5 illustrate several features of the Ames test which make it suitable for mutagenicity screening of PAH-containing samples. First, the combination of five mutant Salmonella strains allows the detection of a wide variety of chemical mutagens. Of the compounds for which the carcinogenic activity is known (Figure 7), the Ames test correctly identified 86 percent (6/7) of them and therefore the rate of false negatives was 14 percent (13-H dibenzo(a,i) carbazole, a weak carcinogen was not detected). Seventy-one percent (5/7) of the non-carcinogens were non-mutagenic, thus the rate of false positive was 29 percent (fluoranthene and perylene were detected as mutagens by one or more strains). In addition to the ability to detect carcinogens with good accuracy, a comparison of the relative carcinogenic activity (Figure 7) and mutagenic activity (Table 5) demonstrates that the magnitude of the mutagenic response of the bacterial strains was closely correlated with the intensity of carcinogenic activity. Examples which display this correlation are the inactive carcinogens, acridine, anthracene, carbazole, phenanthrene, and pyrene, which were non-mutagens; the weakly-to-moderately active carcinogens, benz(a)anthracene, and dibenz(a,h) anthracene which were moderately strong mutagens; and the highly active carcinogens, benzo(a)pyrene, and 7,12 dimethylbenz(a)anthracene were strong mutagens.

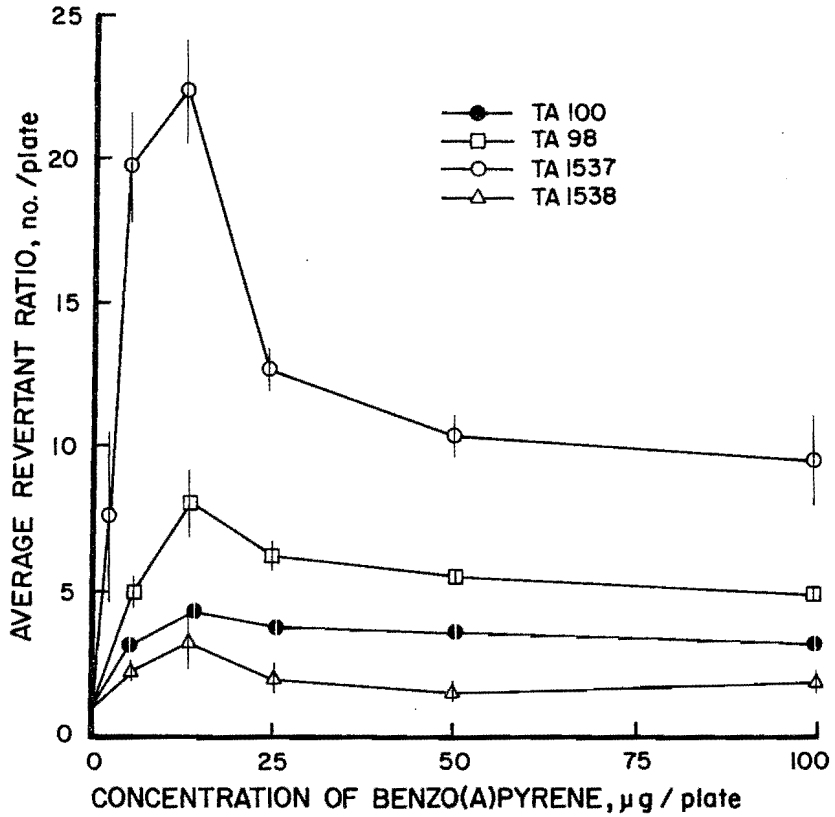

Figure 5. Transformed dose response curves obtained by normalizing the data presented in Figure 3 to the spontaneous reversion rate of each strain. Each point represents an average value from at least two replicates in at least two experiments. The thin vertical lines passing through each point indicate the range of standard error around the mean.

Besides indicating the carcinogenic activity of these compounds, the results in Table 5 suggest the most likely type of mutagen. Some of the compounds, for example benzo(a)pyrene and 1-aminopyrene are general mutagens, i.e., they can cause base pair substitution and frame-shift mutations of various types, while others are specific to the class of mutations initiated. Benzo(ghi)-perylene is one of the latter, and appears to only cause mutations in the specific sequence of bases found in the mutant TA 1537.

The Ames test assay has thus been shown to be a useful tool for mutagen screening; it is a sensitive bioassay and may be used to detect some mutagens which are present in small quantities. The magnitude of the mutagenic response may give some indication of the carcinogenic activity of the compound, and the responding strains may be used to indicate the type of mutation the compound causes.

Mutagenicity Testing of Spent Shale Extracts

Four types of spent shale were used to obtain samples for mutagenicity testing. Two of these shales were processed by the Paraho operation, one (Paraho A) by the indirect mode, the other (Paraho B) by the direct mode. The other two spent shales were processed by TOSCO and Union.[1]

The results of mutagenicity screening of the extracts from these spent shales are compiled in Table 7. The same format that was used to present responding mutant strain and magnitude of mutagenic response for the PAH standards is repeated for the unknown mutagens in the shale extracts. Also included in this table is a brief description of the procedures (extraction, concentration, and separation) used in obtaining each sample.

[1]Commercial process names are used throughout the text for convenient reference. For more information about the physical and chemical properties of the four samples investigated see Maase and Adams (1980). The data reported should be judged as representative only of early surface retorting research and should not be considered reflective of commercial scale processed shales.

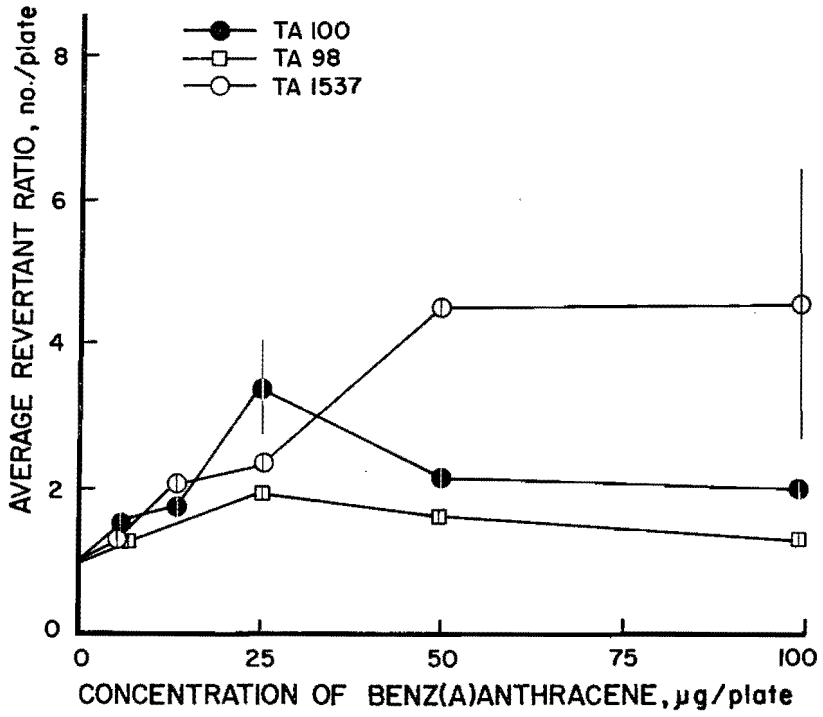

Figure 6. Transformed dose response curves obtained by normalizing the data presented in Figure 4 to the spontaneous reversion rate of each strain. Each point represents an average value from at least two replicates in at least two experiments. The thin vertical lines passing through each point indicate the range of standard error around the mean.

Table 5. Results of Ames test assay of polycyclic aromatic hydrocarbon standards.[a]

Symbol	Compound Name	TA Strain				
		1535	1537	1538	98	100
	Acridine (2)[b]	-[c]	-	-	-	-
	1-Aminopyrene (2)	-	N.T.[d]	+(3.77)[e] MMR 2.0	+++++(39.48) MMR 2.0	++(4.50) MMR 2.0
	w/o S-9[f]	-	N.T.	++(6.45) MMR 6.45	+++++(37.24) MMR 2.0	+ (3.32) MMR (10.0)
A	Anthracene (2)	-	-	-	-	-
	4-Azafluorene (2)	-	-	-	-	-
BA	Benz(a)anthracene (3)	-	++(4.6) MMR 12.5	-	(+) (1.94) MMR 25.0	+ (3.46) MMR 25.0
BP	Benz(ghi)perylene (3)	-	+++(9.76) MMR 5.0	-	-	-
BaP	Benzo(a)pyrene (3)	-	++++(22.43) MMR 2.5	+(3.22) MMR 5.0	+++(8.08) MMR 5.0	++(4.39) MMR 5.0
	Carbazole (2)	-	-	-	-	-
	Dibenz(a,h)anthracene (3)	-	+(2.74) MMR 12.5	-	-	(+) 2.03 MMR 25.0
	13-H-Dibenzo(a,i)carbazole (2)	-	-	-	-	-
	Dibenzothiophene (2)	-	-	-	-	-
DMBA	7,12 Dimethylbenz(a)anthracene (4)	-	+++(8.82) MMR 5.0	-	(+)(2.17) MMR 12.5	+(2.42) MMR 50
F	Fluoranthene (2)	-	+(2.53) MMR 25.	-	+(2.32) MMR 200.	+(2.49) MMR 25.
P	Perylene (3)	-	+++++(45.14) MMR 2.5	-	(+)(2.17) MMR 12.5	-
	Phenanthrene (2)	-	-	-	-	-
	Pyrene (3)	-	-	-	-	-
	Thianthrene (2)	++(4.95) MMR 2.0	-	++(6.65) MMR 2.0	++(4.82) MMR 2.0	-
	w/o S-9	-	-	+(2.62) MMR 2.0	-	-
	Triphenylene (2)	+(2.36) MMR 2.0	-	-	-	-

[a]Data are presented as the revertant ratio, R.R., where

$$R.R. = \frac{\text{total number of revertants}}{\text{number of spontaneous revertants}},$$

determined for at least two experiments for mutagens. Unless indicated the R.R. is calculated from data for response with metabolic activation (S-9).

[b]Number in parentheses indicates minimum number of experiments conducted to obtain R.R. value reported for compound.

[c]Symbols are used to indicate relative mutagenic strength, refer to Table 6.

[d]Numbers refer to revertant ratio, R. R.

[e]Δ indicates toxicity at high concentrations of sample.

[f]N.T. = not tested.

Table 6. Description of symbols used to indicate relative mutagenicity.

Symbol	Average Revertant Ratio Value	Interpretation
-	<2.0	No mutagenic response
(+)	>2.0 ranging <2.0 or <2.0 ranging >2.0	Questionable mutagenic response
+	2.0 - 4.0	Weak mutagenic response
++	4.0 - 8.0	Moderate mutagenic response
+++	8.0 - 16.0	Strong mutagenic response
++++	16.0 - 32.0	Very strong mutagenic response
+++++	>32.0	Extremely strong mutagenic response

The results of Ames test assays indicate that mutagens are present in each of the four types of spent shale and that mutagens can be obtained by a variety of different extraction methods. In general, these mutagens require metabolic activation (addition of enzyme-induced S-9) for their detection as mutagens. The repeated response of TA strains 1537, 1538, and 98, indicate that many of these mutagens cause frame-shift substitutions of bases in bacterial DNA. There were fewer base-pair substitution mutagens detected in these samples. In addition, a toxic response was common and was usually associated with the solvent, benzene, in the sample but not always (Paraho A #2, Paraho B #6, Union #3). This toxic response may have obscured the detection of mutagens in these and other samples (Paraho A #5, Paraho B #5, TOSCO #4). Attempts to separate the complex mixtures for the purposes of removing the chemicals (or solvents) responsible for the toxic response and of allowing more accurate detection and identification of chemical mutagens were unsuccessful. When thin layer chromatography was applied to certain mixtures which had a lot of mutagenic activity (Paraho B #1, TOSCO #2, Union #1), the mutagenic activity of the fractions (Paraho B #3, TOSCO #3, and Union #2, respectively) was weak or non-existent. The weak response may indicate that the compounds present are weak mutagens and/or they are present in low concentrations (i.e. poor recovery and subsequent dilution of sample).

Table 7 also summarizes some of the attempts to obtain mutagens in aqueous leachates of shale. The various methods of concentration using sephadex and XAD resins worked poorly in this regard resulting in questionable mutagenic activity (Union #3, #5). The attempts to use the aqueous sample in the agar media were also unsuccessful (Union #4).

These general results were found for all four sources of spent shale. Although inconsistencies in extraction procedure and sample preparation prevent specific comparisons in the mutagen composition of the spent shales, the technique used on individual shales can be compared in terms of their yield of mutagens.

Paraho A spent shale was subjected to two contrasting extraction regimes. When extracted for a short period (1 day) by each of two solvents (benzene followed by methanol), the sample showed frame-shift mutation activity (Paraho A #1). Another sample, extracted for a longer period (4 days), using the same combination of solvents, showed only a slight base-pair substitute type of mutagenic activity (Paraho A #2, #3). The combination of these two samples (Paraho #4) resulted in the detection of the frame-shift type of mutagen only.

The mutagenic response of samples of Paraho B shale was, overall, greater than that for Paraho A. Of particular interest is the result of assay experiments with samples #1 and #2. These samples were extracted with methanol only without prior extraction with benzene. The mutagenic response is relatively strong, and no toxicity was observed. The chromatographic separation (TLC) of these samples also showed mutagenic activity (Paraho B #3). This activity was of lesser magnitude than the original sample as would be expected if the chemical creating the effect was diluted. The lower concentration of this compound or of others may explain the lack of response of strains TA 1535, 1538, and 98.

Sample #5 (Paraho B) was the only sample which had a definite positive response when assayed without S-9. In fact, the response was somewhat greater when S-9 was absent than when it was present, for those strains which responded in both instances. The toxicity of this sample at high concentrations was, in all likelihood, due to the quantity of benzene (20 percent) in the solvent mixture assayed. Benzene was toxic to the bacterial strains at concentrations exceeding between 5 percent but composing less than 10 percent of the 0.1 ml aliquot solvent volumes added (Figure 8).

Table 7. Results of Ames mutagenicity testing of spent shale extracts.[a]

Spent Shale Type and Sample No.	TA Strain					Extraction Procedure[b]
	1535	1537	1538	98	100	
Paraho A						
#1	(+)[c] 3.06[d]	+ 3.54	(+) 1.80	(+) 1.90	-	Sox. Ext. one day with ØH followed by one day with MeOH, KD concentration, in MeOH
#2	-	-	-	Δ[e]	(+) 1.76	Sox. Ext. four days with ØH followed by four days with MeOH, RE concentration, in MeOH
#3	++ 4.60	-	-	-	(+) 1.86	Sox. Ext. four days with ØH followed by four days with MeOH, KD concentration, in MeOH
#4	N.T.[f]	+ 2.74	+ 3.32	-	-	Samples #2 and #3 combined.
#5	N.T.	Δ	Δ	N.T.	Δ	Sox. Ext. four days with ØH-MeOH solution 1:5 by volume, KD concentration, in ØH-MeOH
Paraho B						
#1	+ 3.30	+++ 9.75	+ 2.85	+ 2.41	-	Sox. Ext. three days with MeOH, KD concentration, in MeOH
#2	N.T.	++ 5.73	+ 3.40	N.T.	(+)	Same as #1
#3	-	(+) 2.25	-	-	-	TLC on silica gel of sample #1, R_f = - eluted with MeOH:p-Dioxane solution 1:1 by vol.
#4	+ 2.75	++ 4.25	-	-	-	Same as #3 except R_f = - eluted with MeOH: p-Dioxane solution 1:4 by vol.
#5 w/S-9	N.T.	++ 5.40 Δ	+ 3.76 Δ	+ 2.46 Δ	Δ	Sox. Ext. days with ØH:MeOH solution 1:5
w/o S-9[g]	N.T.	++ 6.60 Δ	++ 5.67 Δ	Δ	Δ	by vol., KD concentration, in ØH:MeOH
#6	N.T.	Δ	Δ	Δ	Δ	Sox. Ext. one day with ØH followed by one day with MeOH, RE concentration, in MeOH
#7	N.T.	(+)	(+)	-	-	Sox. Ext. one day with ØH followed by two days with MeOH, in MeOH
#8[h]	N.T.	(+)	-	-	-	Sox. Ext. one day with Pentane, KD concentration, TLC on silica gel eluted 5 fractions w/MeOH
Tosco						
#1	N.T.	+++10.43	+++11.54	++5.07	-	Sox. Ext. three days with ØH followed by five days with MeOH, RE concentration, in MeOH
#2	N.T.	+++11.33	+++14.44	++8.17	(+) 2.19	Same as #1 except KD concentration
#3	-	-	-	-	-	TLC on silica gel of sample #2, eluted - fractions with MeOH
#4	N.T.	Δ	Δ	Δ	Δ	Separation of sample #2 on Al_2O_3, eluted with ØH
Union						
#1	N.T.	++ 5.00	+++11.90	+ 3.99	(+)	Sox. Ext. one day with MeOH, KD concentration, in MeOH
#2	N.T.	-	-	-	-	TLC on silica gel of sample #1 eluted - fractions with MeOH
#3	N.T.	(+)	-	Δ	-	AQ. Leach. filtered and passed through Sephadex gel, eluted with MeOH
#4	N.T.	-	-	-	-	AQ. Leach. used in agar media preparation
#5	N.T.	(+)	(+)	-	-	AQ. Leach. filtered and passed through XAD-2 resin eluted with MeOH

[a] Results of plate incorporation assays with rat liver homogenate (S-9) except where indicated, from at least two replicates.

[b] Abbreviations used: Sox. Ext. = Soxhlet Extraction; ØH = Benzene; MeOH = Methanol; KD = Kuderna Danish; RE = Roto-evaporation; TLC = Thin layer chromatography; R_f = R_f value; Vol. = Volume; AQ. Leach. = aqueous leachate.

[c] Symbols are used to indicate relative mutagenic strength, refer to Table 6.

[d] Numbers refer to revertant ratio, R. R.

[e] Δ indicates toxicity at high concentrations of sample.

[f] N.T. = not tested.

[g] w/o S-9 = assay performed without rat liver homogenate.

[h] Of 5 fractions eluted from silica gel, only one R_f = 0.18 showed any mutagenicity.

When Paraho B spent shale was extracted in a similar manner to Paraho A (first with one solvent, benzene, and then with another, methanol), the samples contained few mutagens and these elicited a weak response (Paraho B #6 and #7). This result may have been due to the leaching of chemicals by the benzene or the toxicity of the remaining chemicals and/or solvent masking the mutagenic response.

TOSCO shale was subjected to extraction by two solvents, first benzene followed by methanol. Strong mutagenic activity mostly due to compounds causing frame-shift mutations (TA strains 98, 1537, and 1538), was observed in these samples (TOSCO #1 and #2). The separation procedures used to develop the components of these strongly mutagen samples were unsuccessful at further identifying which components were responsible for the

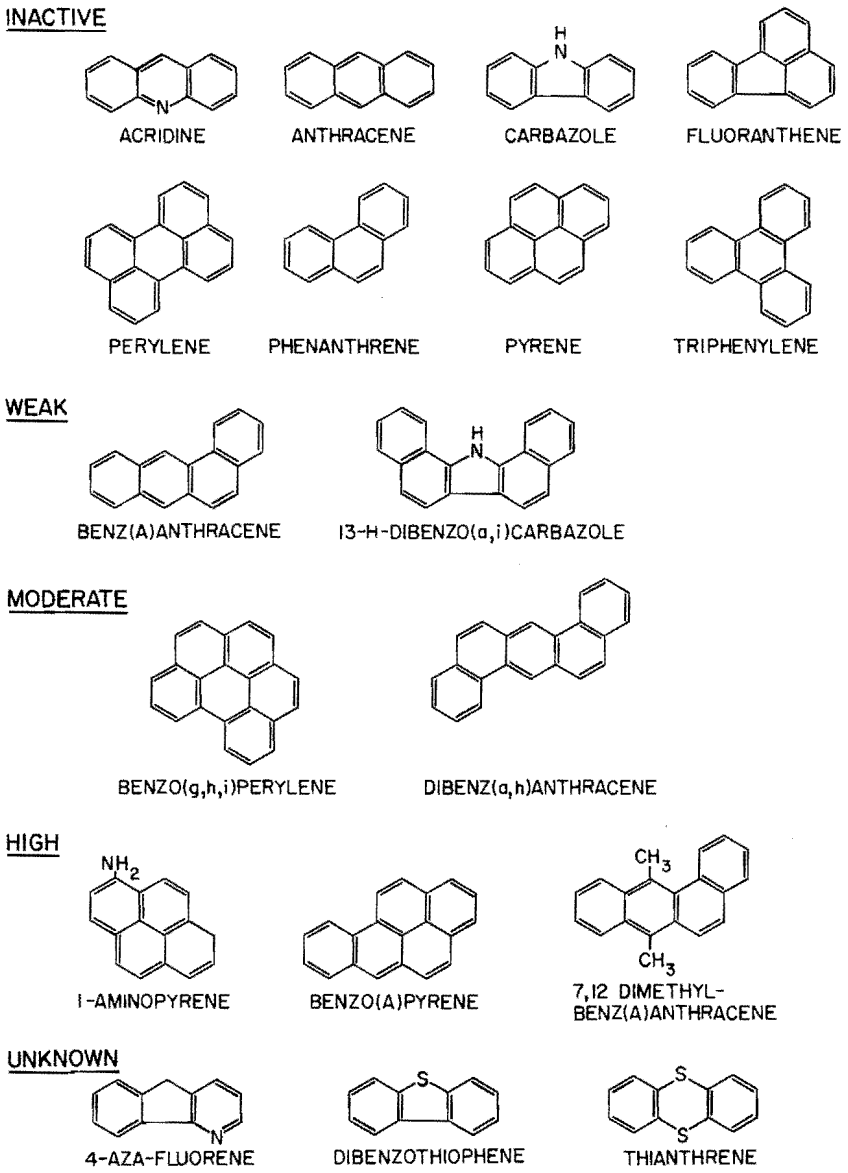

Figure 7. Chemical structure and relative carcinogenic activity of the 18 polycyclic aromatic hydrocarbons assayed in the Ames test. Data complied from Dipple (in Searle 1976).

mutagenic activity, probably due to sample dilution and loss (TOSCO #3) and the presence of benzene (TOSCO #4).

Mutagenic activity was detected in Union spent shale when it was subjected to short extraction periods with methanol (Union #1). Thin layer chromatographic separation of this sample and subsequent assay of fluorescent components gave no further information regarding the compound(s) responsible for the original activity. Three water developed samples of Union spent shale (#3, #4, and #5), in which various methods were applied to concentrate the sample and any mutagens therein, were only marginally successful. A weak mutagenic response was detected in two of the three samples, however the results are questionable without a repeat of this experiment.

Mutagenicity of Chemical Mixtures

The Ames test has been shown effective in detecting a wide variety of PAH, some of which are present in the organic residue of spent oil shale, as mutagens. The selectivity and intensity of the mutagenic dose response curve of the tester strains is seen to uniquely describe the PAH compounds assayed. The environmental samples that are being screened for mutagenicity using this procedure (e.g., surface waters, wastewater treatment plant effluent, and others) are generally mixtures of chemicals, some of which may be mutagenic and others not. Does the mutagenic response of the Ames test mutant bacterial strains reflect the presence of all mutagens in the mixture? If it does, is this response additive, i.e., does the sum of the parts equal the whole, or is it more or less than additive indicating the existence of antagonistic or synergistic interactions?

Assays of simple two component mixtures were performed to assess the capability of the Ames test to integrate the total mutagenic potential of environmental samples. The chemicals paired in these mixtures were chosen from those in the list of standards which gave a unique mutagenic response when assayed with a particular mutant strain. The ideal mutagenic response to this mixture would be one that was additive. The hypothesized response to this mixture was that 1) the Ames testing of a one-to-one mixture of components X and Y would result in a dose-response curve which could be clearly distinguished from either of the dose response curves of compounds X or Y alone, and

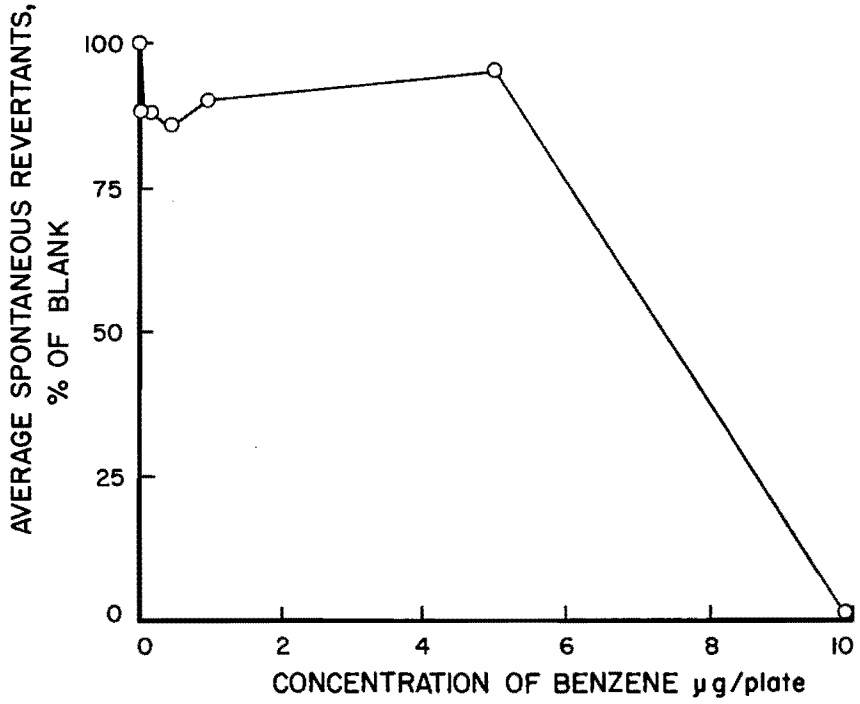

Figure 8. Bacterial response to increasing doses of the solute, benzene, in a fixed volume (0.1 ml) of solution (solvent was DMSO). Data for each of four mutant Salmonella strains (TA 100, 98, 1537 and 1538) were normalized to the DMSO control (blank) and averaged.

that 2) the dose response curve of the mixture (XY) would reflect the mutagenic potential of the individual compounds in an additive way.

Ten pairs of compounds were assayed, in a total of eighteen experiments using the plate incorporation method to test this hypothesis. The resulting dose response curves for compound X alone, compound Y alone and the one-to-one (by weight) mixture, XY, were subject to chi-square analysis for interaction. This statistical test was used to determine whether the set of three dose-response curves were distinguishable and if so, whether the dose-response attributable to the mixture was indistinguishable from the hypothetical response simply calculated by adding the responses of the individual components. In Table 8, the pairs of compounds that were assayed, the particular mutant strain used in each experiment, and the results of the chi-square analysis are shown.

Lower case letters (a, b and c) are used to indicate those dose-response curves in the set of three which are statistically indistinguishable (α=0.01). A symbol is shared among those curves which are similar, a different symbol indicates a curve in that set which is statistically different from the others. To aid in the interpretation of these data and to allow an evaluation of the hypothesis regarding the shape of the dose response curve for mixtures assayed using the Ames test, the relative magnitude of mutagenic strength of the individual compound when assayed with that particular mutant strain, is indicated using the symbols, + or -, used in Table 6.

Figures 9 through 12 display the outcomes that of these experiments. The dose response curve that results when two mutagens are assayed together was found to be one of the following: 1) indistinguishable from the dose-response curves of either of the individual components, which are themselves indistinguishable (a,a,a), 2) distinguishable from the dose response curve of one of the individual components but indistinguishable from the other (a,b,a), or 3) distinguishable from the dose response curves of both of the individual components (a,b,c). The experimental outcomes corresponding to the first case (four pairs: #1, TA 1537; #4, TA 98; #5, TA 98; and #9, TA 98) are not helpful in testing the hypothesis (H1) because the fact that the response curves of the individual components are not statistically different negates the initial premise that the mutagens had unique dose response curves. However, these data serve to reject the hypothesis of an additive response (H2) because the response to the mixture is not statistically different from either component

Table 8. Ames testing of solutions of two mutagens.*

Pair No.	Paired Compounds	TA Strain 1537	98	100
1	Benzo(ghi) perylene, BP	a†+++**		
	Dimethylbenz(a) anthracene, DMBA	a +++		
	BP/DMBA	a		
2	Benzo(a)pyrene, BaP	a ++++	a +++	
	Perylene, P	b +++++	b (+)	
	BaP/P	a	a	
3	Benzo(a)pyrene, BaP	a ++++	a +++	a ++
	Dimethylbenz(a) anthracene, DMBA	b +++	b (+)	b +
	BaP/DMBA	a	a	a
4	Benz(a)anthracene, BA		a (+)	a +
	Dimethylbenz(a) anthracene, DMBA		a (+)	b +
	BA/DMBA		a	c
5	Benz(a)anthracene, BA	a ++	a (+)	
	Fluoranthene, F	b +	a (+)	
	BA/F	a,b	a	
6	Dimethylbenz(a) anthracene, DMBA	a +++	a (+)	
	Perylene, P	b ++++	b (+)	
	DMBA/P	c	a,b	
7	Benzo(ghi) perylene, BP	a +++		
	Perylene, P	b ++++		
	BP/P	c		
8	Fluoranthene, F		a (+)	
	Perylene, P		b (+)	
	F/P		a	
9	Dimethylbenz(a) anthracene, DMBA		a (+)	a +
	Fluoranthene, F		a (+)	b +
	DMBA/F		a	a
10	Benzo(a)pyrene, BaP		a +++	a ++
	Fluoranthene, F		b +	b +
	BaP/F		a	c

*Results of plate incorporation assays with rat liver homogenate (S-9).

†Small case letters (a,b,c), refer to the results of chi-square analysis which was used to test whether the dose response curves of the set were distinguishable (different letters) or not (same letters) at $\alpha \leq 0.01$.

**Symbols are used to indicate relative mutagenic strength, refer to Table 6.

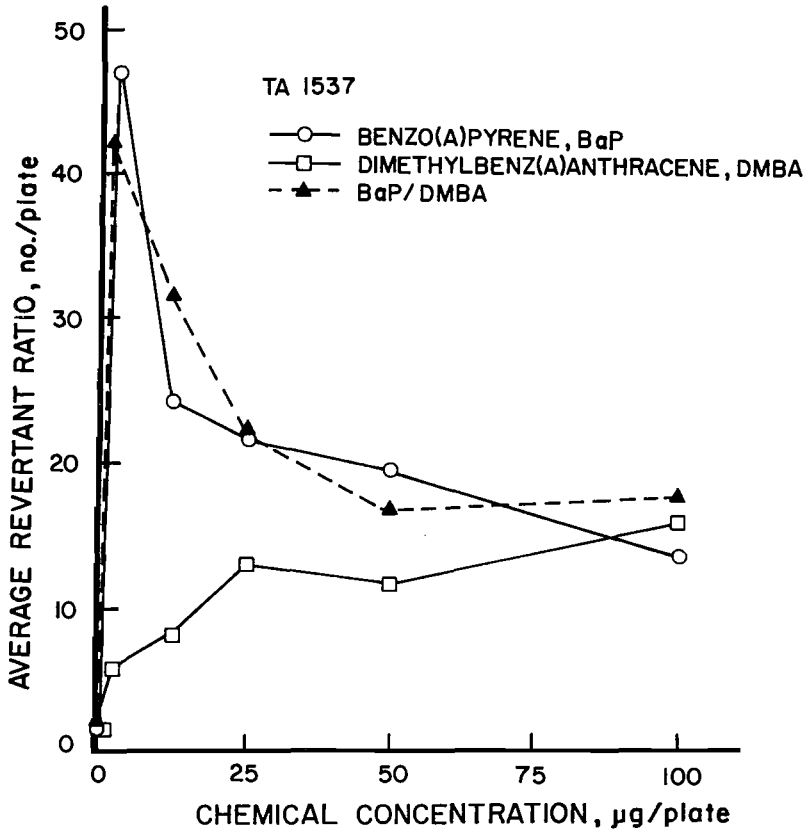

Figure 9. Dose response curves for the mutant Salmonella strain TA 1537 to benzo(a)-pyrene, dimethylbenz(a)anthracene and their one-to-one, by weight mixture. Each point represents the average of two experiments each of which had at least two replicates. The revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

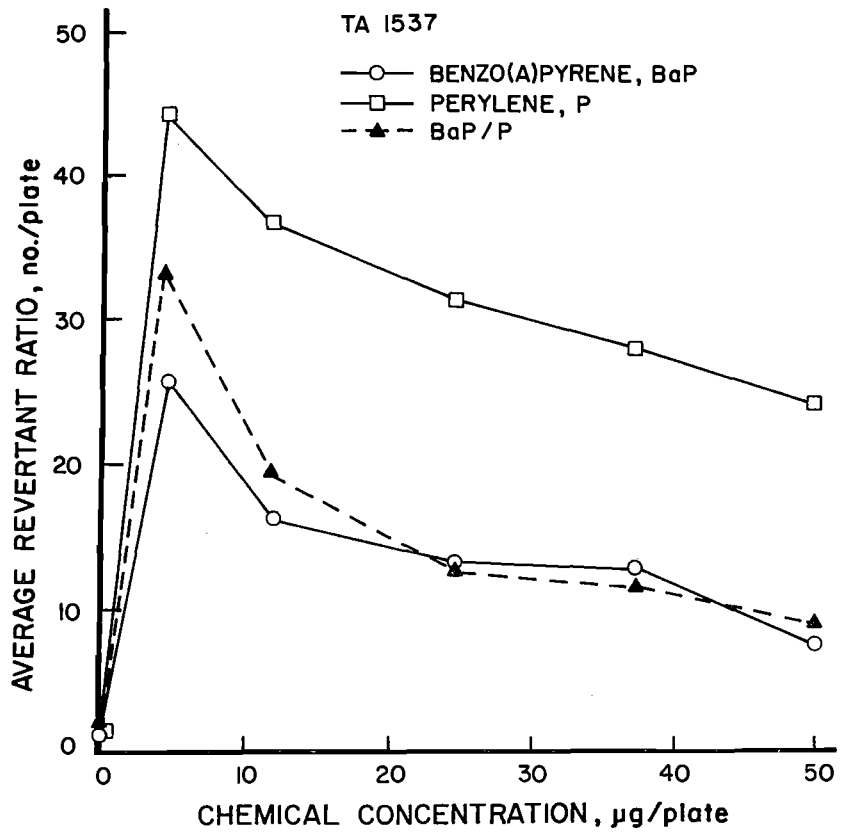

Figure 10. Dose response curves for the mutant Salmonella strain TA 1537 to benzo(a)-pyrene, perylene and their one-to-one, by weight mixture. Each dose of the mixture contains that quantity of both components. Each point represents the average of two experiments each of which had at least two replicates. The revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

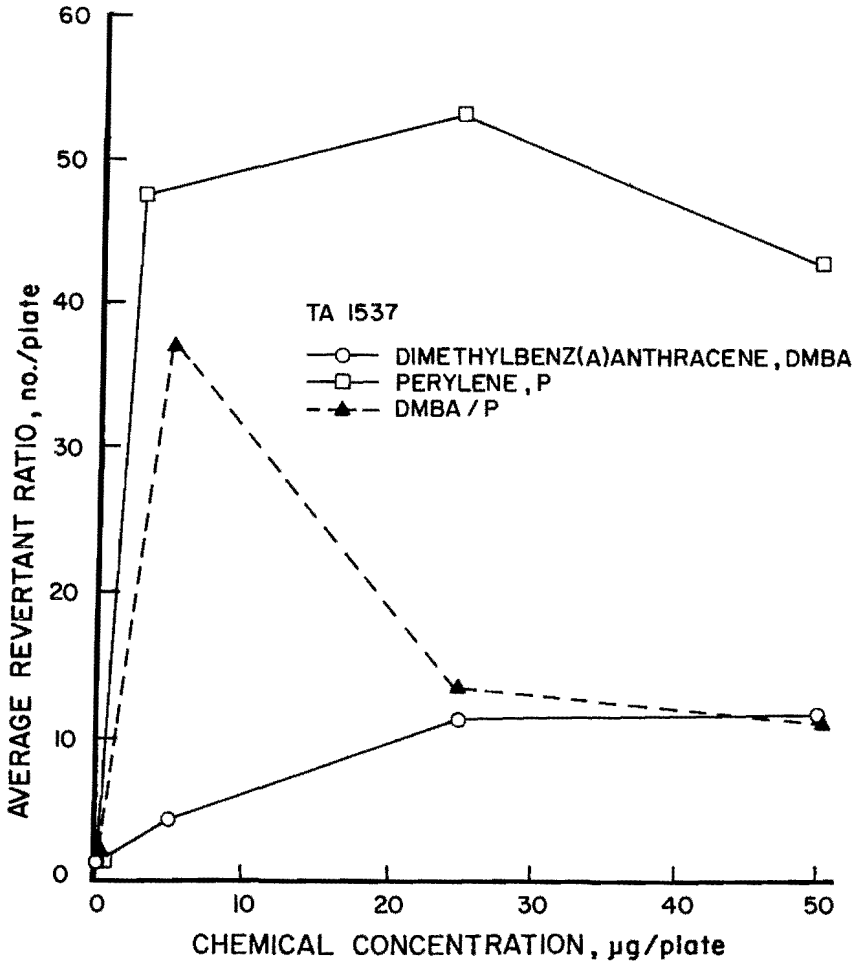

Figure 11. Dose response curves for the mutant Salmonella strain TA 1537 to dimethylbenz(a)anthracene, perylene and their one-to-one, by weight mixture. Each does of the mixture contains that quantity of both components. Each point represents the average of four replicates in one experiment. The revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

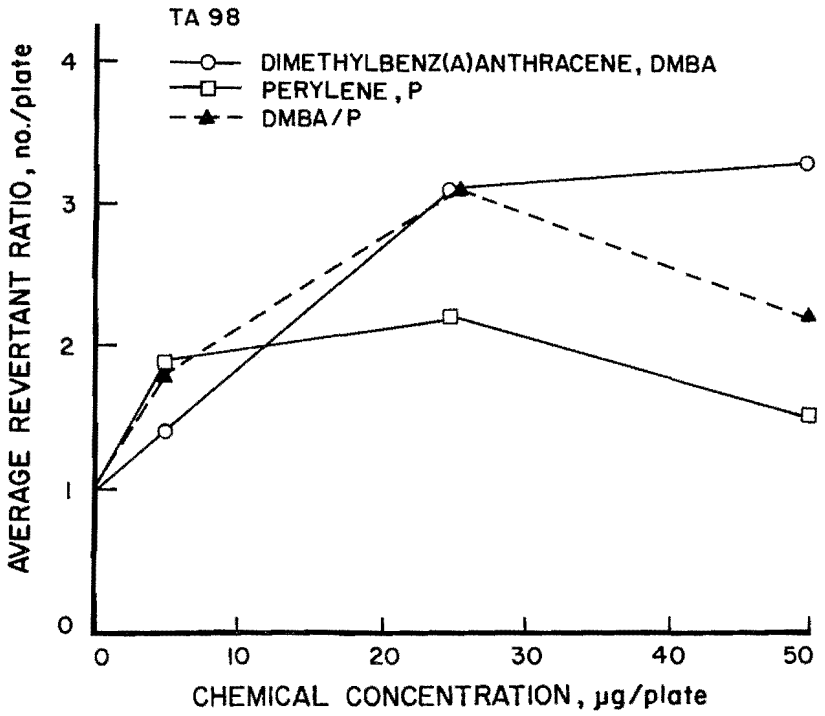

Figure 12. Dose response curves for the mutant Salmonella strain TA 98 to dimethylbenz(a)-anthracene, perylene and their one-to-one, by weight mixture. Each dose of the mixture contains that quantity of both components. Each point represents the average of four replicates in one experiment. The revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

alone and therefore does not reflect the presence of the two mutagens. The outcome corresponding to the second case (eight pairs: #2, TA 1537 and 98; #3, TA 1537, 98, and 100; #8, TA 98; #9, TA 100, and #10, TA 98) again is a rejection of the hypothesis (H2) because the dose-response curve for the mixture does not agree with the response that would be expected if the Ames test could integrate the total mutagens response of the two mutagens present. In fact, not only is the response to the mixture non-additive, it is shown to be statistically indistinguishable from one of the two mutagens in the mixture. As a likely explanation, an interaction exists whereby one chemical effectively masks detection of the other. Generally, the component which "dominates" in the mixture is the mutagen which elicits the mutagenic response of greater magnitude (Figure 9). However, certain compounds (BaP, in particular) appear to dominate in a mixture even when they are the "weaker" of the two mutagens (Figure 10).

The experimental outcomes of the third case (six pairs: #4, TA 100; #5, TA 1537; #6, TA 1537 and 98; #7, TA 1537; #10, TA 100), the results are of two types. While the three-way chi-square analyses indicated that the three dose response curves (X, Y, and XY) were all different when analyzed together, the results of the two-way chi-square analyses (X with XY and Y with XY) did not always substantiate this finding. For the majority of the pairs (4 of 6), the response curve, XY, was shown to be distinguishable from both of the other two curves when paired for analyses with it (Figure 11). These data support the hypothesis (H1) that the response to the mixture is different from the response to either of the components separately. For the other two pairs, the dose response curve of the mixture when paired for chi-square analysis with each curve from the individual components, could not be distinguished (Figure 12) though the three-way chi-square analysis showed the curves for the separate components were distinguishable. Based on an inspection of the data, this distinction of types would appear to be due to the magnitude and shape of the dose response curves of the individual components. The first type of response (a, b, c) occurs when the response varies greatly in magnitude and curve shape. The second type (a, b, ab) occurs when the two component curves are similar in magnitude and the curve of the mixture, being intermediate between the two, is statistically indistinguishable from them because the difference between the actual response and the expected response generated in the chi-square analysis is small. The results from all six pairs provide further information in rejection of the hypothesized additive response (H2) to the mixture.

To further evaluate the phenomenon of dominant interaction which was observed in more than half (8 of 14) of the experiments (the case 1 outcomes were disregarded because one cannot statistically support or reject the existence of a dominance interaction when the dose-response curves of the individual components are not distinct), mutagens and non-mutagens combined in a one-to-one (by weight) solution were assayed. Given the previous behavior of the mutagenic response to the two-mutagen mixture, it was hypothesized that the mutagen would dominate over the non-mutagen in the mixture in the same way.

The results of nine experiments utilizing six pairs of compounds are shown in Table 9. The manner in which the data are presented follows that used in Table 8. Two types of outcomes were identified by chi-square analysis. These were: 1) The case in which all three curves were indistinguishable within random experimental error (a,a,a), and 2) The case in which the mutagen clearly dominated the response to the mixture (a,b, a). The results of the experiments were nearly evenly distributed between these categories. If the first type of response is disregarded based on the fact that one cannot

Table 9. Results of Ames testing of solutions of mutagen/nonmutagen pair.*

	TA Strain		
	1537	98	100
Benzo(ghi)perylene, BP		a†-**	
Dimethylbenz(a)anthracene, DMBA		a (+)	
BP/DMBA		a	
Fluoranthene, F	a +	a +	a +
Anthracene, A	a -	b -	b -
F/A	a	a	a
Benzo(a)pyrene, BaP			a ++
Perylene, P			b -
BaP/P			a
Anthracene, A	a -	a -	
Perylene, P	b ++++	a (+)	
A/P	b	a	
Dibenz(ah)anthracene, DBA		a (+)	
Fluoranthene, F		b -	
DBA/F		b	
Dimethylbenz(a)anthracene, DMBA			a +
Perylene, P			a -
DMBA/P			a

*Results of plate incorporation assays with rat liver homogenate (S-9).

†Small case letters (a,b,c), refer to the results of chi-square analysis which was used to test whether the dose response curves of the set were distinguishable (different letters) or not (same letters) at $\alpha \leq 0.01$.

**Symbols are used to indicate relative mutagenic strength; refer to Table 6.

statistically distinguish between the curves of the individual components and therefore cannot test whether one compound dominates over the other, all of the data remaining support the hypotheses of dominance by the mutagen in a mutagen/non-mutagen mixture.

The Ames test assays of solutions pairing mutagens with mutagens and mutagens with non-mutagens in a one-to-one (by weight) ratio has demonstrated a dominance interaction in which the response to the mixture is indistinguishable from that of one of the two components (generally the stronger mutagen). With environmental samples, however, the one-to-one composition is doubtful. What ratio of dominant to subordinant component is necessary to maintain this interaction? It was hypothesized that the domination of the stronger mutagen would be insignificant when it composed 10 percent of the mixture. The paired compounds chosen to test this hypothesis were those that demonstrated a clearly defined dominance interaction. These pairs were BaP/DMBA (TA 100, 98) and BaP/P (TA 1537). One component (X) of the pair was assayed at three concentrations (0.5, 5, 50 µg/plate) while the other (Y) was kept constant (5 µg/plate). Then the components were switched and the process was repeated (a dose-response curve for Y was determined while X was kept constant). The results of a chi-square analysis for interaction for the three sets of dose-response curves are shown in Table 10. This analysis indicates that in two of the three experiments (BaP/DMBA, TA 98 and 100), the dose response curve for the stronger mutagen (BaP), was indistinguishable from that of the stronger mutagen plus a constant quantity of the weaker mutagen (Figures 13 and 14). The effect of the dominant mutagen on the dose response curve of the lesser mutagen was to make it differ more from the dose response curve of that component alone as the ratio of stronger mutagen:weaker mutagen was increased. The difference between the curves at the highest concentration tested does not support the hypothesis that the dominance interaction by the stronger mutagen would be insignificant when it composed 10 percent of the assayed mixture.

Table 10. Results of Ames test assay of non one-to-one, two component mixture.*

Compound Pairs	TA Strain 1537	TA Strain 98	TA Strain 100
BaP		a† +++**	a ++
BaP w/const DMBA		a	a
DMBA		b (+)	b +
DMBA w/const BaP		c	c
BaP	a ++++		
BaP w/const P	b		
P	c +++++		
P w/const BaP	a		

*Results of plate incorporation assays with rat liver homogenate (S-9).

†Small case letters (a,b,c), refer to the results of chi-square analysis which was used to test whether the dose response curves of the set were distinguishable (different letters) or not (same letters) at $\alpha \leq 0.01$.

**Symbols are used to indicate relative mutagenic strength, refer to Table 6.

The results of the BaP/P pair were more consistent with the stated hypothesis. In this pair, despite the fact that P is the stronger mutagen, BaP is the dominant in a one-to-one solution. This is seen in Figure 15 for solutions in which BaP composes 50 percent or more of the total solution. However, when BaP is present as 10 percent of the solution, it no longer dominates the response. When P is assayed with a constant amount of BaP, the dominance interaction by BaP recurs (Figure 16). When BaP composes 10 percent (25 µg BaP:125 µg P) of the solution, the response to the mixture is similar to that of BaP alone. These data are contradictory, in one case (P with BaP constant, 25 µg) BaP dominates when it is 10 percent, 50 percent, or 90 percent of the solution, in the other case (BaP with P constant, 25 µg) BaP dominates when it is 50 percent or 90 percent of the solution but not when it is only 10 percent (5 µg) of the mixture. It may, therefore, not be the composition in terms of the weight ratio of component X to component Y that is important in determining which compound is detected at the expense of the other, but the absolute concentration of each. This hypothesis appears to be supported by the results shown in Figures 11 and 12. At low concentrations of BaP the response to the mixture is intermediate to that of the components, however, at a theshold concentration (12.5 - 25 µg BaP/plate when paired with P (Figure 10) and > 25 µg P/plate when paired with DMBA (Figure 11)) the dominance interaction is in effect.

Solvent Effect

Four solvents are recommended for use in the Ames test mutagenicity screening. These solvents are dimethylsulfoxide (DMSO), ethanol, methanol, and p-dioxane (Ames et al. 1973). Since the PAH compounds chosen to assay as standards were all soluble in DMSO (solutions were 1 mg/ml with the exception of perylene which was 0.5 mg/ml), and because many investigators have used this solvent, it was assumed DMSO should also be used in the assays of known compounds. It was hypothesized that the choice of solvent would have no significant effect on the mutagenic response of the mutant bacterial strains. Then, because the spent shale extracts were being obtained in more polar solvents, methanol in particular, it was necessary to investigate the effect of the solvent and re-assay the componds, if possible, in methanol to determine whether the choice of solvent made a difference.

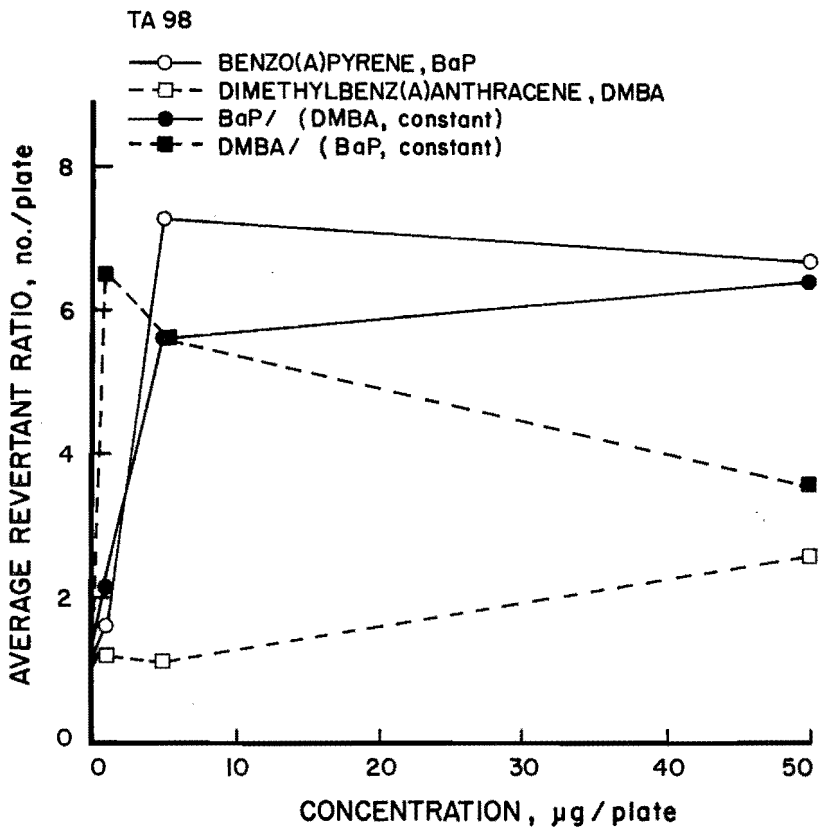

Figure 13. Dose response curves for the mutant Salmonella strain TA 98 to benzo(a)pyrene, dimethylbenz(a)anthracene and two non-one-to-one mixtures of these components. Each dose of the mixtures contains that quantity of the first component listed plus 5 µg of the second component listed. Each point represents the average of four replicates from one experiment. The revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

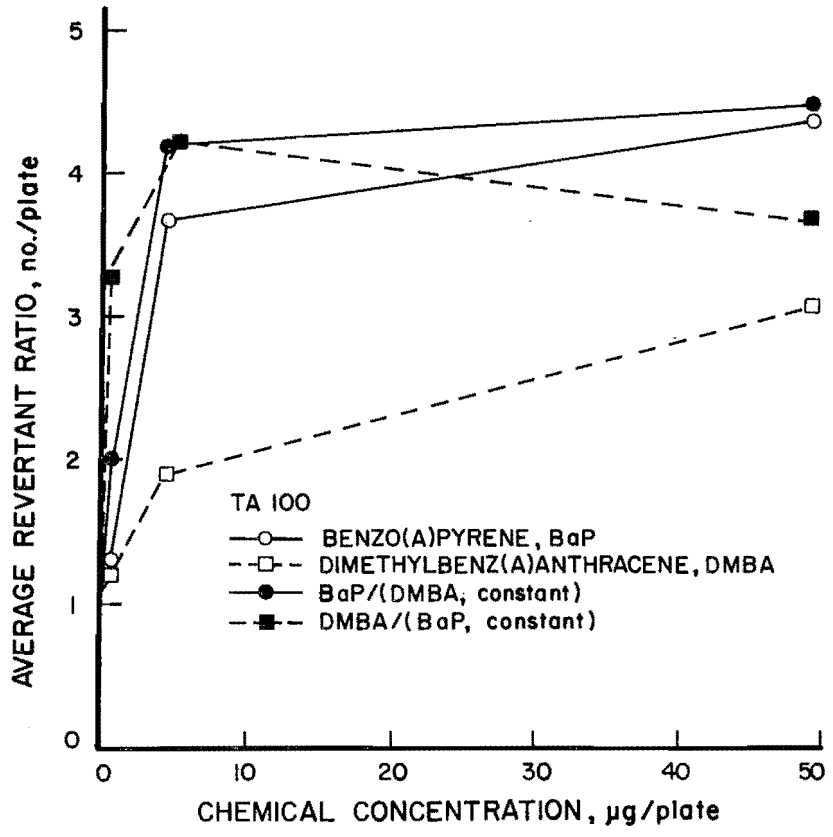

Figure 14. Dose response curves for the mutant Salmonella strain TA 100 to benzo(a)pyrene, dimethylbenz(a)anthracene and two non-one-to-one mixtures of these components. Each dose of the mixtures contains that quantity of the first component listed plus 5 µg of the second component listed. Each point represents the average of four replicates from one experiment. The revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

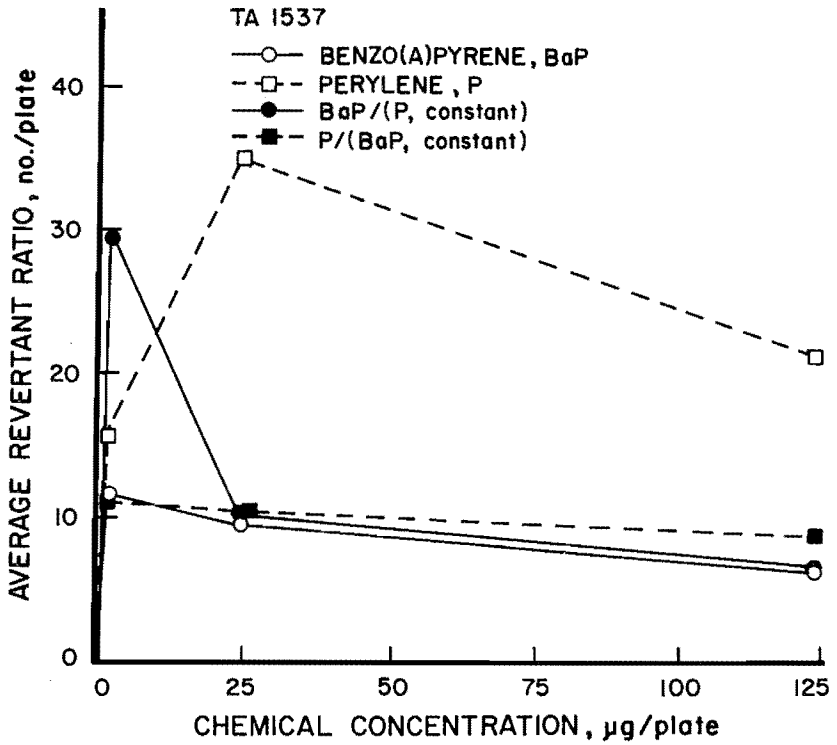

Figure 15. Dose response curves for the mutant Salmonella strain TA 1537 to benzo(a)-pyrene, perylene and two non-one-to-one mixtures of these components. Each dose of the mixtures contains that quantity of the first component listed plus 5 µg of the second component listed. Each point represents the average of four replicates from one experiment. The revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

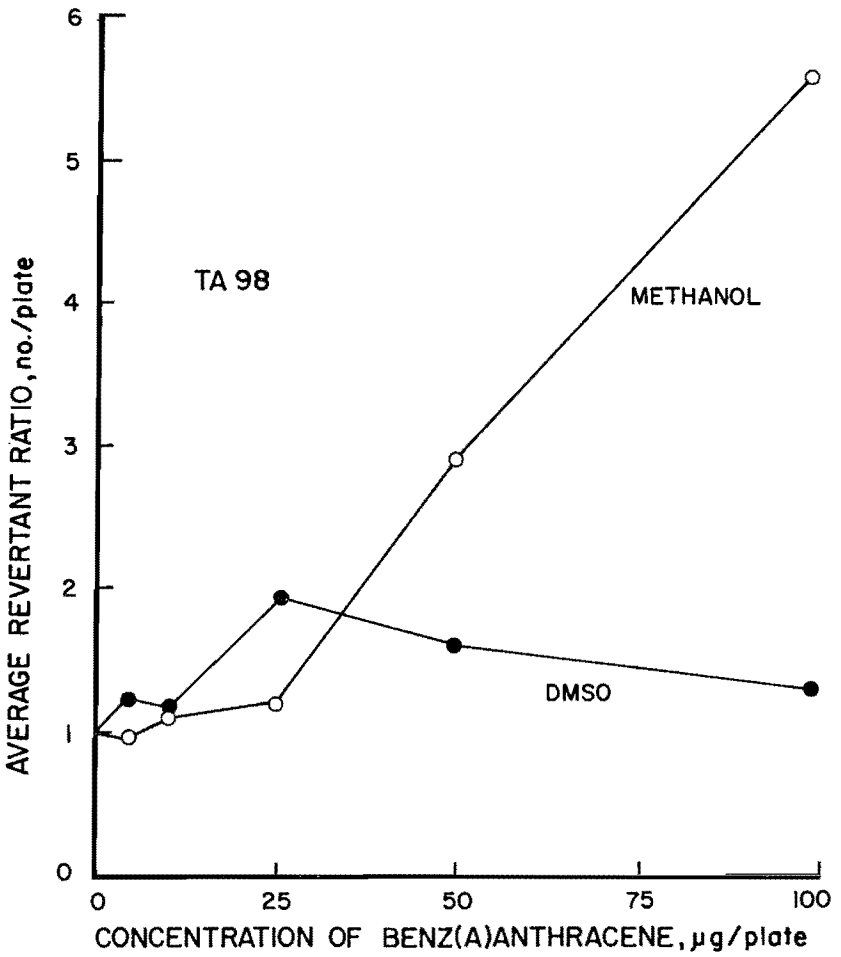

Figure 16. Dose response curves for the mutant Salmonella strain TA 98 to benz(a)anthracene dissolved in two solvents, methanol and DMSO. Each point represents the average of two experiments each of which had at least three replicates. Revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

An attempt was made to dissolve 11 of the 18 compounds used as known PAH (BA, A, BaP, DBA, DMBA, F, BP, P, carbazole, pyrene and phenanthrene) in methanol for Ames testing. Three (BA, DMBA, and F) of these were sufficiently soluble for use in the assay. The resulting dose-response curves were subject to chi-square analysis to test the hypothesis that solvent does not change the response to mutagens in solution. A summary of this analysis is shown in Table 11. It can be seen that in the majority of the experiments (6/8) the solvent did have a significant effect ($\alpha \leq 0.01$) on altering the response of the bacterial strains to mutagens. For the mutagen, BA, methanol had an effect of increasing the magnitude of the mutagenic response above that for the mutagen

Table 11. Effect of solvent on mutagenic response.*

	TA Strain		
	1537	98	100
Fluoranthene in:			
Dimethylsulfoxide		a†	a
Methanol		b	b
Dimethylbenz(a)anthracene:			
Dimethylsulfoxide	a	a	a
Methanol	a	a	b
Benz(a)anthracene:			
Dimethylsulfoxide	a	a	a
Methanol	b	b	b

*Results of plate incorporation assays with rat liver homogenate (S-9).

†Lower case letters (a,b) refer to the results of chi-square analysis which was used to test whether the dose response curves of the pair were distinguishable (different letters) or not (same letters) at $\alpha \leq 0.01$.

dissolved in DMSO (Figures 16 and 17). With fluoranthene, however, methanol as a solvent had an effect on increasing the sensitivity of the assay (i.e. greater magnitude of response at small concentrations of F), but at high concentrations of F dissolved in methanol, the solution was toxic to the bacteria. This result led to the testing of F dissolved in the two other recommended solvents, p-dioxane and ethanol. The results of all of these experiments are shown in Figures 18 and 19. The data presented here differ depending on strain used. The chi-square analyses for interaction indicates that the mutagenic response of TA 98 to F in DMSO, methanol, or p-dioxane are indistinguishable within random experimental error. Methanol, on the other hand, has a pronounced effect on the response at high concentrations (Figure 18). The dose response curves of TA 100 to F in the four solvents (Figure 19) show that the choice of solvent can be important; all of these dose-response curves are distinctly different ($\alpha \leq 0.01$).

Solvent Effect on Dominance Interaction

The pronounced effect of the solvent, methanol, on the response of TA 98 to fluoranthene prompted an experiment to test the effect of solvent on the mutagenic response to a one-to-one mixture of mutagens. In DMSO, BaP clearly dominates the response of TA 98 to the mixture of BaP and F (Table 9). Would this dominance interaction by BaP be effective when assayed with F dissolved in methanol? The results of one experiment indicated that this dominance interaction may continue to exist, but appears to shift from dominance by BaP at low concentrations of both compounds (5 µg F: 5 µg BaP, per plate), to dominance by F at high concentrations (25 µg F: 25 µg BaP and 100 µg F:100 µg BaP, per plate) where the toxic effect of F is evident (Figure 20). The chi-square analysis of the five dose-response curves shown in the figure indicates that each curve, except for the pair BaP and BaP with F (in DMSO) is distinctly different.

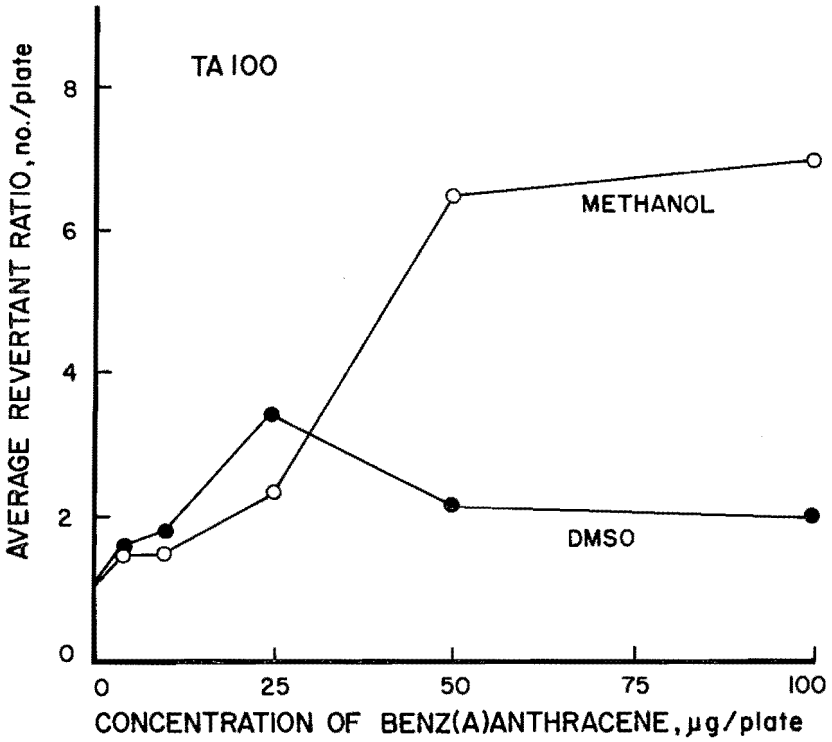

Figure 17. Dose response curves for the mutant Salmonella strain TA 100 to benz(a)anthracene dissolved in two solvents, methanol and DMSO. Each point represents the average of two experiments each of which had at least three replicates. Revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

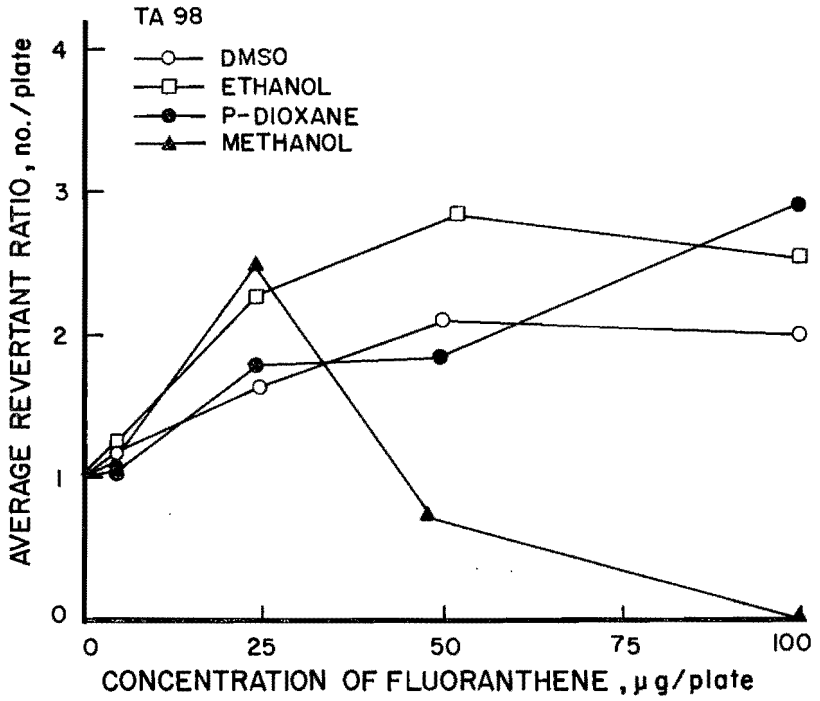

Figure 18. Dose response curves for the mutant Salmonella strain TA 98 to fluoranthene dissolved in four solvents, DMSO, ethanol, p-dioxane and methanol. Each point represents the average of four replicates in one experiment. Revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

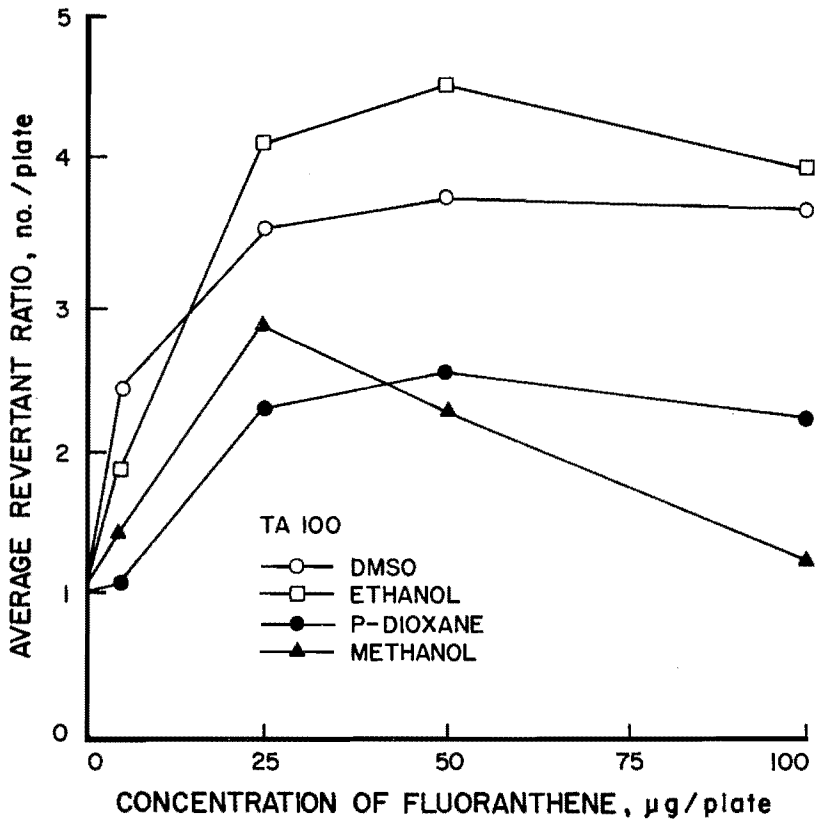

Figure 19. Dose response curves for the mutant Salmonella strain TA 100 to fluoranthene dissolved in four solvents, DMSO, ethanol, p-dioxane and methanol. Each point represents the average of four replicates in one experiment. Revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

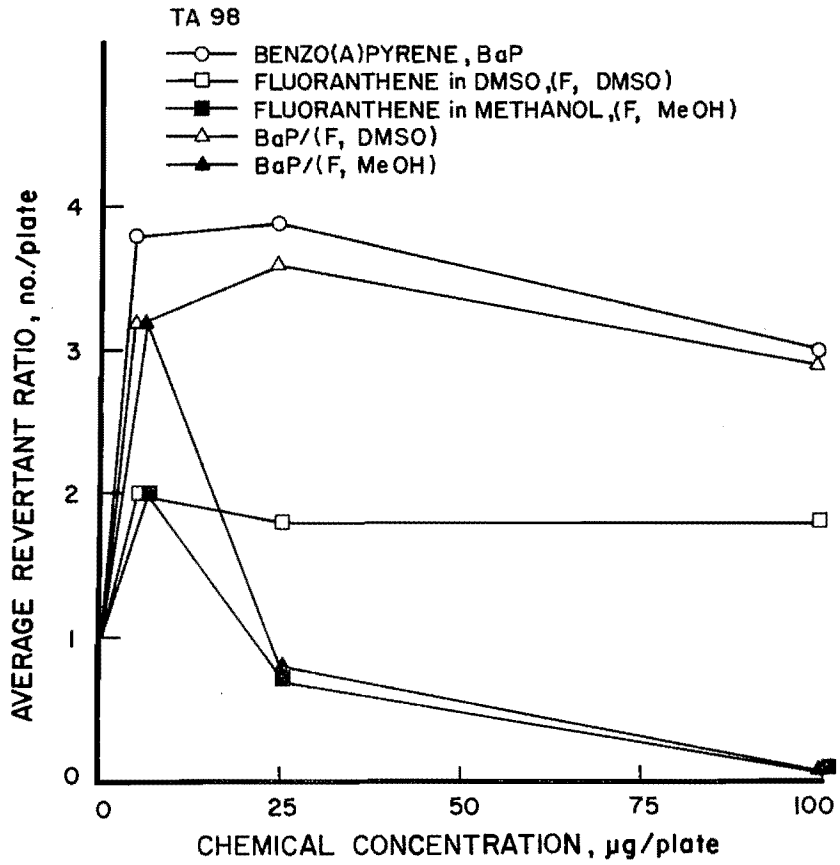

Figure 20. Dose response curves for the mutant Salmonella strain TA 98 to benzo(a)pyrene and fluoranthene (dissolved in DMSO) assayed separately and then together in a one-to-one, by weight mixture, and to benzo(a)pyrene and fluoranthene (dissolved in methanol) assayed separately and then together in a one-to-one, by weight mixture. BaP was always dissolved in DMSO. Each point represents an average of two experiments each having four replicates. Revertant ratio refers to the normalization of the data to the spontaneous reversion rate.

DISCUSSION

Much controversy exists concerning the proper use of short term mutagenesis assays in screening environmental samples for potential carcinogens. Therefore this study made a special effort to characterize one particular assay, the Ames test, in terms of its strengths and limitations regarding its application to extracts from spent oil shale and petroleum-related effluents in general.

Several PAH compounds were obtained for Ames testing initially to determine whether these procedures were correct and later to validate the assay. The consistency with which the magnitude and shape of the dose response curves could be reproduced using this assay, allowing a unique characterization of each known compound, indicated that the procedures were reliable. Generally, when comparisons could be made, the data correlated well with those reported in the literature (McCann et al. 1975; Kraybill 1979; Belser, personal communication). For some of the stronger mutagens (1-aminopyrene, benz(a)anthracene and benzo(a)pyrene) our data were conservative (Table 12), whereas for the weaker mutagens the data agree. The discrepancy could be due to differences in the viability of the bacterial strains or the liver microsomal fraction or in the failure to assay the concentration given the maximum response, all of which affect the magnitude of the mutagenic activity observed. It is also possible that the spontaneous reversion rate determined in these experiments was different from that used to calculate the R.R. in Table 12. (As noted previously, the spontaneous reversion rate is increased by the presence of the liver homogenate and therefore the control used should be the one with S-9 added and no chemical.)

Unfortunately, the three data sets (Tables 5, 12, and 13) do not overlap enough to allow more comparisons. More importantly, while the magnitude of response may vary, this has little effect on predicting the carcinogenicity of strong mutagens. The difficulty lies in distinguishing weak mutagens (and therefore likely weak carcinogens) from non-mutagens. This problem is seen in the two different categorizations (both weak mutagen and non-mutagen) for benzo(e)pyrene and pyrene (Tables 12 and 13).

The Ames test was used with mixed success in detecting mutagens in extracts from spent oil shale. The best results were

Table 12. Ames test results of selected polycyclic aromatic hydrocarbons.[a]

Compound Name	Maximum Revertant Ratio[b]	Relative Mutagenic Strength[c]
1-amino pyrene	119 (TA 98)	++++
Anthracene	<1 (TA 100,98,1537,1538)	-
Benz(a)anthracene	16.5 (TA 100)	+++
Benzo(a)pyrene	15.0 (TA 100)	+++
Benzo(e)pyrene	<1 (TA 100)	-
Dibenz(a,h)anthracene	2.5 (TA 100)	+
7,12 Dimethylbenz-(a)anthracene	9.1 (TA 100)	+++
Phenanthrene	<1 (TA 100,98,1537,1538)	-
Pyrene	<1 (TA 100,98,1537,1538)	-

[a] From McCann et al. (1975).

[b] Revertant Ratio based on spontaneous values reported in Ames et al. (1975); TA 100 (160); TA 98 (45). The strain or strains indicated in parentheses are those for which the response (revertant ratio) is reported.

[c] Symbols: - inactive; + weak, ++ moderate, +++ strong, ++++ very strong, and +++++ extremely strong mutagenic activity. All compounds which were mutagenic, required mammalian activation.

Table 13. Mutagenicity of selected polycyclic aromatic hydrocarbons found in freshwater based on Ames test.[a]

Compound Name	Salmonella TA Strain[b]
I. Known Mutagens	
Benz(a)anthracene	TA 98, 100 (TA 1537, 1538)
Benzo(a)pyrene	TA 98, 100, 1537, 1538
Benzo(e)pyrene	TA 100
Chrysene	TA 98, 100
Dibenz(a,h)pyrene	TA 98, 100, 1537 (TA 1538)
II. Suspected Mutagens	
Benzo(ghi)perylene	TA 98
Pyrene	TA 100

[a] From Kraybill et al. (1978). All compounds that were mutagenic required mammalian activation.

[b] Strains in parentheses gave a weakly positive mutagenic response.

obtained when soxhlet extraction was performed using organic solvents. The first few samples obtained in methanol following benzene extraction (Table 7, Paraho #1-4; Tosco #1,2) were shown to produce dose response curves similar to those obtained for the known standards. Then, in order to get a more detailed description of the chemical composition of these extracts and the mutagenically-active fractions, separation techniques were employed. For the most part, these procedures did not enable further characterization of the mutagenic potency of these extracts. Dilution and solvent incompatibility appeared to be major problems. Subsequently, it was necessary to determine whether the mutagenic response to the complex mixtures being obtained prior to separation and purification was indicative of the total response contributed by each mutagenic component.

The results of experiments using two component mixtures indicated that the response of the bacterial strains to two mutagens assayed together was non-additive. In the majority of cases, the response to the mixture was indistinguishable from the response to one of the components assayed separately (masking of one component in the presence of another, more dominant compound). In a few instances, the response to the mixture was intermediate to that of the two separate components. These observations conflict with other reported results. Epler and coworkers (1979) reported that the mutagenic activity of the neutral fraction of two different oils could be totally accounted for by the simple sum of the mutagenic activities of the four subfractions of the neutral fraction. This confirmation allowed comparison of the relative mutagenic strengths of these two oils by summing the activities from 14 fractions weighted according to the contribution each makes toward the total sample. The phenomenon of co-mutagenesis (i.e., the enhancement of the mutagenicity of one compound by the addition of another compound which may or may not be a mutagen), has also been reported with the Ames test (Sugimura 1977). The data presented in this report indicate that the mutagenic potential of a mixed sample may be underestimated.

While the spent shale extracts obtained using soxhlet extraction and organic solvents were assayed with fairly good success, with results indicating the potential for groundwater pollution from mutagens present in spent shale, the assays of aqueous leachates did not support the observation by others (Schmidt-Collerus 1974) that they could be washed out of the shale. If mutagens were present in these leachates, they were either in very small concentrations or were lost during the procedures of extraction, purification, and handling. Neither the standard Ames plate incorporation assay nor the two alternate methods for assaying aqueous samples indicated a sample having positive mutagenic activity. Another obstacle encountered was that one of the solvents, methylene chloride, which is recommended for use in sample separation, is a mutagen to the bacterial strains, thus preventing many fractions from being assayed.

In conclusion, it can be said that mutagens, many of which are certainly carcinogens are present or at least can be formed from precursors in processed shale and that they can be extracted in organic solvents through soxhlet extraction. The failure to obtain positive evidence of mutagens in the aqueous leachates does not negate the possibility that they were extracted. Due to limitations in the quantity of spent shale with which to experiment and in the procedures used to concentrate and prepare the extracts for testing, the compounds may have been present but in concentrations below the sensitivity level of the assay. This speculation is corroborated by the GC/MS data (Maase and Adams 1980), indicating that many of the PAH previously determined to be mutagenic are present in the aqueous extracts at the limit of their solubility (ng/l). It is likely, given this information, that further biological testing of concentrated leachates from processed shale would successfully demonstrate that the disposal of spent oil shale can be a source of mutagens to the environment through leaching.

CONCLUSIONS AND RECOMMENDATIONS

The results of Ames mutagenicity testing of extracts from spent oil shale waste derived from three different processes (Paraho, Tosco, and Union), indicate that mutagens can be collected and/or formed from precursors through soxhlet extraction using organic solvents. The high rate of carcinogen prediction demonstrated by this assay supports the speculation that carcinogenically-active compounds from spent shale can be expected to enter the environment. The Ames test on these wastes failed to show evidence that mutagens could be extracted in aqueous leachates from spent shale; however, due to limited quantities of processed shale available for testing and inadequate techniques to allow both concentration and biological testing of leachates, leaching of weathered spent shale should not be discounted on the basis of these results as a potential pathway for mutagens and/or carcinogens to become mobile in the environment.

The Ames test was found to be a sensitive and reliable indicator of potentially carcinogenic compounds in energy-related effluents as long as attention is given to understanding the limitations of this short term assay. In particular, the information obtained through the course of this study has identified at least two areas of caution. First, the response of the Ames test to environmental mixtures in which chemical interactions may occur is unpredictable. The presence of mutagens may be masked by the dominating influence of another mutagen. This non-additive response almost necessarily means the screening assay will underestimate the potential hazard to humans. This problem can possibly be avoided by using a sophisticated fractionation scheme prior to testing. Second, the appropriate choice of solvents is essential for minimizing toxicity, chemical interactions, and excess mutagenicity, to the tester strain.

Although the Ames test is highly recommended as a screening procedure for identifying sources of environmental carcinogens, its findings should always be confirmed by one or more short term assay to identify any false positive or false negative samples prior to in vitro and in vivo mammalian testing.

LITERATURE CITED

Ames, B. N., W. E. Durston, E. Yamasaki, and F. D. Lee. 1973. Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc. Natl. Acad. Sci. USA 70(8):2281-2285.

Ames, B. N., H. O. Kammen, and E. Yamasaki. 1975. Hair dyes are mutagenic: Identification of a variety of mutagenic ingredients. Proc. Natl. Acad. Sci. USA 72(6):2423-2427.

Andelman, J. B., and M. J. Suess. 1970. Polynuclear aromatic hydrocarbons in the water environment. J. Bull. WHO 43:479-508.

Ander, P., K.-E. Eriksson, M.-C. Kolar, K. Kringstad, and U. Rannug. 1977. Studies on the mutagenic properties of bleaching effluent. Svensk Papperstidning 80(14):454-459.

Atlas, R. M., and R. Bartha. 1972. Biodegradation of petroleum in seawater at low temperatures. Can. J. Micro. 18:18511855.

Atwood, M. T., and R. M. Coomes. 1974. The question of carcinogenicity in intermediates and products in oil shale operations. Unpublished Colony Development Operation Paper. The Oil Shale Corporation, Rocky Flats Research Center.

Badger, G. M. 1962. The chemical basis of carcinogenic activity. Charles C. Thomas, Springfield, Ill. 72 p.

Bailey, E. J., and N. Dungal. 1958. Polycyclic aromatic hydrocarbons in Icelandic smoked food. Brit. J. Cancer 12:348-350.

Barnes, W. S., and E. J. Klekowski, Jr. 1978. Testing the environment for dispersed mutagens: Use of plant bioconcentrators coupled with microbial mutagen assays. Environmental Health Perspective 27:61-67.

Barnsley, E. A. 1975. The bacterial degradation of fluoranthene and benzo(a)-pyrene. Can. J. Micro. 21:1004-1008.

Bartsch, H. 1976. Predictive value of mutagenicity tests in chemical carcinogenesis. Mutat. Res. 38:177-190.

Berenblum, I. 1941. The cocarcinogenic action of croton resin. Can. Res. 1:44-47.

Blum, A., and B. N. Ames. 1977. Flame-retardant additives as possible cancer hazards. Science 195:17-23.

Blummage, K. G. 1975. The sources of oil entering the sea. In: Background papers for a workshop on inputs, fates, and effects of petroleum in the marine environment. Nat. Acad. Sci., Washington, D.C. pp. 1-6.

Borneff, J. 1963. Carcinogenic substances in water. Munch. Med. Wschr. 105:1237-1242. (German) Cited in Andelman and Suess, 1970.

Borneff, J. 1969. The removal of carcinogenic polycyclic aromatic hydrocarbons during water treatment. Gas-u. Wass Fach 110:29-34. (German) Cited in Andelman and Suess, 1970.

Borneff, J. and R. Fischer. 1963. Carcinogenic substances in water and soil. XII Polycyclic aromatic hydrocarbons in surface water. Arch. Hyg. Bakterial 146:572-585. (German) Chem. Abst 59:5604d.

Borneff, J. and R. Knerr. 1960. Carcinogenic substances in water and soil: III. Quantitative investigation of solublity, filtration, adsorption and penetration depth. Arch. Hyg. Bakteriol 144:81-94. Chem. Abst. 54:25413h.

Borneff, J. and H. Kunte. 1964. Carcinogenic substances in water and soil. Detection of polycyclic aromatic hydrocarbons in water samples by direct extraction. Arch. Hyg. 148:585. Chem Abst. 62:10958b.

Borneff, J. and H. Kunte. 1965. Carcinogenic substances in water and soil: XVII. On the origin and evaluation of polycyclic aromatic hydrocarbons in water. Arch. of Hyg. 149:227.

Bridges, B. A. 1974. The three-tier approach to mutagenicity screening and the concept of radiation equivalent dose. Mutat. Res. 26:335-340.

Bridges, B. A. 1976. Short term screening tests for carcinogens. Nature 261:195-200.

Buncher, C. R. 1975. Cincinnati drinking water--an epidemiologic study of cancer rates. University of Cincinnati Medical Center, Cincinnati, Ohio.

Burnham, A. K., G. V. Calder, J. S. Fritz, G. A. Junk, H. J. Svec and R. Willis. 1972. Identification and estimation of neutral organic contaminants in potable water. Anal. Chem. 44(1):139-142.

Chrisp, C. E., G. L. Fisher, and J. E. Lammert. 1978. Mutagenicity of filtrates from respirable coal fly ash. Science: 199(4324):73-75.

Cleave, M. L. 1979. Effects of oil shale development on freshwater phytoplankton. PhD dissertation, Utah State University, Logan, Utah.

Commoner, B. 1976a. Reliability of bacterial mutagenesis techniques to distinguish carcinogenic and noncarcinogenic chemicals. Report to United States Environmental Protection Agnecy, Office of Research and Development, Washington, D.C. April.

Commoner, B. 1976b. Tests of the reliability with which the bacterial mutagenesis technique can distinguish between carcinogenic and noncarcinogenic synthetic organic chemicals. Report to Environmental Protection Agency, Contr. #68-01-2471. May.

Commoner, B. 1977. Carcinogens in the environment. Chem-Technol. 7(2): 76-82.

Cook, G. B., and F. R. Watson. 1966. The geographic locations of Missourians with multiple cancer. Mo. Med. 32:997.

Coomes, R. M. 1976. Health effects of oil shale processing. Presented at the Ninth Oil Shale Symposium. In: Quarterly of the Colo. School of Mines 71(4): 101-123.

Corner, E.D.S., C. C. Kilvington, and S. C. M. O'Hara. 1973. Qualitative studies on the metabolism of naphthalene in Maia squinado (Herbst). J. Mar. Biol. Assn., UK, 53:81832.

Dassler, G. L. 1976. Assessment of possible carcinogenic hazards created in surrounding ecosystems by oil shale development. MS thesis, Utah State University, Logan. 99 p.

Drake, J. W., S. Abrahamson, J. F. Crow, A. Hollaender, S. Lederberg, M. S. Legator, J. V. Neel, M. W. Shaw, H. E. Sulton, R. C. von Borstel, and S. Zimmering. 1975. Environmental mutagenic hazards. Science 187(4176):503-514.

Dungal, N. 1961. The special problem of stomach cancer in Iceland. J. Amer. Med. Assn. 178:789-798.

Durston, W. E., and B. N. Ames. 1974. A simple method for the detection of mutagens in urine: Studies with the carcinogen 2-acetylaminofluorene. Proc. Natl. Acad. Sci. 71(3):737-741.

Ekwall, P., and L. Sjöblöm. 1952. Butyric acid and lactic acid in aqueous solutions as solubilizers for carcinogenic hydrocarbons. Acta. Chem. Scand. 6:96-100.

Environmental Protection Agency (EPA). 1972. Industrial pollution of the lower Mississippi River in Louisiana. EPA, Dallas, Texas.

Epler, J. L. 1979. Evaluation of mutagenicity testing of shale oil products and effluents. Environ. Health Perspect. 30:179-184.

Epler, J. L., F. W. Larimer, T. K. Rao, C. E. Nix, and T. Ho. 1978a. Energy-related pollutants in the environment: Use of short term tests for mutagenicity in the isolation and identification of biohazards. Environ. Health Perspect. 27:11-20.

Epler, J. L., J. A. Young, A. A. Hardigree, T. K. Rao, M. R. Guerin, I. B. Rubin, C.-H. Ho and B. R. Clark. 1978b. Analytical and biological analyses of test materials from the synthetic fuel technologies. I. Mutagenicity of crude oils determined by the Salmonella typhimurium/microsomal activation system. Mutat. Res. 57(3):265-276.

Garner, R. C., E. C. Miller, and J. A. Miller. 1972. Liver microsomal metabolism of aflatoxin B1 to a reactive derivative toxic to Salmonella typhimurium TA 1530. Can. Res. 32:2058-1066.

Gerarde, H. W. 1960. Toxicology and biochemistry of aromatic hydrocarbons. Amsterdam, Elservier Publ. Co.

Glatz, B. A., C. D. Chriswell, M. A. Arguello, H. J. Svec, J. S. Fritz, S. M. Grimm, and M. A. Thomson. 1978. Examination of drinking water for mutagenic activity. Jour. AWWA, 70(8): 465-468.

Gräf, W. and H. Diehl. 1966. About the natural background of carcinogenic PA and its cause. Arch. Hyg. (Berl) 153:49-59. (German).

Gräf, W. and G. Nothhafft. 1963. Drinking water chlorination and benzo(a)pyrene. Arch. Hyg. (Berl.) 147:13335-146. (German) Chem. Abstr. 1963:59:11102g.

Grant, E. L., R. H. Mitchell, P. R. West, L. Mazuch, and M. J. Ashwood-Smith. 1976. Mutagenicity and putative carcinogenicity tests of several polycyclic aromatic compounds associated with impurities of the insecticide methoxychlor. Mutat. Res. 40:225-228.

Hadeed, S. J. 1978. Early organic detection system established on the Ohio River. Jour. Water Poll. Contr. Fed. 50(3): 41418.

Harris, R. H. 1974. Implications of cancer-causing substances in Mississippi River water. Environmental Defense Fund, Washington, D.C.

Harrison, R. M., R. Perry, and R. A. Wellings. 1975. Polynuclear aromatic hydrocarbons in raw, potable and waste waters. Water Res. 9:331-346.

Higginson, J. 1972. Environment and cancer. R. L. Clark (ed.) p. 69, Williams and Wilkins, Baltimore.

Hites, R. A., K. Bieman. 1972. Water pollution: organic compounds in the Charles River, Boston. Science 178 (4057):158-160.

Hoffmann, D., I. Schmeltz, S. S. Hecht, and E. L. Wynder. 1976. On the identification of carcinogens, tumor promotors, and cocarcinogens in tabacco smoke. In: Smoking and Health, 1:125-145. DHEW Publ. No. (NIH) 72-1221.

Hoffmann, D., I. Schmeltz, S. S. Hecht, and E. L. Wynder. 1978. Polynuclear aromatic hydrocarbons in tobacco carcinogenesis. In: Polycyclic hydrocarbons in cancer: Chemistry, molecular biology and environment. Gilboin, H., and P. O. Tso, eds. Acad. Press, N.Y.

Hoffmann, D. and E. L. Wynder. 1962. A study of air pollution carcinogenesis. Part II: The Isolation and Identification of polycyclic aromatic hydrocarbons from gasoline exhaust condensate. Cancer 15:93-102. Chem. Abst. 57:1225b.

Hueper, W. C. 1960. Cancer hazards from natural and artificial water pollutants. In: Proceedings of a conference on Physiological Aspects of Water Quality, Wash., D.C., U.S. Public Health Service, pp. 181-193.

Hueper, W. C., and H. J. Cahnman. 1958. Carcinogenic bioassay of Benzo(a)pyrene-free fractions of American shale oils. Amer. Med. Assoc. Arch. of Path. 65:608-614.

Hueper, W. C., and W. D. Conway. 1964. Chemical carcinogenesis and cancers. Charles C. Thomas, Springfield, Illinois.

Hueper, W. C., and W. W. Payne. 1963. Carcinogenic effects of adsorbates of raw and finished water supplies. Amer. J. Clin. Pathol. 39:475.

Hutton, J. J., and C. Hackney. 1975. Metabolism of cigarette smoke condensates by human and rat homogenates to form mutagens detectable by Salmonella typhimurium TA 1538. Can. Res. 35: 82461-2468.

Ilnitsky, A. P., and S. N. Varshavskaya. 1964. Water as a factor in spreading carcinogens in the environment (Russian). English Translation Hyg. Sanit. p. 88-96.

Jobson, A., F. D. Cook, and D. W. S. Westlake. 1972. Microbial utilization of crude oil. Appl. Micro. 23(6):1082-1089.

Jobson, A., M. McLaughlin, F. D. Cook, and D. W. S. Westlake. 1974. Effects of amendments on the microbial utilization of oil applied to soil. Appl. Micro 27(1):16171.

Junk, G. A., and S. E. Stanley. 1975. Organics in drinking water. Part I: listing of identified compounds. 15:3671 NTIS. July.

Kier, L. D., E. Yamasaki, and B. N. Ames. 1974. Detection of mutagenic activity in cigarette smoke. Condensate. Proc. Natl. Acad. Sci. U.S.A. 71(10):4154163.

Kloeper, R. D., and B. J. Fairless. 1972. Characterization of organic components in a municipal water supply. Environ. Sci. & Tech. 6:1036-1037.

Knorr, M. and D. Schenk. 1968. Synthesis of polycyclic aromatic compounds by bacteria. Arch. Hyg. Bakteriol. 152(3):282-285 (German) Chem. Abst. 69:74645k.

Kraybill, H. F. 1969. Food contaminants and gastro-intestinal or liver neoplasia. Survey of Experimental Observations. Environ. Res. 2:231-246.

Kraybill, H. F. 1975. Origin, classification and distribution of chemicals in drinking water with an assessment of their carcinogenic potential. Proceedings of the Conference on Environmental Impact of Water Chlorination. Oak Ridge Natl. Lab., Oak Ridge, Tenn., Rept. Conf. 751096.

Kraybill, H. F. 1977. Global distribution of carcinogenic pollutants in water. Ann. N. Y. Acad. Sci. Conference on Aquatic Pollutants and Biological Effects with Emphasis on Neoplasia. Sept. 27-29, 1976.

Kraybill, H. F., C. T. Helms, and C. C. Sigman. 1978. Biomedical aspects

of biorefractories in water. Paper presented at the Second International Symposium on Aquatic Pollutants. Amsterdam, The Netherlands. Sept. 26-28, 1977. In: Aquatic Pollutants Transformation and Biological Effects. (Hutzinger, O., L. H. Van Leylveld and B. C. J. Zacteman, eds.) Pergamon Press. New York, New York. pp. 419-459.

Lawerenz, A. 1967. Demonstration of carcinogenic materials in water. Presented by two examples of water treatment procedures. Z. Ges. Hyg. 13:844-846. (German) Cited in Andelman and Suess, 1970.

Lee, R. F., R. Sauerheber, and G. H. Dobbs. 1972a. Uptake, metabolism and discharge of polycyclic aromatic hydrocarbons by marine fish. Mar. Biol. 17:201-208.

Lee, R. F., R. Sauerheber, and A. A. Benson. 1972. Petroleum hydrocarbons: Uptake and discharge by the marine mussel Mytillus edulis. Science 177:344-346.

Lee, R. F., C. Ryan, and M. L. Neuhauser. 1976. Fate of petroleum hydrocarbons taken up from food and water by the Blue Crab Callinectes sapidus. Mar. Biol. 37:363-370.

Legator, M. S. 1972. Chemical mutagens. Ann Rev. of Med. 23:413-428.

Lijinsky, W., and J. H. Quastel. 1956. Metabolism of carcinogenic hydrocarbons by soil microorganisms. Arch Biochem. Biophys. 63:16164.

Loper, J. C., D. R. Lang, R. S. Schoeny, B. B. Richmond, P. M. Gallaghe, and C. C. Smith. 1978a. Residue organic mixtures from drinking-water show in vitro mutagenic and transforming activity. J. Tox. Env. Health 4(5-6):919-938.

Loper, J. C., R. S. Schoeny, and R. G. Tardiff. 1978b. Evaluation of organic extracts of drinking-water by bacterial mutagenesis. Mut. Res. 53(2):223-224 (abstract).

Maase, D. L., and V. D. Adams. 1980. An evaluation of polycyclic aromatic hydrocarbons from processesd oil shale. Utah Water Research Laboratory. Logan, Utah.

MacKenzie, M. J., and J. V. Hunter. 1977. Sources and fates of aromatic compounds in urban stormwater runoff. Environ. Sci. and Tech. 13(2):17183.

McCann, J., and B. N. Ames. 1976. The Salmonella/microsome mutagenicity test: Predictive value for animal carcinogenicity. Origins of Human Cancer, Cold Spring Harbor Laboratory, N.Y. 11724. Proceedings of Conference, September 7-14.

McCann, J., N. B. Spingarn, J. Kobori, and B. N. Ames. 1975. Detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids. Proc. Natl. Acad. Sci., U.S.A. 72(3):979-983.

Meselson, M., and K. Russell. 1977. Comparisons of carcinogenic and mutagenic potency. In: Origins of human cancer Book Co. Human Risk Assessment. Hiatt, H. H., J. D. Watson, and J. A. Winsten, eds., Cold Spring Harbor Laboratory.

Metcalf, R. L. 1975. Evaluation of a laboratory microcosm for study of toxic substances in the environment. NSF RANN (ESR 74-22760).

Middleton, F. M., and A. A. Rosen. 1956. Organic contaminants affecting the quality of water. Public Health Rep. 71:1125.

Miller, E. C. and J. A. Miller. 1971. The mutagenicity of chemical carcinogens: Correlation, problems and interpretation. In: Chemical Mutagens: Principles and Methods for Their Dectection. Vol. 1. A Hollander, ed. Plenum Press, N.Y. pp. 83-119.

Miyaji, T. 1971a. Pathological study on toxicity of furylfuramide. I. Acute and chronic toxicity of furylfuramide in rats and mice. Tohoku J. Exp. Med. 103(4):331-369.

Miyaji, T. 1971b. Pathological study on toxicity of furylfuramide. III. Effect of furylfuramide on reproduction and malformation. Tohoku J. Exp. Med. 103(4):381-388.

Mottram, J. C. 1944. A developing factor in experimental blastogenesis. J. Path. Bact. 56:181-187.

Mueller, G. C., T. W. Kensler, and K. Kajiwara. 1978. Mechanism of DNA and chromatin replication: Possible targets for cocarcinogenesis. In: Carcinogenesis, Vol. 2. Mechanisms of turmor promotion and carcinogenesis. (Slaga, T. J., A. Sivah, and R. K. Boutwell, eds.) Raven Press, New York. pp. 79-90.

Nau, C. A., J. Neal, and V. A. Stembridge. 1962. Physiological effects of carbon black. III. Adsorption and elution potentials, subcutaneous injections. Arch. Environ. Health 1:512-533.

Neff, J. M., B. A. Cox, D. Dixit, and J. W. Anderson. 1976. Accumulation and release of petroleum derived AHC by marine animals. Mar. Biol. (Berl) 38(3):27289.

Page, T., R. H. Harris, and S. S. Epstein. 1976. Drinking water and cancer

mortality in Louisiana. Science 193 (4247):55-57.

Parry, J. M., D. J. Tweats, and M. A. J. Al-Mossawi. 1976. Monitoring the marine environment for mutagens. Nature 264:538-540.

Payne, W. W., and W. C. Hueper. 1960. The carcinogenic effects of single and repeated doses of benzo(a)pyrene. Amer. Industr. Hyg. Ass. J. 21:35355.

Pelon, W., B. F. Whitman, and T. W. Thomas. 1977. Reversion of histidine-dependent mutant strains of Salmonella typhimurium by Mississippi River water samples. Environ. Sci. Technol. 11(6):619-623.

Pitts, J. N., Jr., K. A. van Cauwenberghe, D. Grosjean, J. P. Schmid, D. R. Fitz, W. L. Belser, Jr., G. B. Knudson, and P. M. Hynds. 1978. Atmospheric reactions of polycyclic aromatic hydrocarbons: Facile formation of mutagenic nitro derivatives. Science 202(4367):515-519.

Poel, W. E. 1963. The alimentary tract: A route for carcinogenic exposure. J. Occup. Med. 5:22-23.

Purchase, I. F. H., E. Longstaff, J. Ashby, J. A. Styles, D. Anderson, P. A. Lefevre, and F. R. Westwood. 1976. Evaluation of six short term tests for detecting chemical carcinogens and recommendations for their use. Nature 264:624-627.

Rao, T. K., et al. 1977. Correlation of mutagenic activity of energy related effluents with organic constituents. 8th Annual Meeting, Environ. Mutagen. Soc., Colorado Springs, Colo. p. 47-48.

Reichert, J. K. 1968a. Carcinogenic substances in water and soil XXI. Quantitative results on the removal of polycyclic aromatic compounds from drinking water by chlorine dioxide treatment. Arch. Hyg. Bakteriol. 152(1):37-44 (German). Chem. Abst. 69:21B27q.

Reichert, J. K. 1968b. Carcinogenic substances in water and soil. XXIII. Removal of polycyclic aromatic compounds in the treatment of tap water with chlorine dioxide: Isolation and identification of products of the reaction with 3,benzopyrene. Arch. Hyg. Bakteriol. 152(3):265-276 (German). Chem. Abst. 69:89651h.

Reichert, J. K. 1968c. Carcinogenic substances in water and soil. XXIV. Removal of polycyclic aromatic compounds in drinking water treatment with chlorine dioxide: Identification of previously unknown products of the reaction of 3,4-benzopyrene and chlorine dioxide. Arch. Hyg. Bakteriol. 152(3):277-279 (German). Chem. Abst. 69:89650g.

Rubin, I. B., M. R. Guerin, A. A. Hardigree, and J. L. Epler. 1976. Fractionation of systematic crude oils from coal for biological testing. Environ. Res. 12:356-365.

Saxena, J., and D. J. Schwartz. 1979. Mutagens in wastewaters renovated by advanced wastewater treatment. Bull. Environ. Contam. Toxicol. 22:319-326.

Scheiman, M. A., R. A. Saunders, and F. E. Salfeld. 1974. Organic contaminants in the District of Columbia water supply. Bio. Mass. Spec. 1(4):209-211.

Schmidt-Collerus, J. J. 1974. The disposal and environmental effects of carbonaceous solid wastes from commercial oil shale operations. First Annual Report to NSF, NSFGI 34282 x 1, Denver Research Institute. 169 p.

Schmidt-Collerus, J. J., F. Bonomo, K. Gala, and L. Leffler. 1976. Polycondensed aromatic compounds and carcinogens in the shale ash of carbonaceous spent shale from retorting of oil shale. pp. 115-156. In: T. F. Yen (ed.) Science and Technology of Oil Shale. Ann Arbor Science Publishers, Inc., Ann Arbor, Michigan. 226 p.

Searle, C. E. (ed.). 1976. Chemical carcinogens. American Chemical Society. Washington, D.C. 788 p.

Sheldon, L. S., and R. A. Hites. 1978. Organic compounds in the Delaware River. Environ. Sci. & Tech. 12(10):1188-1194.

Shirasu, Y., M. Moriya, K. Kota, A. Furuhash and T. Kada. 1976. Mutagenicity screening of pesticides in the microbial system. Mutat. Res. 40:130.

Simmon, V. F., and R. G. Tardiff. 1976. Mutagenic activity of drinking water condensates. (Abstr.) Mutat. Res. 38:389-390.

Slawson, G. C., Jr. (ed.). 1979. Groundwater quality monitoring of western oil shale development: Identification and priority ranking of potential pollution sources. EPA-600/7-79-023. January.

Strong, L. C. 1976. Theories of pathogenesis: Mutation and cancer. In: The Genetics of Human Cancer. (J. Mulvihill, ed.) Raven Press, New York.

Suess, M. J. 1967. The behavior and fate of 3,4-benzopyrene in aqueous systems.

Suess, M. J. 1972. The environmental load and cycle of polycyclic aromatic hydrocarbons. Science of the Total Environment 6(3):239-250.

Ph.D. dissertation, Graduate School of Public Health, Univ. of Pittsburgh, Pittsburgh, Pa., USA.

Sugimura, T., T. Kawachi, T. Matsushima, M. Nagao, S. Sato and T. Yahagi. 1977. In: Progress in Genetic Toxicology. (D. Scott, B. A. Bridges and F. H. Sobels, eds.) Elsevier/North Holland Biomedical Press, Amsterdam, 1977. pp. 126-154.

Svec, H. J., J. S. Fritz, and G. V. Calder. 1973. Trace soluble organic compounds in potable water supplies. Department of the Interior, Washington, D.C.

Swain, A. P., J. E. Cooper, and R. L. Stedman. 1969. Large scale fractionation of cigarette smoke condensate for chemical and biologic investigations. Can. Res. 29:579-583.

Teranishi, K., K. Homada, and H. Wantanabe. 1975. Quantitative relationship between carcinogenicity and mutagenicity of polyaromatic hydrocarbons in Salmonella typhimurium mutants. Mutat. Res. 31:97-102.

Tornabene, T. G. 1977. Microbial formation of hydrocarbons. In: Microb. Energy Convers. Proc. Semin. H. G. Schlegel and J. Barnea, eds. Pergamon Press, Oxford, Engl. pp. 281-299.

Turner, J. V., A. D. Ward, and C. G. Freeman. 1978. The mutagenic screening of fourteen imidazo compounds using a modified Ames test. Mutat. Res. 57:135-139.

Van Duuren, B. L., C. Katz, and B. M. Goldschmidt. 1973. Cocarcinogenic agents in tobacco carcinogenesis. J. Natl. Cancer Inst. 51:703-705.

Vogel, F., and G. Röhrborn. 1970. Chemical mutagenesis in mammals and man. Springer, Berlin-Heidelberg-New York.

Voll, M. J., J. D. Isbistee, L. I. Isaki, and M. D. McCommas. 1977. Mutagenic potential of petroleum byproducts in Chesapeake Bay waters. Water Res. Research Center, Univ. of Maryland, College Park, Md. Completion Report A-034-Md 14-34-0001-6021. Tech Report #39.

Wakeham, S. G. 1977. A characterization of the sources of petroleum hydrocarbon in Lake Washington. Journal Water Poll. Contr. Fed. 49(7):1681687.

Walker, J. D., and R. R. Colwell. 1974. Microbial degradation: Use of mixed hydrocarbon substrates. Appl. Micro. 27(6):1053-1060.

Walker, J. D., R. R. Colwell, and L. Petrakis. 1975a. Degradation of petroleum by an alga Prototheca zopfii. Appl. Micro. 30(1):79-81.

Walker, J. D., R. R. Colwell, and L. Petrakis. 1975b. Evaluation of petroleudegrading potential of bacteria from water and sediment. Appl. Micro. 30(6):1036-1039.

Wallcave, L., H. Garcia, R. Feldman, W. Lijinsky, and P. Shubik. 1971. Skin tumorigenesis in mice by petroleum asphalt and coal-tar pitches of known polynuclear aromatic hydrocarbon content. Tox. and Appl. Pharm. 18:41-52.

Ward, J. C. 1971. Water pollution potential of spent oil shale residues. Prepared for Environmental Protection Agency, Grant No. 14030EDB.

Wedgwood, P., and R. L. Cooper. 1955. The detection and determination of traces of polynuclear aromatic hydrocarbons in industrial effluents and sewage. Part III: The examination of some gasworks effluents. Analyst. 80:652-655.

Wynder, E. L., and K. Mabuchi. 1972. Etiological and preventive aspects of human cancers. Prev. Med. 1:30334.

Yamasaki, E., and B. N. Ames. 1977. Concentration of mutagens from urine by adsorption with the nonpolar resin XAD-2: Cigarette smokers have mutagenic urine. Proc. Natl. Acad. Sci. USA 74(8):3555-3559.