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**Short Term Bioassay and Bioassay
Chemistry of Materials Related to
Synthetic Fossil Fuels**

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OAK RIDGE NATIONAL LABORATORY
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SHORT TERM BIOASSAY AND BIOASSAY CHEMISTRY OF MATERIALS RELATED TO SYNTHETIC FOSSIL FUELS

Compilation of ORNL Papers Presented at the Symposium on Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures, Williamsburg, Virginia, February 21-23, 1978

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A symposium entitled "Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures" was held in Williamsburg, Virginia under the sponsorship of the U.S. Environmental Protection Agency in February of this year. This report compiles contributions of the Oak Ridge National Laboratory staff to that symposium. Proceedings of the complete symposium will be available through the USEPA.

CELL-MEDIATED MUTAGENESIS BY CHEMICAL CARCINOGENS

Eliezer Huberman and Robert Langenbach

Biology Division

Research jointly sponsored by the National Cancer Institute under Inter-agency Agreement 40-636-77 and the Division of Biological and Environmental Research, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

INTRODUCTION

A significant proportion of human cancers have been shown by epidemiological surveys to be related to exposure to environmental chemicals (8). In view of the difficulties of studying carcinogenesis in the whole animal, there are advantages in employing simpler systems such as mammalian cells *in-vitro* for carcinogenesis studies. Cell culture systems are useful for elucidating the mechanisms involved and may also provide an expedient means of screening for environmental chemicals that are potentially hazardous to humans.

While the mechanism by which chemicals induce cancer is unknown, one of the simplest explanations is a somatic mutation caused by the interaction of the carcinogen with the cellular DNA (7, 13, 22). In view of this possibility, it is important to study the mutagenic activity of chemical carcinogens in cultured mammalian cells. However, most naturally occurring chemical carcinogens are chemically nonreactive and must be metabolically activated in order to exert their biological effects (6, 7, 10, 11, 21, 22). Many microorganisms and mammalian cell lines which are suitable for studies on mutagenesis are not able to activate these chemicals metabolically (1, 4, 11). In such systems, chemicals have been tested by supplying either the chemically reactive metabolites or a microsomal enzyme system to activate the chemicals (1, 14, 16, 17, 20, 21, 26). However, microsomes are prepared by disrupting cells and may not necessarily metabolize the carcinogens as do intact cells (3, 5, 23, 24). In the intact cells, the amount and nature of the reactive metabolites produced from the chemical carcinogens depend on the balance between the enzymatic

detoxification. Therefore, a system which utilizes viable normal mammalian cells to activate the chemicals may have an advantage over cellular enzyme preparations.

We have developed a cell-mediated mutagenesis assay in which cells with the appropriate markers for mutagenesis are co-cultivated with either lethally irradiated rodent embryonic cells that can metabolize carcinogenic hydrocarbons (11, 12) or with primary rat-liver cells that can metabolize chemicals carcinogenic to the liver (19). Conversion from ouabain susceptibility to ouabain resistance, a mutation associated with the surface membrane Na^+/K^+ ATPase, was used as the genetic marker (2).

METHODS

Fibroblast- and Liver-Cell-Mediated Mutagenesis Assay

The fibroblast- and liver-cell-mediated assays were performed as described earlier (11, 12, 19). Chinese hamster V79 cells were seeded at 3×10^5 cells in 4 ml of medium into a 50-mm Petri dish containing 2×10^6 lethally irradiated (5000 R) secondary golden hamster embryo cells which are capable of metabolizing polycyclic aromatic hydrocarbons. The hydrocarbins were added in 1 ml of medium 5 hr later. In some experiments, aminophylline at 0.1 mM was added 2 hr before treatment with hydrocarbons.

Primary rat-liver cells were prepared from 6- to 8-week-old male Sprague-Dawley rats by enzymatic perfusion of the liver (18). Liver cells at about 10^7 cells/8 ml medium were then seeded into 25-cm² T-flasks which had been seeded 18 hr earlier with 2×10^5 V79 cells. The plating efficiency of the liver cells was about 20%, and the maximum number of viable liver cells was attached by 3 hr after seeding. At this time the medium was changed to 8 ml of fresh medium containing the compound to be tested.

Forty-eight hours after addition of the chemical, the V79 cells, which had been co-cultivated with either the fibroblasts or liver cells, were dissociated with trypsin-EDTA, counted with a hemocytometer, and reseeded into 50-mm Petri dishes at 200 cells/dish for determination of cloning efficiency and at 10^5 cells/dish for selection of ouabain-resistant colonies. The mutation frequency for resistance to ouabain was calculated per 10^6 survivors, based on cloning efficiency and number of cells seeded for mutation selection. In the fibroblast-mediated system, cells were incubated in Eagle's medium with a fourfold concentration of amino acids and vitamins plus 10% fetal calf serum in 10% CO_2 in air. For the liver-cell-mediated mutagenesis studies, the cells were incubated in William's medium E supplemented with 10% fetal calf serum in the presence of 5% CO_2 in air.

RESULTS

Cell-Mediated Mutagenesis by Chemical Carcinogens

We have developed a cell-mediated mutagenesis assay in which mutable Chinese hamster V79 cells are co-cultivated with embryonic fibroblasts that are capable of metabolizing various polycyclic aromatic hydrocarbons (11, 12) or with primary cultures of liver cells that are capable of metabolizing various liver carcinogens (19). During co-cultivation the reactive metabolites are apparently transferred from the metabolizing cell to the V79 cells, where they induce mutations.

To determine the relationship between mutagenic activity and carcinogenicity of various polycyclic aromatic hydrocarbons, eleven hydrocarbons with different degrees of carcinogenicity were tested by the fibroblast-

mediated mutagenesis assay for induction of ouabain-resistant mutants (Table 1). After co-cultivation, four carcinogenic hydrocarbons [7,12-dimethylbenz(α)anthracene, benzo(α)pyrene, 3-methylcholanthrene, and 7-methylbenz(α)anthracene] induced ouabain-resistant mutants, whereas five noncarcinogenic hydrocarbons [benzo(e)pyrene, benz(α)anthracene, phenanthrene, pyrene, and chrysene] were not mutagenic (Table 1). Dibenz(α, c)-anthracene and dibenz(α, h)anthracene, which have been reported as non-carcinogenic in golden hamsters (25), showed a weak mutagenic effect. In the presence of aminophylline, which enhanced polycyclic hydrocarbon metabolism (15), there was a two- to fourfold increase in mutagenicity with benzo(α)pyrene and 3-methylcholanthrene. Dibenz(α, c)anthracene, which showed a low degree of mutagenicity without aminophylline, showed a less than twofold increase of mutagenicity with aminophylline. Dibenz(α, h)-anthracene, which exhibited mutagenic activity similar to that of dibenz(α, c)anthracene without aminophylline, showed a tenfold increase in mutagenicity with aminophylline (Table 2). These results indicate that there is a relationship between mutagenesis and the degree of carcinogenicity of polycyclic aromatic hydrocarbons after enhancement of their metabolism by aminophylline.

Although the embryo cells are capable of metabolizing carcinogenic hydrocarbons to mutagenic intermediates, they are not capable of activating some other classes of chemical carcinogens, such as those which cause cancer of the liver. Therefore, to expand the spectrum of compounds which can be studied in the cell-mediated assay, we have conducted studies using primary cultures of rat-liver cells to activate the carcinogen. The data in Table 3 demonstrate that in the presence of liver cells, three liver

Table 1.

Induction of Ouabain-Resistant Mutants in the Fibroblast-Mediated Assay by Different Carcinogenic Hydrocarbons^a

Hydrocarbon	Concentration of hydrocarbon (μg/ml)	No. of ouabain-resistant mutants/10 ⁶ survivors
Control	0	1
Benzo(<i>e</i>)pyrene	1	1
Phenanthrene	1	1
Pyrene	1	1
Benz(<i>α</i>)anthracene	1	2
Chrysene	1	2
Dibenz(<i>α, c</i>)anthracene	1	3
Dibenz(<i>α, h</i>)anthracene	1	4
7-Methylbenz(<i>α</i>)anthracene	1	24
3-Methylcholanthrene	1	108
Benzo(<i>α</i>)pyrene	1	121
7,12-Dimethylbenz(<i>α</i>)anthracene	0.1	66

^aData are based on results from Huberman (9) and Huberman and Sachs (12).

Table 2.

Induction of Ouabain-Resistant Mutants in the Fibroblast-Mediated Assay by Carcinogenic Polycyclic Aromatic Hydrocarbons After Treatment With or Without Aminophylline^a

Hydrocarbon	No. of ouabain-resistant mutants/10 ⁶ survivors	
	Without aminophylline	With aminophylline
Control	1	1
Pyrene	1	1
Phenanthrene	1	1
Dibenz(a,c)anthracene	3	5
Dibenz(a,h)anthracene	4	46
3-Methylcholanthrene	108	413
Benzo(a)pyrene	121	214

^aCells were treated with 1 µg/ml of the polycyclic aromatic hydrocarbons and 0.1 mM aminophylline. Data are based on results from Huberman and Sachs (12).

Table 3.

Induction of Ouabain-Resistant Mutants in the Liver-Cell-Mediated Assay by Different Liver Carcinogens^a

	Concentration (mM)	No. of ouabain- resistant mutants/10 ⁶ survivors
Control	0	1
Dimethylnitrosamine	1.4	84
Diethylnitrosamine	4.5	19
Methyl- <i>tert</i> -butylnitrosamine	4.5	1
Aflatoxin B ₁	3.2 x 10 ⁻³	24
Aflatoxic G ₂	3.2 x 10 ⁻³	1

^aData are based on results from Langenbach *et al.* (19).

carcinogens (dimethylnitrosamine, diethylnitrosamine, and aflatoxin B₁) were metabolized to intermediates which were mutagenic to V79 cells. None of these compounds were mutagenic to the V79 cells in the absence of liver cells. Methyl-tert-butylnitrosamine and aflatoxin G₂, which are noncarcinogenic analogues, were not mutagenic to the V79 in either the presence or absence of the liver cells. Thus, the compounds tested in the liver-cell-mediated mutagenesis system, as in the case of polycyclic aromatic hydrocarbons, showed a correlation between the degree of mutagenicity and the degree of carcinogenicity.

CONCLUSION

In the present studies, we have shown that the cell-mediated mutation system, with the proper choice of metabolizing cells, can be used to detect the mutagenic activities of different classes of chemical carcinogens. When fibroblastic cells were used as the metabolizing cells, a correlation between the *in-vivo* carcinogenic activity and in the *in-vitro* mutagenic activity of eleven aromatic polycyclic aromatic hydrocarbons was observed. When primary liver cells were used as the metabolizing cells, three known liver carcinogens were demonstrated to be mutagenic by the cell-mediated assay, while two noncarcinogenic analogues were nonmutagenic. These results from the cell-mediated system suggest that the reactive intermediates of the carcinogens are stable enough to be transferred from the metabolizing cells to the V79 cells. The cell-mediated mutagenesis system is a simple *in-vitro* assay which may simulate the *in-vivo* situation. This approach could be extended to the co-cultivation of cells from other organs or tissues with mutable mammalian cells.

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SHORT-TERM BIOASSAY OF COMPLEX ORGANIC MIXTURES:
PART I. CHEMISTRY*

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Analytical Chemistry and Biology Divisions

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INTRODUCTION

A multidisciplinary program to elucidate the health and ecological effects of advanced fossil fuels utilization is currently underway at the Oak Ridge National Laboratory. The development of short-term bioassays applicable to the characterization of complex mixtures is an important aspect of the program. Studies reported here have been designed to identify constituents responsible for the mutagenicity of energy related materials. Observations made in the course of these studies may prove useful for designing biotesting methods suitable for the quantitative routine application to complex mixtures.

PREPARATION OF COMPLEX MIXTURES FOR SHORT-TERM BIOTESTING

The preparation of complex mixtures for in-vitro bioassay is complicated by at least two concerns: (a) the relevance of the material applied to the test system and, (b) the compatibility of the material with the test system. Chemical "relevance" is achieved when the test system is dosed with a material whose chemical composition mimics that which reaches the natural (man, plant, animal, soil, water, etc.) point of impact. Difficulties with "compatibility" are encountered when the material being bioassayed contains constituents which interfere with the test organisms' ability to respond to the effect of interest. Physical properties of the test material may inhibit the release, for example, of its mutagenic constituents to the test bacteria. High concentrations of mildly toxic constituents or small quantities of highly toxic constituents can mask the more subtle effect of mutagenic constituents.

Complex mixtures are generally entities of poorly defined and continuously changing chemical composition. If, by virtue of sample history (generation, storage, handling), the material is altered in its content of bioactive constituents or undergoes other changes which affect the biological test system, the results of the bioassay may be invalidated. Whole animal inhalation bioassays of cigarette smoke, for example, are designed with considerable attention (11, 12, 15, 18) to the compatibility of the "smoke" being bioassayed to that which is freshly generated by the cigarette under conditions comparable to those representative of the human situation. Pellet implantation, while highly successful for the application of pure compounds to respiratory tract epithelium, is questionably applicable to the study of complex mixtures because (19) the constituents may be released to the test organism at differing rates depending on their chemical properties.

Methods for the preparation of complex materials for bioassay must meet different requirements than those designed for chemical characterization. Methods for quantitative chemical analyses seek to recover 100% of the individual constituent being determined without regard to the remaining constituents. Differing degrees of recovery are known. For bioassay purposes, however, quantitative recovery is important only because it provides the maximum amount of material for bioassay from the starting material. The critical objective is to produce a material whose constituents have been recovered equally. Recovering the constituents to an equal degree ensures that the composition of the material bioassayed is the same as that which was sampled.

"Compatibility" with the test system becomes of greater concern when the researcher moves from whole animal models to in-vitro bioassay systems. Mechanisms of selective adsorption, metabolism, and of detoxification, for example, embodied in whole animal models may be absent in in-vitro test systems. Observation of a highly toxic reaction to a test material need not imply the absence of constituents capable of producing the more subtle biological effects.

The requirements of relevance and compatibility are themselves incompatible. Any steps taken to remove toxic constituents or to otherwise make the material compatible with the test system necessarily involves a change in physical or chemical nature of the test material. The objective of methods development here has been to prepare materials in a form suitable for biotesting but with a minimal or at least interpretable impact on the relevance of the test material.

The approach used most successfully to date is to separate the constituents of the mixture into a manageable number of chemically distinct fractions. Each fraction is subsequently bioassayed to determine whether mutagenic constituents of any type are present independent of synergism, toxic interferences, or other complicating factors due to the interaction of constituents of widely differing properties. If it is assumed (or experimentally demonstrated) that the mutagenicities of the fractions are additive, the activities of the fractions may be summed to estimate the activity of the starting material. The primary chemical requirement is that all of the constituents present in the starting material are recovered in the fractions to be biotested.

Acid-base extractive fractionation and gel chromatographic fractionation have been used in an attempt to identify constituents responsible for the mutagenicity of synthetic crude oils and to assess their utility for preparing the oils for bacterial mutagenesis testing.

CHEMICAL CLASS FRACTIONATION

Procedures. Acid-base fractionation involves the liquid-liquid partitioning of the sample between an immiscible organic solvent and an alkaline or acidic aqueous phase. The procedure (6, 20) used here first subjects the sample to partitioning between ether and 1N NaOH. Acidic constituents of the sample concentrate in the aqueous phase while alkaline and neutral constituents concentrate in the organic phase. The organic phase is subsequently contacted with 1N HCl which preferentially extracts the alkaline constituents. The constituents of the aqueous phase can be back-extracted into ether following pH adjustment. Four fractions (acids, bases, neutrals, and any insoluble residue which may be formed) thus result for biological testing.

Additional discrimination is possible by subjecting the primary fractions to further separation. The procedure (Fig. 1) used here was chosen because of the extensive literature (1-4,14,24-26) available dealing with its application to chemical and biological characterizations of condensed tobacco smokes. Basic constituents are further separated into those which are water soluble at pH 11, those which are ether soluble, and those which are insoluble under these conditions of separation. Acidic constituents are divided into weakly acid (presumably phenolic), strongly acidic/water soluble, strongly acidic/ether soluble, and two insoluble residues. Neutral constituents are divided into any number of subfractions by Florisil column chromatography or other chromatographic methods.

Fig. 1. Acid-base extractive separation of complex mixtures (21).

The extractive fractionation procedure is advantageous in that most fractions contain chemically similar constituents of predictable types, the procedure is applicable to large sample sizes (25,26), and that it has been demonstrated (1,2,14) to be effective for elucidating the biological properties of a complex mixture. An additional advantage is that it can be applied to both hydrophilic (e.g., an aqueous effluent) and lipophilic (e.g., a crude oil) materials. A practical disadvantage is that the procedure is highly labor-intensive and therefore both costly and time-consuming when a high discrimination is required. A potentially more serious concern is that contact with highly acidic and highly alkaline environments can lead to chemical reactions which may alter the nature and quantities of bioactive constituents present thus invalidating the bioassay.

A new procedure (Fig. 2), optimized to fractionate materials which are predominantly lipophilic, has been developed (13) for comparison with the extractive method. The sample is dissolved in hexane and added to a column of Sephadex LH-20 gel previously equilibrated with methanol (85 v%)/water (15 v%). Lipophilic constituents are eluted from the column using hexane. Hydrophilic constituents are subsequently eluted from the column using methanol and/or acetone. In the second step of the procedure, the lipophilic constituents are separated into "polymeric", "sieved", and hydrogen-bonding fractions by eluting the lipophilic fraction from a column of Sephadex LH-20 eluted with isopropanol. Because the pore size of the gel is somewhat smaller when equilibrated with isopropanol than tetrahydrofuran, additional "polymeric" material elutes prior to the aliphatic constituents in this third step of the procedure. The gel chromatographic procedure thus produces the following six fractions: hydrophilic, polymeric, hydrogen-bonding, aliphatic, simple aromatic, and polyaromatic.

Fig. 2. Gel chromatographic separation of complex mixtures (14).

The primary advantage of the gel chromatographic procedure is that separation is affected by gentle mechanisms. The gel acts as an inert support for the methanol/water phase in the first step of the procedure yielding separation of the lipophilic constituents from the hydrophilic constituents by an essentially continuous liquid-liquid partition mechanism. Molecular-sieving and hydrogen-bonding are the primary mechanisms associated with the second step of the procedure. Final separation of the "sieved" subfraction obtained in the second step of the procedure is effected by a combination of size exclusion, π -bonding to the gel matrix, and hydrogen-bonding. Being a chromatographic procedure, additional precautions such as excluding light, deoxygenating solvents, and operating under inert gas blankets could be taken to further minimize the likelihood of chemical changes occurring.

Discrimination and Reproducibility. Class fractionation procedures are optimized to recover all of the starting material rather than to quantitatively isolate one class of constituents free of all others. This factor, combined with the extreme chemical complexity of most natural or anthropogenic mixtures, generally results in fractions which themselves contain a variety of chemical types. As an example, the "aliphatic" fraction of Shale Oil B (10) obtained using the gel chromatographic procedure was further separated (8) into acids, bases, and neutrals by acid-base partition. The neutral constituents were then chromatographed on Alumina (neutral activity I) using stepwise elution with hexane, benzene, methylene chloride, and methanol. Essentially all (~98 w/w%) of the "aliphatic" fraction was recovered in the "neutral" subfraction as would be expected. Only ~50 w/w% of the "neutrals" were found in the hexane eluate from

Alumina with an additional ~ 10 w/w% being found in the benzene eluate. Up to 40 w/w% of the mass of the aliphatic fraction would, therefore, be expected to contain aromatic or polar moieties or to consist of very high molecular weight species.

Gas chromatographic retention, mass spectra, and reference to a detailed study (7) of shale oil composition suggest (8) the presence of a complex variety (Table 1) of hydrocarbons in the aliphatic fraction derived from shale oil B. Aromaticity is noted in the benzene elutables and might be expected to increase in proportion to aliphatic character in fractions eluted by methylene chloride and methanol. The aliphatic fraction would thus be considered primarily "aliphatic" in character but also contains constituents with aromatic moieties. Fractions are not distinctly different in composition but rather are "enriched" in a given class of constituents relative to the other fractions.

Preparative scale class fractionations have generally been carried out (20,25) in a semiquantitative manner. As is illustrated in Table 2 for studies carried out here, quantitative reproducibility is particularly poor for fractions constituting less than 5 w/w% of the starting material. It must be recalled, however, that the purpose of these (1,2,6) studies has been to identify constituents contributing to the biological activity of the starting material rather than to quantitate that activity. Little attention has yet been given to improving the quantitative reproducibility of fractionation procedures. Reduced sample sizes, simplified fractionation procedures, and a reduction in the number of manual operations may contribute to the development of a procedure sufficiently reproducible for quantitative biotesting.

Table 1.

Some Constituents of the "Aliphatic" LH-20
Fraction of a Shale Derived Oil

Alumina/cyclohexane elutables of neutral subfractions

N C₁₀ - n C₃₀ (C₁₈ - C₂₃ predominating)

Phytane, Pristane

Micellaneous

mw = 558

Terpanes

tricyclics

n = 2-8, mw = 276-360

mw = 398+

pentacyclics

mw = 412

Steranes

mw = 288-400

Alumina/benzene elutables of neutral subfraction

R = C₁ C₁₀

R = C₁, C₂

NMR Average

Table 2.

Typical Reproducibility and Recoveries of Preparative
Scale Class Fractionation

Extractive Fractionation ¹			Sephadex Gel Chromatographic Fractionation		
Designation	Fraction %(w/w)	Rel. std. deviation (%)	Designation	Fraction %(w/w)	Rel. std. deviation (%)
NaOH Insol.	0.9	34	Lipophilic ²	92.4	1.4
WA _I	0.2	86	Hydrophilic ³	6.5	12
WA _E	1.9	18	Total	100.2	1.5
SA _I	0.2	41			
SA _E	1.1	50			
BI _a	1.6	82			
B _{Ib}	0.2	29	Polymeric ⁴	1.2	34
B _E	2.2	38	Sieved ⁵	92.5	17
B _W	7.3	16	H-Bonding ⁵	7.7	23
N/Hexane	74.2	2	Total ⁵	101	19
N/Hex-Bz	4.9	47			
N/Bz-Ether	4.7	17			
N/MeOH	2.4	17			
Total	102	9			

¹Coal derived crude oil "D" (10), four repetitions, sample sizes of 4.4 - 11.9 grams (20).

²Shale derived oil "B" (10), five repetitions, samples sizes of 17.5 - 302.4 grams (13).

³Shale derived oil "B", three repetitions, samples of 17.5 - 302.4 grams (13).

⁴From lipophilic fraction of coal derived oil "D" (10), five repetitions, 4 gram samples (13).

⁵From lipophilic fraction of coal derived oil "D" (10), eight repetitions, 4 gram samples (13).

Mutagenicity. Methods used and significant observations concerning the mutagenicity of fractionated samples are summarized elsewhere in these proceedings. Table 3 summarizes the results of mutagenicity testing of fractions from the extractive and chromatographic separations of Shale Oil B for an evaluation of the efficacy of the fractionation procedures. The mutagenicities of the individual fractions and their calculated contributions to the mutagenicity of the starting material are tabulated.

The basic fractions are seen (Table 3) to exhibit the highest mutagenic activities for Shale Oil B separated by the extractive fractionation method. The neutral fraction is of substantially lower specific activity but makes the largest contribution to the calculated activity of the starting material because it constitutes the largest part (~87 w/w%) of the material. The gel chromatographic procedure produces three fractions of high specific activity. Importantly, these fractions are those which would be expected to concentrate constituents suggested as mutagenic by the extractive fractionation procedure. Basic constituents would be expected to concentrate in the hydrophilic and hydrogen-bonding fractions while polycyclic aromatic hydrocarbons and azaarenes would be expected to accumulate in the polyaromatic fraction. The aliphatic fraction, a somewhat refined version of the neutral fraction provided by the extractive procedure, is seen to be the major contributor to the calculated activity of the starting material by virtue of its quantitative contribution (60 w/w%) to the mass of the material. The gel chromatographic data thus tends to confirm the utility of the acid-base extraction fractionation procedure.

Table 3.

Distribution of Mutagenic Activity by Fractionation Procedures

Extractive Fractionation			Sephadex Gel Chromatographic Fractionation		
Fraction ¹	Activity ² rev/mg	Weighted activity ³ rev/mg	Fraction ¹	Activity ² rev/mg	Weighted activity ³ rev/mg
NaOH Insol.	256	3	Hydrophilic	1300	78
Weak Acid (WA _I)	185	<1	Polymeric 1	54	3
Weak Acid 2 (WA _E)	52	<1	H-Bonding	1040	52
Strong Acid 1 (SA _I)	0	0	Polymeric 2	0	0
Strong Acid 2 (SA _E)	159	<1	Aliphatic	180	108
Strong Acid 3 (SA _W)	160	<1	Mono/Diaromatic	24	3
Basic 1 (B _{Ia})	1377	3	Tri/Tetraaromatic	132	7
Basic 2 (B _{Ib})	800	2	>Tetraaromatic	1220	49
Basic 3 (B _E)	952	68			
Basic 4 (B _W)	223	1			
Neutral	112	97			
TOTAL		178			300
Unfractionated		---			204,268,196

¹Shale derived crude oil "B", separate aliquots 6 months apart.

²Revertants per milligram of fraction using TA98, S-9, and Aroclor 1254 (6).

³Revertants per milligram of starting material computed by multiplying activity of fraction by weight percentage of total recovered weight constituted by the fraction (6).

The studies yielding the results given in Table 3 were carried out for qualitative rather than quantitative purposes. The weighted activities are tabulated and summed, however, to illustrate the degree of additivity and quantitative reproducibility accompanying these procedures. Summation of weighted activities from the extractive method indicates that Shale Oil B exhibits a mutagenicity of 178 rev/mg. The gel chromatographic method yields a calculated result of 300 rev/mg as compared to an average of 223 rev/mg from three tests of the unfractionated oil carried out at the same time. Considering that the oil sample used for the gel chromatographic study was first subjected (13) to azeotropic distillation and other substantial differences in procedures, agreement between the methods is good.

The efficacy of summing mutagenicities of fractions to determine the mutagenicity of the starting material is yet to be systematically studied. Additivity observed (Table 4) to date has, however, been generally good.

IDENTIFICATION OF MUTAGENIC CONSTITUENTS

Acid-base fractionation has consistently shown (6) basic constituents of synthetic crude oils to exhibit a high mutagenicity relative to those from petroleum crude oils. The basic fractions of condensed cigarette smoke have also been reported (14) to exhibit high mutagenicities. Recent reports which demonstrate a high correlation of factors such as tobacco type (17), degree of fertilization (18), and stalk position (16) with the mutagenicity of the resulting cigarette smoke condensate further suggest (9) that nitrogenous constituents are important contributors to mutagenicity. Candidate nitrogenous constituents are known to be present in synthetic oils (21,22), cigarette smoke condensate (23) and airborne particulate matter (5).

Table 4.

Comparison of Mutagenicity Calculated by Summing
Fractions with that Experimentally Determined

Separation	Revertants per milligram					
	A ¹	B ²	C ³	D ⁴	E ⁵	F ⁶
Calculation	300	260	138	167	252	112
Determined	223	223	196	100	350	109

¹Shale oil separated by gel chromatographic procedure (13).

²Shale oil separated into lipophilic and hydorphilic fractions.

³Lipophilic fraction separated into polymeric, aliphatic, hydrogen bonding fractions.

⁴Sieved fraction separated into polymeric, aliphatic, aromatic, polyaromatic fractions.

⁵Shale oil separated into isopropanol and acetone elutables from Sephadex LH-20.

⁶Neutral fraction separated into hexane, hexane/benzene, benzene/ether, and methanol eluatables from Florisil.

The general approach of fractionation and mutagenicity testing has been used (6) to better define the nature of the constituents responsible for the observed mutagenic activity. A procedure (Fig. 3) optimized to isolate mutagenic constituents of the ether soluble basic (B_E or ESB) fraction has been developed. Approximately one gram of the ESB from shale oil B and coal synthoil C ["synfuel A" (10)] were eluted from a basic alumina column using benzene followed by ethanol. For both oils, the benzene fraction contained (Table 5) from 75-80 w/w% of the mass but no more than 2% of the mutagenic activity. The ethanol fraction, containing essentially all of the mutagenic activity, was then separated into isopropanol and acetone subfractions using Sephadex LH-20 gel. For both oils, the acetone subfraction was found to contain approximately 90% of the mutagenic activity of the ESB in approximately 10% (w/w) of its mass.

The acetone subfraction constitutes approximately 0.5% (w/w) of the original oils. The subfraction is extremely complex in spite of the 20-fold enrichment. The gas chromatogram (Fig. 4) resulting from the analysis of acetone subfraction from coal synthoil C reveals the presence of at least one hundred individual constituents.

The compositions of the benzene, isopropanol, and acetone subfractions from both oils are being examined. Table 6 summarizes observations to date based on gas chromatographic and mass spectral analysis. All of the fractions are seen to contain nitrogen heterocyclics. Pyridines and quinolines predominate in the inactive benzene fraction while constituents of greater structural complexity are enriched in the isopropanol and acetone fraction. A general feature of these materials is that they contain a large variety of alkyl-substituted and partially hydrogenated derivatives of the parent heterocyclics.

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Fig. 3. Isolation of mutagenic constituents of ether soluble bases.

Table 5.

Distribution of Mutagenic Activity in ESB Subfractions

Subfraction	Shale Derived Oil B		Coal Derived Oil C	
	Average weighted activity ¹ (%)	Average relative weight (%)	Average weighted activity ² (%)	Average relative weight (%)
Benzene	0	78	2	76
Isopropanol	1	13	0	12
Acetone	<u>92</u>	<u>9</u>	<u>88</u>	<u>12</u>
TOTAL	93	100	90	100

¹Percentage of mutagenic activity [TA98, S9, Aroclor 1254 (6)] of the ether soluble bases (ESB) fraction accounted for in the subfraction.

²Percentage by weight of the ESB.

Fig. 4. Gas chromatogram of a mutagenically enriched acetone subfraction. Coal synthoil C, 20' x 1/8" glass column of 3% Dexsil 400 on 100/120 mesh Chromosorb 750, programmed from 100°C to 320°C at 1°C/min following an eight minute isothermal hold at 100°C hydrogen flame ionization detection.

Table 6.

Constituents of the Ether Soluble Base Fraction

Shale Oil Subfractions		Synthoil Subfraction	
Benzene	Isopropanol	Acetone	Acetone
	Azaindanes	Azaindanes	C ₁ -C ₄ Diazines
C ₁ -C ₁₃ Pyridines	C ₂ -C ₅ Dihydropyridines	C ₃ -C ₅ Dihydropyridine	C ₂ -C ₆ Pyridines
	C ₄ -C ₁₁ Pyridines	C ₁ -C ₅ Pyridines	
	C ₁ Diazaindane	C ₁ Diazaindane	
C ₂ Tetrahydroquinoline	C ₁ -C ₂ Azaindane	C ₁ -C ₂ Azaindane	C ₁ -C ₄ Tetrahydroquinoline
	C ₁ -C ₅ Tetrahydroquinoline	C ₁ Tetrahydroquinoline	
C ₁ -C ₄ Quinolines	Tetrahydroquinoline	Tetrahydroquinoline	Tetrahydroquinoline
	C ₂ -C ₄ Quinolines	C ₁ -C ₂ Quinolines	C ₁ -C ₆ Quinolines
		Quinoline	
		C ₃ -C ₄ Dihydropyridine	
		Vinylquinoline	
		Phenylpyridines	
C ₂ Phenylpyridine	C ₂ Phenylpyridine	C ₁ -C ₆ Phenylpyridines	C ₂ -C ₄ Phenylpyridines
	C ₃ -C ₆ Dihydrodiazanaphthalene	C ₃ Dihydrodiazanaphthalene	
		C ₁ hydroazafluorene	Azadihydrofluorene
C ₂ Azafluorene		C ₁ -C ₂ Azafluorene	C ₁ -C ₂ Azafluorene
	Azabenzo(def)fluorene		
	C ₅ Diazanaphthalene		
C ₁ -C ₃ Acridines	C ₁ Acridines	C ₁ -C ₂ Acridines	C ₁ -C ₂ Acridines
		Acridine	Acridine
		Tetrahydroacridine	
			C ₁ Tetrahydroacridine
			C ₁ -C ₃ Azabenzo(def)fluorene
		C ₁ -C ₅ Diazafluorenes	
			C ₃ Diazabenzo(def)fluorene
	Diazabenzo(ghi)fluorene		
	C ₄ Tetrahydrodiazanaphthalenes		

Table 6 (Cont'd)

Constituents of the Ether Soluble Base Fraction

Shale Oil Subfractions			Synthoil Subfraction
Benzene	Isopropanol	Acetone	Acetone
		Azapyrenes	Azapyrenes
		C ₁ -C ₃ Azapyrenes	C ₁ -C ₃ Azapyrenes
Diazapyrenes		C ₂ -C ₄ Diazapyrenes	
C ₁ Diazapyrenes		Benzacridine	Benzacridine
		C ₁ -C ₃ Benzacridines	C ₁ Benzacridines
		Azabenzopyrene	C ₁ Indenoquinoline
		C ₁ Azabenzopyrene	Azabenzopyrene
			Azadibenzopyrene
			Azabenzoperylene
			Azaindenopyrene
			Azaocronene

The primary difference between the acetone, isopropanol, and benzene fractions is that the acetone (highly mutagenic) fraction contains higher molecular weight azaarenes. Constituents such as benzacridines and aza-benzpyrenes are found in the acetone fraction. Perhaps importantly, the exceptionally mutagenic acetone fraction from the coal derived oil contains a larger variety of these constituents than does the shale derived acetone fraction. Mass spectral evidence suggests the presence of azacoronene in coal derived fraction.

SUMMARY

Petroleum substitutes produced from coal and shale are among the materials requiring biological evaluation to assess environmental and health impacts of new energy technologies. Intractability and the presence of highly toxic constituents are among the physical and chemical properties of products and process streams which complicate short term biotesting for subtle health effects. An effective approach is to separate the starting material into chemically well-defined fractions. Bioassay results obtained for the separated fractions may be summed to estimate the biological activity of the starting material. Biological activities of individual fractions provide evidence as to the types of constituents responsible for the biological activity of the starting material.

Liquid-liquid partition from strongly acidic and alkaline solutions has proven viable for the testing of coal- and shale-derived oils. A theoretically more gentle separation procedure based on Sephadex LH-20 gel chromatography has been found viable for lipophilic materials.

Studies suggest that alkaline constituents of petroleum substitutes are major contributors to their Ames Test activity. Subfractionation of ether soluble bases from a shale-derived and coal-derived oil has concentrated the bioactive constituents in a fraction constituting approximately 0.5 wt% of the starting oil. Nitrogen heterocyclics are found to be the predominant constituents of this active subfraction.

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SHORT-TERM BIOASSAY OF COMPLEX ORGANIC MIXTURES:

PART II. MUTAGENICITY TESTING

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INTRODUCTION

The feasibility of using short-term mutagenicity assays to predict the potential biohazard of various crude and complex test materials has been examined in a coupled chemical and biological approach. The principal focus of the research has involved the preliminary chemical characterization and preparation for bioassay, followed by testing in the *Salmonella* histidine reversion assay described by Ames (1). The mutagenicity tests are intended to (a) act as predictors of profound long-range health effects such as mutagenesis and/or carcinogenesis, (b) act as a mechanism to rapidly isolate and identify a hazardous biological agent in a complex mixture, and (c) function as a measure of biological activity correlating baseline data with changes in process conditions. Since complex mixtures can be fractionated and approached in these short-term assays, information reflecting on the actual compounds responsible for the biological effect may be accumulated. Thus, mutagenicity tests will (d) aid in identifying the specific hazardous compounds involved and in establishing priorities for further validative testing, testing in whole animals, and more definitive chemical analysis and monitoring.

Our work has emphasized test materials available from the developing synthetic fuel technologies (2). However, the procedures are applicable to a wide variety of industrial and natural products, environmental effluents, and body fluids. The general applicability of microbial test systems has already been demonstrated with, for example, the use of the assay as a prescreen for potential genetic hazards of complex environmental effluents

or products, e.g., tobacco smoke condensates (3), natural products (4,5), hair dyes (6), soot from city air (7), fly ash (8), and, in our work with synthetic fuel technologies, oils and aqueous wastes (9, 10).

BIOASSAY METHOD

For the study of application of mutagenicity testing to environmental effluents and crude products from the synthetic fuels technology we performed preliminary screening with the highly sensitive Ames histidine reversion strains known to respond to a wide variety of known mutagens/carcinogens. The working hypothesis was that sensitive detection of potential mutagens in *fractionated* complex mixtures could be used to isolate and identify the biohazard. In addition, the information could be helpful in establishing priorities for further testing, either with other genetic assays or carcinogenic assays.

The *Salmonella* strains used in the various assays are listed below. All strains were obtained through the courtesy of Dr. Bruce Ames, Berkeley, California.

Salmonella typhimurium Strains

TA1535 *hisG46*, *uvrB*, *rfa* (missense)

TA100 *hisG46*, *uvrB*, *rfa* (missense plus R factor)

TA1537 *hisC3076*, *uvrB*, *rfa* (frameshift)

TA1538 *hisD3052*, *uvrB*, *rfa* (frameshift)

TA98 *hisD3052*, *uvrB*, *rfa* (frameshift plus R factor)

In the screening of fractionated materials, the two strains TA98 and TA100 were generally employed. Standard experimental procedures have been given by Ames *et al.* (7). Briefly, the strain to be treated with the

potential mutagen(s) is added to soft agar containing a low level of histidine and biotin along with varying amounts of the test substance. The suspension containing approximately 2×10^8 bacteria is overlaid on minimal agar plates. The bacteria undergo several divisions with the reduced level of histidine, thus forming a light lawn of background growth on the plate and allowing the mutagen to act. Revertants to the wild-type state appear as obvious large colonies on the plate. The assay can be quantitated with respect to dose (added amount) of mutagen and modified to include "on-the-plate" treatment with the liver homogenate required to metabolically activate many compounds.

Fractions and/or control compounds to be tested were suspended in dimethyl sulfoxide (supplied sterile, spectrophotometric grade from Schwarz/Mann) to concentrations in the range of 10-20 mg/ml solids. The potential mutagen was in some cases assayed for general toxicity (bacterial survival) with strain TA1537. Normally, the fraction was tested with the plate assay over at least a 1000-fold concentration range with the two tester strains TA98 and TA100. Revertant colonies were counted after 48 hr incubation. Data were recorded and plotted versus added concentration, and the slope of the induction curve was determined. It is assumed that the slope of the linear dose-response range reflects the mutagenic activity. Positive or questionable results were retested with a narrower range of concentrations. All studies were carried out with parallel series of plates plus and minus the rat liver enzyme preparation (7) for metabolic activation. Routine controls demonstrating the sterility of samples, enzyme or rat liver S-9 preparations, and reagents were included. Positive controls with known mutagens were also included in order to

recheck strain response and enzyme preparations. All solvents used were non-mutagenic in the bacterial test system.

SAMPLES

Samples that have been tested and their sources are listed below:

(1) coal liquefaction product from a process under development, courtesy of the Pittsburgh Energy Research Center (Synfuel A), or Coal A from ORNL repository; (2) coal-liquefaction product from the COED Pyrolysis Process, courtesy of FMC (Synfuel B), or Coal B from ORNL repository; (3) Louisiana-Mississippi sweet crude oil, courtesy of Dr. J. A. Carter of the Analytical Chemistry Division, Oak Ridge National Laboratory; (4) composite crude oil sample from materials obtained through the courtesy of Dr. Dee Latham of the Laramie Energy Research Center; (5) a crude shale-oil sample (B) from the above-ground simulated *in-situ* oil-shale retorting process; (6) the aqueous product water consisting of the centrifuged water of combustion from the same process (both samples 5 and 6 courtesy of the Laramie Energy Research Center); (7) a coal-gasification aqueous condensate from a process under development, courtesy of Pittsburgh Energy Research Center; and (8) a separator liquor from a coal-liquefaction process, courtesy of FMC. The authors recognize the possibility that these samples may bear no relationship to the process as it may exist in the future, nor should it be construed that these materials are representative of all natural crudes, synthetic or shale-oil processes. They are used here simply as appropriate and available materials for the research.

*BIOASSAY RESULTS**Class Fractionation*

Oil Samples. The bulk of the samples listed above were subjected to the fractionation scheme described by Swain *et al.* (11), as modified by Bell *et al.* (12). The scheme is described in detail as applied to oils in Rubin *et al.* (13) and in Part I of this presentation. As an example, a summary of the results from a sample of Synfuel A-2 (9) is given in Table 1. Subfractionation results are shown with the neutral fraction chromatographed on a Florisil column. The column was eluted with the solvents shown and, with this sample, collected in one fraction. The data includes the analytical weight analysis of the sample (column 1) along with the specific mutagenic activity (slope of dose-response curve) of each fraction (column 2). The product of these (column 3) represents a weighted value of each fraction relative to the contribution to the starting test material. Mutagenic activity is seen in both the acidic and basic fractions along with the neutral subfractions. However, the major contributors to the mutagenicity appear to occur in the basic fractions, with activities also consistently present in the neutral materials.

A comparison of these activities and the total mutagenic potential of the various oil and aqueous samples is given in Table 2. Reasonable reproducibility is seen in similar samples, e.g., Synfuel A-1 and A-2; Synfuel B-1 and B-2. Synfuel A-3 represents the same material without prior centrifugation of the solids. The consistency of activities seen in all oils considered is illustrated. On a relative scale, the synthetic fuels show more mutagenic activity than the natural crude "control"

Table 1.
Distribution of Mutagenic Activity of Synthetic Oil^a
(Synfuel A-2)

Fraction ^b	Relative weight (% of total)	Specific activity ^c (rev/mg)	Weighted activity ^d (rev/mg)
1. NaOH _I	20.9	1700	356
2. WA _I	2.2	180	4
3. WA _E	4.9	1260	62
4. SA _I	0.1	30	0
5. SA _E	0.4	130	1
6. SA _W	0.4	120	1
7. B _{Ia}	6.8	38700	2633
8. B _{Ib}	0.1	1270	1
9. B _E	2.0	36200	725
10. B _W	0.6	570	3
Neutral	69.2	583 (570) ^e	403
TOTAL	107.6		4189
<u>Neutral Subfractions</u>			
Hexane	72.7	340	244
Hexane/benzene	5.0	710	35
Benzene/ether	19.8	1360	270
Methanol	2.3	1460	34
Subtotal	99.8		583
Initial sample, g	26.166		
Chromatographed, g	10.664		

Table 1. (cont'd)

^aAll assays carried out in the presence of crude liver S-9 from rats induced with Aroclor 1254.

^bI = insoluble (fractions a and b), E = ether soluble, W = water soluble, WA = weak acid, SA = strong acid, B = base.

^crev/mg = revertants/mg, the number of histidine revertants from *Salmonella* strain TA98 by use of the plate assay with 2×10^8 bacteria per plate. Values are derived from the slope of the induction curve extrapolated to a milligram value. NT = not tested.

^dWeighted activity of each fraction relative to the starting material is the product of columns one and two. The sum of these products is given as a measure of the total mutagenic potential of each material.

^eComparable to "specific activity", but based on the activity of the total neutral fraction rather than the summation of the individual fractions.

Table 2.

Summary of Mutagenicity Testing Results with Synthetic Oils and Aqueous
Samples - Class Fractionation Scheme^a

Sample	SA _W		B _E		Neutral		Total Wt. act. (rev/mg)	Ref.
	Rel. wt. (%)	Spec. act. (rev/mg)	Rel. wt. (%)	Spec. act. (rev/mg)	Rel. wt. (%)	Spec. act. (rev/mg)		
Composite Crude-1	0.1	400	0.2	150	84.2	277	241	(9)
Composite Crude-2	0.1	750	0.2	500	84.2	166	147	(10)
La-Miss Crude	0.1	240	0.2	180	80.7	90	76	(9)
Shale Oil	0.6	160	7.1	952	86.7	112	178	(10)
Synfuel A-1	0.3	240	2.0	28900	73.6	517	4032	(9)
Synfuel A-2	0.4	120	2.0	36200	69.2	583	4189	(9)
Synfuel A-3	0.3	1010	3.1	43300	56.4	1094	7308	(9)
Synfuel B-1	0.4	0	2.6	1500	82.3	560	516	(9)
Synfuel B-2	1.6	0	1.8	3800	89.3	465	484	(9)
Separator Liquor 1.3 w/v%	53.9	0	0.5	850	1.5	0	17	unpubl.
Gasifier Condensate 0.9 w/v%	30.8	0	0.5	4000	1.9	100	211	(14)
Process Water (shale oil) 1.0 w/v%	65.0	0	2.7	1575	2.4	52	68	(10)

^aStrain TA98 with metabolic activation with Aroclor-induced preparation.

samples shown. Shale oil appears to be only slightly higher than the natural crudes. References are given to the complete published compilations on these samples. See Table 2.

Each determination represents the slope of the dose-response curve. All testing was carried out in the presence of the rat-liver microsomal activation system. Slight mutagenic activity without enzyme treatment was occasionally noted.

The routine screening utilized strains TA100 (missense) and TA98 (frameshift); however, complete strain-specificity tests were carried out with selected materials. Fractions giving a positive response with strain TA98 were, in general, also positive with the other frameshift strains, TA1537 and TA1538. Additionally, positive results were routinely noted with the sensitive missense strain TA100; however, reversion of the missense strain TA1535 was rare. TA98 appeared to be the best general indicator of mutagenic activity of these materials. Furthermore, liver preparations from rats induced with Aroclor 1254 (a gift of Monsanto) showed the best *general* applicability. However, individual differences in effectiveness do occur; for example, variously induced preparations show obvious differences between basic fractions, and e.g., the neutral/methanol fraction (9). An Aroclor-induced preparation reacts best with the neutral fraction (polynuclear aromatic hydrocarbons?), while a phenobarbital-induced preparation works more efficiently with the basic fraction (heterocyclic nitrogen compounds?).

Primary candidates for the mutagens (and carcinogens?) responsible for activity in the basic fractions include quinoline, substituted quinolines, alkyl pyridines, acridine, naphthylamines, aza-arenes,

benzacridines, and aromatic amines; in the neutral fractions potential threats may consist of benzanthraces, dibenzanthracenes, substituted anthracenes, benzopyrenes, benzofluorenes, pyrene, substituted pyrenes, and chrysenes [see Ho *et al.* (15)]. Thus, work with these pure compounds is being carried out concurrently.

Reproducibility of results was shown by comparison of data from similar samples. Although discrepancies exist from fraction to fraction, the general trend is apparent, and the sum of activities appears to be roughly reproducible. Again, when the major-component, neutral fraction is assayable, as with the Synfuel A, the summation of the subfraction values of the neutrals reflects the approximate additivity of the individual mutagenic determinations. For example, 570 revertants/mg with a direct assay of the neutrals from Synfuel A-2 compares with 583 revertants/mg based on the summation (Table 1).

An overview of the results points to a number of consistencies: (1) all crudes and Synfuels showed some mutagenic potential; (2) the neutral and basic fractions showed activities regardless of the source of the sample; and (3) the relative total mutagenic potentials varied over two orders of magnitude. Whether these results reflect a comparative biohazard of processes still under development is not the point in question here. The results simply show that biological testing - genetic reversion assays in this case - can be carried out with the newly developed tester systems, but only when coupled with the appropriate analytical separation schemes. Conceivably, the use of this approach could provide rapid information concerning health effects.

Aqueous Samples. Table 2 also lists sample results from a group of aqueous samples subjected to the class fractionation procedure ("Stedman procedure"). In general, greater activity is seen in the more polar, more water soluble fractions rather than in the non-polar neutral materials. Caution has to be extended on work with any aqueous material because of the high potential for instability. Although we have used organic extraction here, techniques with resin concentration, e.g., XAD-2, may prove useful with aqueous samples (16,17). Only in exceptional cases is the mutagenic activity directly observable in an unconcentrated sample.

Liquid Chromatographic Fractionation

In the initial studies with coal liquefaction products, the crude oils were fractionated using the scheme originally developed for cigarette smoke condensates ("Stedman procedure"). The scheme yields class separations based on the relative acid-base properties of the components. The samples are partitioned between ethyl ether and 1 *N* NaOH in a single-stage, continuous procedure to yield an aqueous acid fraction and organic phase basic and neutral fractions. The organic fraction is extracted with 1 *N* HCl to yield an aqueous basic fraction and an organic basic fraction and an organic neutral fraction. The neutral material is subsequently subfractionated on a Florisil column. These primary subfractions are then subjected to mutagenicity testing.

Realizing the potential for modification of the components within the procedure, we moved to consideration of a number of other methods. The fractionation procedure using Sephadex LH-20 can provide a gentle and large-scale class separation for (initially) crude oils from shale oil

and coal liquefaction processes. The procedure involves three steps using the gel in different modes: (1) lipophilic-hydrophilic partitioning; (2) molecular size separation; and (3) aliphatic-aromatic separation. The procedure (18) was designed by Jones, Guerin, and Clark of the Analytical Chemistry Division. Using fractions prepared as above, we have started a comparison of this procedure and the Stedman procedure for usefulness in preparation for bioassay. The preliminary mutagenicity studies confirm the suitability and utility of the method. Table 3 summarizes some of the results from shale oil. The method appears to be generally applicable to complex organic mixtures and achieves the goal of presenting a gentle and rapid separation scheme, useful with large-scale samples.

Subfractionation of Neutral Components. The polycyclic aromatic hydrocarbons (PAH's) presumably occurring in the neutral fractions of the various schemes noted have been listed as major contributors to mutagenicity of the test materials. With natural crudes, these components appear to account for the bulk of the activity. With synthetic crudes, the contributions of both the basic and neutral fractions must be considered. Further work is needed to define (isolate and identify) the mutagenic components of these important classes.

We have carried out a preliminary study with synfuel PAH's subfractionated and detected by the short-term mutagenicity assay. As shown in Table 4, shale oil (Sample B) can be separated into lipophilic and hydrophilic fractions with the Sephadex LH-20 partition chromatography described previously (18, 19). Further separation of the lipophilic fraction is achieved by neutral alumina and LH-20 using various solvents. The various subfractions can then be assayed for mutagenicity with the

Table 3.

Sephadex LH-20 Fractionation of Shale Oil Coupled With
Mutagenicity Testing

Test Material-Fraction	% of Total	Specific ^a Activity (rev/mg)
Crude Oil	100	233
Hydrophilic	6	1300
Lipophilic	93	196
-Polymer	5	54
-Hydrogen-Bonding	5	1040
-Sieved	84	100
-Polymer	1	0
-Aliphatics	60	180
Aromatics		
-1 and 2 ring	14	24
-3 and 4 ring	5	132
-Polynuclear	4	1220

^aSlope of dose-response curve with *Salmonella* strain TA98 plus rat-liver preparation induced with Aroclor 1254.

Table 4.

Subfractionation of Neutral Components from Shale Oil:
Distribution of Polycyclic Aromatic Hydrocarbons and Mutagenic Activity

Subfraction	Weight %	Specific activity ^a rev/mg of fraction	
		Without S-9	With S-9
Aromatic Fraction	100	60	170
I (polymeric)	5.7	0	0
II (1-ring)	47.0	0	0
III (2-ring)	33.7	0	0
IV (3-ring)	8.0	0	1000
V (4-ring)	2.7	1600	4000
VI (5-ring)	0.6	2600	3800
VII (>5-ring)	0.5	600	1500
TOTAL	98.2	62	214

^aNumber of histidine revertants from *Salmonella* strain TA98 by use of plate assay with 2×10^8 bacteria per plate. Values derived from slope of the induction curve. "With S-9" indicates test carried out in presence of crude enzyme preparations from rats induced with Aroclor 1254.

Salmonella histidine reversion system. As seen in Table 4, activity seems to peak in the 4- and 5-ring subfractions, those containing known carcinogens/mutagens as benzo(a)pyrene, benzo(e)phenanthrene, and 3-methylcholanthrene.

Subfractionation of Basic Components. Again, considering the results with class fractionation procedures, we developed a procedure (20) specifically designed for subfractionation of the basic materials, now realized to be a major contributor to mutagenic activity. An elution sequence using alumina and Sephadex LH-20 gel with a combination of solvents isolates 90% of the mutagenic activity from basic compounds into a 0.5 wt% fraction of crude oil.

A basic alumina column eluted first with benzene and then ethanol isolates the mutagenic components of the ether soluble base fractions (ESB) of synthetic crude oils into a fraction of about 25 wt% of the ESB. A further separation is achieved by eluting the ethanol isolate through a Sephadex LH-20 gel column with isopropanol followed by acetone. About 90% of the basic mutagenic activity is recovered in the acetone subfraction which comprises ~0.5 wt% of the crude oil. Development of this separation scheme was made possible using the Ames microbial mutagenesis assay as the detector during exploratory liquid chromatographic separations. Table 5 lists some of the preliminary data from these studies.

COMPARATIVE MUTAGENESIS

In order to validate and compare the results accumulated in the Ames system with complex test materials from synthetic fuel technologies, we selected specific fractions or subfractions on the basis of their activity

Table 5.

Subfractionation of Basic Fraction:
Distribution by Weight and Mutagenic Activity

	Shale Oil		Synfuel A-3	
	wt%	rev/mg ^a	wt%	rev/mg ^a
Basic Fraction (A)	100	2,500	100	30,000
<u>Alumina</u>				
Benzene (B)	78	600	76	0
Ethanol (C)	---	---	---	---
<u>LH-20</u>				
Isopropanol (D)	12	0	12	0
Acetone (E)	10	20,000	12	222,000

^aAssayed with Strain TA98 with Aroclor-induced enzyme preparation.

in the histidine reversion assay for further testing in the various other tests designed to detect mutagenicity. Preliminary results have been published in the Proceedings of the Second International Conference on Environmental Mutagens, Edinburgh, 1977 (21). For the purposes of a qualitative comparison, the results are given in Table 6. The selected fractions or subfractions utilized were basic and neutral isolates from synthetic crude oils from coal liquefaction processes [Synfuel A and B as described in Epler *et al.* (9)]. With *Drosophila* (22) and in the mammalian cell gene mutation assay (23), the detection has been a function of newly developed fractionation schemes, e.g., the use of LH₂₀, (39, 40), that result in higher specific activity (more highly purified) mutagenic subfractions. In general, the results validate the initial screening carried out in the *Salmonella* assay but these other systems have not as yet been used to exhaustively test materials that are negative in the Ames system. Note, also, however, that the preliminary results of Generoso (personal communication) show that the crude synthetic fuel does induce dominant lethals in mice although the *basic* fraction alone appears to be negative.

For the comparative studies with microbial systems given here, we selected four synfuel fractions. The results with the frameshift strain TA98 with metabolic activation were considered. Fractions 6 [strong acid, water soluble (SA_w)]; 7 [base insoluble, fraction A (B_{Ia})]; 9 [base, ether soluble (B_E)]; and 14 (neutrals/methanol) were selected on the basis of their ability to revert the Ames strains. In order to validate the mutagenicity results obtained from the *Salmonella* histidine-reversion system, we extended the treatment with the selected test fractions to the *E. coli* 343/113 system of Mohn (24). The results obtained in the forward (gal⁺)

Table 6.
Comparative Mutagenesis of Fractions From
Synthetic Crude Oils^a

Test system	Assay	Basic fraction	Neutral fraction	Crude ^b synfuel
<i>Salmonella</i>	his ⁻ →his ⁺	+	+	+
<i>E. coli</i>	arg ⁻ →arg ⁺	+	+	NT
	gal ⁻ →gal ⁺	+	+	NT
Yeast	his ⁻ _S →his ⁺ _r	+	+	NT
	CAN ⁻ →can ^r	+	+	NT
<i>Drosophila</i>	SLRL	+	-	NT
CHO cells	6-thioguanine resistance	+	NT	NT
Human leukocytes	chromatid aberrations	P	+?	NT
	sister chromatid exchange	+	NT	NT
Mouse	dominant lethals	-	P	+
Carcinogenesis	skin painting ^c	P	P	P

^aFor references to published work or work in progress see text. The fractions utilized were generally those from Synfuel A-3, or Synfuel B-2. + = mutagenic; - = nonmutagenic; NT = not tested; and P = in progress.

^bCrude synfuels are generally too toxic to test in most systems.

^cWork of J. M. Holland, Oak Ridge National Laboratory, in progress.

and reverse-mutation (arg^+) assays with *E. coli* support the results obtained with *Salmonella*. Both the basic fraction (#9) and the neutral subfraction (#14) are mutagenic upon metabolic activation with Aroclor-induced rat-liver homogenate (S-9).

Further validation of the bacterial results was obtained by assaying for both forward mutation and reverse mutation in the yeast system (21, 25). The Synfuel A fractions tested were weakly mutagenic and were effective *without* metabolic activation. Some antagonistic effects were encountered when metabolic activation was incorporated. The most active fraction, the ether soluble bases (B_E), also reverted the putative frame-shift marker, *hom3-10*. This fraction may contain acridines and other nitrogen heterocyclics. Unpublished results from our group have pointed to similar effectiveness without activation in the *Salmonella* system when suspension tests rather than plate assays are used with crude mixtures.

To ascertain the comparative effectiveness in the human leukocyte chromatid aberration assay, we treated the cells with selected test fractions from Synfuel B. The test materials used were the neutral subfractions and represent largely polycyclic aromatic hydrocarbons. The coal fractions were suspended in DMSO at a concentration of 20 mg/ml total solids. Two hours of control treatment with 5% DMSO and treatment with the four subfractions (Neutrals as Hexane, Hexane/benzene, Benzene/ether, and Methanol subfractions) over a concentration range of 0.1 - 1.0% (20 μg - 200 μg) were ineffective in producing chromatid aberrations (1600 cells scored). However, metabolic activation was not included with any exogenous enzyme source nor are the assumed constituents - PAH's, effective as chromosome breaking agents. Work with other fractions and the inclusion of metabolism is in progress.

Selected fractions from Synfuel B were tested in the *Drosophila* sex-linked recessive-lethal assay (SLRL). Fraction 13 (neutral/benzene/ether) is slightly effective as a mutagen for *Drosophila* at the higher concentrations fed.

Several other syncrude fractions which were scored as mutagenic in the *Salmonella* assays were tested in *Drosophila*. (All of the fractions require metabolic activation in the *Salmonella* assay.) Of the five fractions tested, only 12 and 13 gave any indication of an effect. Additionally, the highly active (in *Salmonella*) basic subfraction from the procedure previously described was tested. This basic material showed a significant dose-dependent response in the *Drosophila* SLRL assay. (See discussion by Nix and Brewen, these proceedings, reference 22.)

In conclusion, short-term tests with bacterial and fungal mutagenicity assays appear to detect effectively the mutagenic potential of complex environmental or industrial effluents; however, chemical fractionation is necessary to reduce toxicity and concentrate hazardous materials. Extension of the results to higher organisms, i.e., mammalian cells, *Drosophila*, and the mouse appears to be valid but needs more testing.

CONCLUSIONS

In these initial feasibility studies, the purpose has not been to reflect on whether a biohazard exists in comparison with other materials or processes. The results show that biological testing - within the limits of the specific system used - can be carried out with complex organic materials but perhaps only when coupled with the appropriate analytical separation schemes. An extrapolation to relative biohazard

at this point would be, at least, premature. The primary use that such combined chemical and biological work may serve is to aid in isolating and identifying the specific classes or components involved. A number of precautions are listed below.

The detection or perhaps the generation of mutagenic activity may well be a function of the chemical fractionation scheme utilized. The inability to recover specific chemical classes or the formation of artifacts by the treatment could well corrupt the results obtained in addition to the possibility of an inability to detect the specific biological endpoint chosen. Along with the obvious bias that could accompany the choice of samples and their solubility or the time and method of storage, a number of biological discrepancies can also enter into the determinations. For example, concomitant bacterial toxicity can nullify any genetic damage assay that might be carried out; the choice of inducer for the liver enzymes involved can be wrong for selected compounds; the choice of strain could be inappropriate for selected compounds; and additionally, the applicability of the generally used *Salmonella* test to other genetic endpoints and the validation of the apparent correlation between mutagenicity and carcinogenicity still remains a point of significant fundamental research. Furthermore, the short term assays chronically show negative results with, certain substances, e.g., heavy metals and certain classes of organics. Similarly, compounds involved in or requiring co-carcinogenic phenomena would presumably go undetected.

However, as a prescreen to aid the investigators in ordering their priorities, the short-term testing appears to be a valid testing approach

with complex mixtures. Over-interpretation at this stage of research especially with respect to relative hazard or negative results should be avoided.

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QUANTITATIVE MAMMALIAN CELL GENETIC TOXICOLOGY:
STUDY OF THE CYTOTOXICITY AND MUTAGENICITY
OF 70 INDIVIDUAL ENVIRONMENTAL AGENTS RELATED
TO ENERGY TECHNOLOGIES AND 3 SUBFRACTIONS OF
A CRUDE SYNTHETIC OIL IN THE CHO/HGPRT SYSTEM

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As science and technology advance, an extraordinary quantity of natural and synthetic chemicals is introduced continuously into our environment. Through conventional animal tests, some of these environmental chemicals have been found to be either highly toxic, mutagenic, carcinogenic, or tetraogenic. Epidemiological studies have shown that among these harmful chemicals, a few also exhibit such detrimental effects in the human population. Because of the high cost and length of time required for the animal experiments, such tests have been confined to only a very small fraction of these environmental agents. Thus, the biological effects of the great majority of these chemicals, including ingredients of our daily foods and drugs, remain either incompletely tested or unknown.

During the past few years, evidence has accumulated that a high percentage (80-90%) of human cancer is linked to exposure to industrial and environmental chemicals identifiable as carcinogens (23, 44). Since the expense of animal tests preclude their routine use to identify environmental carcinogens, many short-term assays have been developed as initial carcinogen screening tests. Studies of mutagenesis and DNA-repair in microorganisms, especially *Salmonella typhimurium* and *Escherichia coli*, have established that approximately 90% of chemical carcinogens cause mutation induction or DNA damage in these bacteria (2, 3, 26, 27, 38, 39, 42, 45, 46). Such findings imply that the microbial tests are useful to identify not only potential mutagens but also carcinogens in the environment.

In view of the intrinsic limitation of the microbial assay to respond to certain classes of chemicals, such as the apparent failure of the *Salmonella* assay to demonstrate that carcinogenic halogenated hydrocarbons and metallic compounds are mutagenic (27), it appears that no single test system will give 100% correlation between mutagenicity and carcinogenicity. The use of a battery of tests rather than any single test in isolation has thus been proposed to reduce the probability of false negatives (i.e., known carcinogens are not mutagenic) and false positives (i.e., known noncarcinogens are mutagenic) (4, 37).

It has been recognized that studies of mutagenesis in prokaryotes may not reveal some fundamental mechanisms of mutagenesis in mammals, because mammals differ from prokaryotes in their level of organization and repair of DNA, mechanisms of metabolism of chemicals, and other related functions. Some bacterial mutagens such as caffeine and hydroxylamine do not appear to be mutagenic in mammalian cells, while agents such as nickel and beryllium compounds are mutagenic in mammalian cells but not in the *Salmonella* system (Couch, San Sebastian, and Hsie, unpublished; 27). In addition, it is well known that chromosomal abnormality is a major cause of inheritable human diseases and is often associated with the process of malignancy. The great majority of chemical carcinogens are known to induce chromosomal aberrations (1, 24) or sister-chromatid exchange (1). Diethylstilbestrol, a synthetic hormone associated with cancer in women, causes chromosomal aberrations in cultured mammalian cells (24), but does not cause mutation induction in *Salmonella* (27).. Clearly, mammalian cell systems offer advantages over bacterial systems for studying genetic toxicity at the chromosome and chromatid level.

Since the observation that treatment of mammalian somatic cells with conventional mutagens such as ethyl methanesulfonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) causes an increase in the number of cell variants that differ from parental cells in either nutritional requirement (5, 36) or drug sensitivity (5), there has been much interest in utilizing a quantitative mammalian cell mutation system for studying mechanisms underlying the process of mammalian mutation and, additionally, for assessing the genetic hazard of environmental agents to the human population. Several mammalian cell mutation systems, especially those utilizing resistance to purine analogues such as 8-azaguanine (AG) and 6-thioguanine (TG) as a genetic marker (6), have been developed for such purposes. The selection for mutation induction to purine analogue resistance is based on the fact that the wild-type cells containing hypoxanthine-guanine phosphoribosyl transferase (HGPRT) activity are capable of converting the analogue to toxic metabolites, leading to cell death; the presumptive mutants, by virtue of the loss of HGPRT activity, are incapable of catalyzing this detrimental metabolism and, hence, escape the lethal effect of the purine analogue (Table 1).

The near-diploid Chinese hamster ovary (CHO) cell line has been chosen for our study because a mutation assay, referred to as the CHO/HGPRT system, has been well defined (7-10, 16-22, 29-33). We have used CHO cells because these are perhaps the best characterized mammalian cells genetically (35, 40). They exhibit high cloning efficiency, achieving nearly 100% under normal growth conditions, and are capable of growing in a relatively well-defined medium on a glass or plastic substratum or in suspension with a population doubling time of 12-13 h. In addition,

Table 1.

CHO/HGPRT Mutation Assay^a

(1) Enzyme system:

(2) Mutation induction and selection for variants and revertants:

(a) wild type	mutation (induced by physical or chemical agents)	variant cell
genotype HGPRT ⁺		HGPRT ⁻
phenotype TG ^S ,		TG ^r ,
aminopterin positive		aminopterin negative

(b) Variant selection is based on resistance to TB

(c) Selection of revertants is based on growth in the presence of aminopterin.

(3) Characterization of TG^r variants:

(a) Direct enzyme assay for conversion of [³H]hypoxanthine to [³H]IMP.

(b) Cellular incorporation of [³H]hypoxanthine into cellular macromolecules as revealed by either direct radioactivity measurement or autoradiographic determination.

(c) Sensitivity of clonal growth to aminopterin (10 μ M) in medium F12FCM5 which contains hypoxanthine (30 μ M), glycine (100 μ M), and thymidine (3 μ M).

^aTable II of ref. 21.

the cells have a stable, easily recognizable karyotype of 20 or 21 chromosomes (depending on the subclone) (11) and are suitable for studying mutagen- or carcinogen-induced chromosome and chromatid aberrations (1) and sister-chromatid exchanges (1, 24) (Table 2).

METHODS AND MATERIALS

Cell Culture

All studies to be described have employed a subclone of CHO-K₁ cells (25), designated as CHO-K₁-BH₄ (16). It was isolated following selection in F12 medium containing aminopterin (10 μ M) (16). Cells are routinely cultured in Ham's F12 medium (Pacific Biological Co.) containing 5% heat-inactivated (56°C, 30 min), extensively dialyzed fetal calf serum (Pacific Biological Co.) (medium F12FCM5) in plastic tissue culture dishes (Falcon or Corning Glass Works) under standard conditions of 5% CO₂ in air at 37°C in a 100% humidified incubator. These cells grow in medium which contains aminopterin as well as in regular medium with 5 or 10% dialyzed fetal calf serum with a population doubling time of 12-13 h. Cells are removed with 0.05% trypsin for subculture, and the number is determined with a Coulter counter (model B, Coulter Electronics).

Treatment with Chemicals

We have standardized treatment procedures which are found to be suitable for various chemicals (16, 29). Briefly, CHO cells are plated at 5×10^5 cells/25 cm² bottle in medium F12FCM5. After a 16- to 24-h growth period (cell number = ~ 1.0 - 1.5×10^6 cells/plate), the cells are washed once with saline G, and sufficient serum-free F12 medium is added to bring the final volume to 5 ml after the addition of various amounts

Table 2.
Characteristics of CHO cells^a

-
- (1) Exhibit a stable karyotype over 20 years with a modal chromosome number of 20 which has a distinctly recognizable morphology.
 - (2) Have a colony-forming capacity of nearly 100% in a defined growth medium.
 - (3) Grow well in either monolayer or suspension with a relatively short population doubling time of 12-14 h.
 - (4) Are genetically and biochemically well characterized, with many genetic markers available, including auxotrophy, drug resistance, temperature sensitivity, etc.
 - (5) Respond well to various synchronization methods, including the mitotic detachment procedure which facilitates cell cycle study.
 - (6) Are useful in somatic cell hybridization experiments because they readily hybridize with different cell types, including human cells; when the CHO-human cell hybrid is formed there is subsequent rapid, preferential loss of human chromosome, which facilitates the assignment of marker genes to specific chromosomes or linkage groups in the human karyotype.
 - (7) Quantitatively respond to various physical and chemical mutagens and carcinogens with high sensitivity.
 - (8) Adapt to mutation induction either through coupling with a microsome activation system or through host (mouse) mediation.
 - (9) Are capable of monitoring induced mutation to multiple gene markers, chromosome aberration, and sister chromatid exchange in the same mutagen-treated cell culture.
-

^aTable I of ref. 21.

of microsome preparation (up to 1 ml) and 50 μ l of chemical, usually dissolved in dimethyl sulfoxide. Chemicals and/or microsomes are omitted from some plates to provide controls. The microsomal preparation has been prepared in this laboratory according to the method of Ames *et al.* (3) from livers of Aroclor 1254-induced male Sprague-Dawley rats; the microsome mix for biotransformation contains (per ml) 33 μ moles KCl, 8 μ moles $MgCl_2$, 4 μ moles NADP, 5 μ moles glucose-6-phosphate, 100 μ moles phosphate buffer (pH 7.4), and 0.2 ml microsome fraction. Cells are then incubated for 5 h and washed 3 times with saline G before 5 ml of F12FCM5 are added. Following overnight incubation, cells are trypsinized and plated for cytotoxicity and specific gene mutagenesis to be described below. Treatment with physical agents has been described in detail elsewhere (17, 19, 29, 30).

Cytotoxicity

The effect of chemicals on the cellular cloning efficiency is determined by use of the treated cells described above. For an expected cloning efficiency higher than 50%, 200 well-dispersed single cells are plated, and for an expected survival lower than this, the number of cells plated is adjusted accordingly to yield 100-200 surviving colonies after standard incubation in medium F12FCM5 for 7 days. At the end of the incubation period, the plates are fixed with 3.7% Formalin and stained with a dilute crystal violet solution before the colonies are enumerated. A cluster of more than 50 cells growing within a confined area is considered to be a colony. Control cells, which do not receive treatment with mutagen, usually give 80% or higher plating efficiency under this condition. Neither the solvent-microsome mix nor these agents individually affect the cellular

cloning efficiency. The effect of carcinogen on the cloning efficiency is expressed as percent survival relative to the untreated controls.

Specific Gene Mutagenesis

The CHO/HGPRT system has been defined in terms of medium, TG concentration, optimal cell density for selection (and, hence, recovery of the presumptive mutants), and expression time for the mutant phenotype (16, 29). For the determination of mutation induction, the treated cells are allowed to express the "mutant phenotype" in F12 medium for 7-9 days, at which time mutation induction reaches a maximum which is maintained thereafter (as long as 35 days examined) for several agents (EMS, MNNG, ICR-191, X-ray, and UV) irrespective of concentration or intensity of the mutagen (29-32). Routine subculture is performed at 2-day intervals during the expression period, and at the end of this time the cells are plated for selection in hypoxanthine-free F12FCM5 containing 1.7 $\mu\text{g/ml}$ (10 μM) of TG at a density of 2.0×10^5 cells/100-mm plastic dish (Corning or Falcon), which permits 100% mutant recovery in reconstruction experiments (29). We find the use of dialyzed serum particularly important, presumably due to potential competition between hypoxanthine and TG for transport into the cells and for catalysis by HGPRT (29). After 7 to 8 days in the selective medium, the drug-resistant colonies develop; they are then fixed, stained, and counted. Such a protocol permits the maximum yield by various physical and chemical agents of TG-resistant variants, >98% of which have highly reduced HGPRT activity (7-10, 16-22, 29-33). Mutation frequency is calculated based on the number of drug-resistant colonies per survivor at the end of the expression period.

RESULTS

Characteristics of the CHO/HGPRT System: Evidence of the Genetic Basis of Mutation at a Specific Locus

Conclusive, direct proof of the genetic origin of mutations in somatic cells should theoretically rely on demonstration that the affected hereditary alteration has resulted in a modified nucleotide sequence of the specific gene, causing modified coding properties which result in the production of altered protein with changes in the amino acid sequence. In the absence of such proof, one must rely on indirect criteria which are consistent with the concept that the observed phenotypic variations are genetic in nature. Such criteria include stability of altered phenotype, mutagen-induced increase in occurrence of stable variants, biochemical and physiological identification of the variant phenotype, chromosomal localization of the affected gene, etc. (6, 35, 40, 43).

Over the past 4 years, we have used the assay protocol described (16, 29) and have found in approximately 400 experiments that the spontaneous mutation frequency lies in the range of $1-5 \times 10^{-6}$ mutant/cell. Various physical and chemical agents are capable of inducing TG resistance. Among all chemical mutagens examined, mutation induction occurs as a linear function of the concentration (7-10, 16-22, 29-33). For example, mutation frequency increases approximately linearly with EMS concentration in this near-diploid cell line, conforming to the expectation that mutation induction occurs in the gene localized at the functionally monosomic X chromosome. However, in the tetraploid CHO cells, EMS does not induce an appreciable number of mutations, even at very high concentrations, as predicted theoretically (18).

We have been unable to detect any spontaneous reversion with 13 TG-resistant mutants, all of which contain low, yet detectable, HGPRT activity. More than 98% of the presumptive mutants isolated either from spontaneous mutation or as a result of mutation induction are sensitive to aminopterin, incorporate hypoxanthine at reduced rates, and have less than 5% HGPRT activity (29). Studies in progress have also shown that mutants containing temperature-sensitive HGPRT activity can be selected, suggesting that mutation resides in the HGPRT structural gene (O'Neill and Hsie, unpublished observations).

The CHO/HGPRT system appears to fulfill the criteria for a specific gene locus mutational assay (Table 3) and should be valuable in studying mechanisms of mammalian cell mutagenesis and as a system to determine the mutagenicity of various physical and chemical agents.

Mutagenicity of 70 Individual Energy-Technology-Related Environmental Agents

Polycyclic Hydrocarbons (Total of 27). Some of the most ubiquitous environmental organic pollutants in our environment are polycyclic hydrocarbons, many of which are carcinogenic. Coal- and synthetic-fuel-related energy technologies and gasoline-driven engines often generate high levels of polycyclic hydrocarbons which are detectable in urban air and water. We have studied the mutagenicity of benzo(α)pyrene [B(α)P] and its 19 metabolites, including 11 phenols, 3 epoxides, 3 diols, and 2 diolepoxides. For comparison, benzo(e)pyrene [B(e)P] and pyrene were added to this study. Also included were benz(α)anthracene (BA) and 4 related compounds (7,12-dimethyl BA, anthracene, and two phenolic derivatives of BA). The carcinogenic polycyclic hydrocarbons B(α)P, BA, and 7,12-dimethyl BA

Table 3.

CHO/HGPRT Mutation Assay: Genetic Basis of Mutation at
HGPRT Locus in TG-Resistance Selection^a

-
- (1) Spontaneous mutation frequency at $1-5 \times 10^{-6}$ mutant/cell.
 - (2) Mutation induction by physical and chemical agents with linear dose-response relationship.
 - (3) Frequency of spontaneous reversion at less than 10^{-7} reversion/cell.
 - (4) Failure to induce mutation in near-tetraploid cell lines.
 - (5) Altered HGPRT activity in mutants.
 - (a) 1170/1189 (98.4%) mutant colonies are aminopterin negative.
 - (b) 121/122 (99.2%) mutant colonies show reduced hypoxanthine incorporation by autoradiography studies.
 - (c) 81/83 (97.6%) isolated mutant clones show reduced HGPRT enzyme activity.
-

^aTable III of ref. 21.

require metabolic activation to be mutagenic. The weak carcinogen B(e)P is less mutagenic than B(a)P. The noncarcinogenic polycyclic hydrocarbons pyrene and anthracene are nonmutagenic even with metabolic activation. B(a)P-4,5-epoxide and B(a)P-7,8-diol, 9-10-epoxide(*syn*) are mutagenic. Since CHO cells cannot activate procarcinogens such as B(a)P, these cells appear to be most useful in screening for the mutagenicity of metabolites such as those of B(a)P (Hsie and Brimer, unpublished). Because of the limited availability of B(a)P derivatives, some of the experiments remain to be pursued in detail.

Metallic Compounds (Total of 15). The carcinogenic and mutagenic potential of certain toxic metallic compounds has become an environmental concern, especially with the increasing large-scale coal mining and coal firing of power plants. We found that $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{cis-Pt}(\text{NH}_3)_2\text{Cl}_2$ (an antitumor agent) are mutagenic, while $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{BeSO}_4 \cdot 4\text{H}_2\text{O}$, and CdCl_2 are weakly mutagenic. Determination of metal mutagenicity is apparently complicated by the ionic composition of the medium. For example, we found that the mutagenicity and cytotoxicity of MnCl_2 were abolished by the excess of MgCl_2 . The unusual environment required for demonstration of mutagenicity of MnCl_2 makes assessment of its biological hazard difficult. This too may account in part for varying results obtained in studying the mutagenicity of AgNO_3 , CaCl_2 , $\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$, RbCl , H_2SeO_3 , TiCl_4 , and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (10; Couch, San Sebastian, Forbes, and Hsie, unpublished).

Nitrosamines and Related Compounds (Total of 16). Nitrosamines are potent carcinogens for various animal species. They are of environmental concern because it is known that oxides of nitrogen produced at high

temperature in internal combustion engines and in coal-fired power plants can react with atmospheric water to form nitrosamines. Nitrosamines can also be formed in the human stomach by a reaction between a common meat preservative, sodium nitrite, and various secondary and tertiary amines, many of which are often used as counter or prescription drugs.

Nitrosamines generally require metabolic activation to be cytotoxic and/or mutagenic. In addition to investigating two common aliphatic nitrosamines dimethylnitrosamine (DMN) and diethylnitrosamine (DEN), we have studied the mutagenicity of 11 cyclic nitrosamines, including 3 nitrosopiperidines, 3 nitrosopyrrolidines, 3 nitrosopiperazines, and 2 nitrosomorpholines. Our studies also include the nitrosamine-related chemicals dimethylamine, formaldehyde, and sodium nitrite. We have found that all 9 carcinogenic nitrosamines (DMN, DEN, 2-methyl-1-nitrosopiperidine, 3,4-dichloro-1-nitrosopiperidine, nitrosopyrrolidine, 3,4-dichloronitrosopyrrolidine, 1,4-dinitrosopiperazine, 1,5-dinitrosohomopiperazine, nitrosomorpholine) are mutagenic and all 4 noncarcinogenic nitrosamines (2,5-dimethylnitrosopiperidine, 2,5-dimethylnitrosopyrrolidine, 1-nitrosopiperazine, nitrosophenmetrazine) are nonmutagenic. Formaldehyde and sodium nitrite are nonmutagenic, and dimethylamine is mutagenic at high concentrations (San Sebastian, Couch, and Hsie, unpublished). Variable carcinogenicity data on the latter 3 chemicals existed in the literature.

Quinoline Compounds (Total of 5). One class of potential environmental contaminants from fossil-fuel energy is heterocyclic compounds such as quinolines. Quinoline, a known carcinogen, is mutagenic with metabolic activation. Another carcinogen, 4-nitroquinoline-1-oxide,

is highly mutagenic; its mutagenicity decreases when assayed in the presence of the activation system. The carcinogenicity of 8-hydroxy-, 8-amino-, and 8-nitroquinoline is not known, but these compounds exhibit variably weak mutagenicity in preliminary experiments (San Sebastian and Hsie, unpublished).

Physical Agents (Total of 7). The mutagenicity of both ionizing radiation such as X-ray and nonionizing physical agents such as UV light has been demonstrated. Fluorescent white, black, and blue lights are slightly cytotoxic and mutagenic. Sunlamp light is highly cytotoxic and mutagenic, exhibiting the biological effects within 15 sec of exposure under conditions recommended by the manufacturer for human use. Cytotoxic and mutagenic effects are observed after 5 min of sunlight exposure; responses vary with hourly and daily variations in solar radiation. In view of man's constant exposure to various light sources, demonstration of their genetic toxicity suggests that daily exposure to these light sources, especially sunlight, should be minimized (17, 19, 30). The demonstration that the CHO/HGPRT system is capable of quantifying the cytotoxic and mutagenic effect of sunlight recommends it as a model mammalian cell system for studies of the genetic toxicology of sunlight per se and of the interactive effects between sunlight and other physical and chemical agents, leading ultimately to a better understanding of the effects of sunlight on humans and the environment.

Mutagenicity of 39 Other Chemicals

Direct-Acting Alkylating Agents and Related Compounds (Total of 11).

Included are 10 alkylating agents {2 alkyl sulfates [dimethyl sulfate (DMS), diethyl sulfate (DES)], 3 alkyl alkanesulfonates [methyl

methanesulfonate (MMS), EMS, and isopropyl methanesulfonate (iPMS)], 2 nitrosamidines [MNNG and *N*-ethyl-*N'*-nitrosoguanidine (ENNG)], 3 nitrosamides [*N*-methyl-*N*-nitrosourea (MNU), *N*-ethyl-*N*-nitrosourea (ENU), and *N*-butyl-*N*-nitrosourea (BNU)] and a structural analogue of MNNG, *N*-methyl-*N'*-nitroguanidine (MNG). Among the alkyl sulfates and alkanesulfonates, cytotoxicity was found to decrease with the size of the alkyl group: DMS>DES; MMS>EMS>iPMS. The mutagenicity based on mutants induced per unit mutagen concentration was DMS>DES; MMS>EMS>iPMS. However, when comparisons were made at 10% survival, mutagenic potency was: DES>DMS; EMS>MMS>iPMS. Among the nitroso compounds, the order of the mutagenicity based on 10% survival was MNNG>ENNG>MNU>ENU>BNU. This is the same order of potency as observed for mutation induction per unit concentration of mutagen. MNG is not mutagenic (7-9; Couch, San Sebastian, and Hise, unpublished).

Heterocyclic Nitrogen Mustards - ICR Compounds (Total of 10). A

series of heterocyclic nitrogen half-mustards, the ICR-compounds, has been developed at the Institute for Cancer Research as antitumor agents. Apparently, the biological activities of these compounds are associated with their ability to intercalate and covalently bind nucleic acid. Ten ICR-compounds (ICR-191, -170, -292, -372, -340, -191-OH, -170-OH, -292-OH, -372-OH, and -340-OH) have been studied. The 2-chloroethyl side chain of the first 5 compounds (e.g., ICR-191, etc.) has been replaced by a hydroxy group in the latter 5 (e.g., ICR-191-OH, etc.). The 10 compounds differ in the heterocyclic nucleus (methoxyacridine for ICR-191 and -170, benz(*a*)acridine for -292, and azaacridine for -372 and -340) and the alkylating side chain (the same secondary amine for ICR-191 and -372,

and the same tertiary amine for -170, -292, and -340). Those with 2-chloroethyl side chains are highly mutagenic, with the tertiary amines 3 to 5 times more mutagenic than the secondary amines. The 5 hydroxy derivatives are nonmutagenic, but remain highly toxic, indicating that although the 2-chloroethyl group (nitrogen half-mustard) is needed for mutagenicity, its replacement with a hydroxy group does not alter cytotoxicity. Cytotoxicity and mutagenicity of ICR-compounds appear to be dissociable (32, 33; Fuscoe, O'Neill, and Hsie, unpublished).

Aromatic Amines (Total of 5). Many aromatic amines are human carcinogens. We have shown that the carcinogens 2-acetylaminofluorene and its -hydroxy- and -acetoxy derivatives are mutagenic, while fluorene, a noncarcinogenic analogue, is nonmutagenic. 1-hydroxy-2-acetylaminofluorene appears to be mutagenic at a very high concentration in one preliminary experiment (Hsie, Sun, and Brimer, unpublished).

Miscellaneous Compounds (Total of 13). Three commonly used organic solvents (acetone, dimethyl sulfoxide, and ethanol) are noncarcinogenic and do not appear to be mutagenic. All four metabolic inhibitors (cytosine arabinoside, hydroxyurea, caffeine, and cycloheximide) are nonmutagenic in a preliminary study without coupling with the metabolic activation system. Hydrazine and hycanthone appear to be direct-acting mutagens. N^6, O^2 -dibutyryl adenosine 3':5'-phosphate, an analogue of adenosine 3':5'-phosphate and an important effector of growth and differentiation in many biological systems, is not mutagenic. The pesticides Captan and Folpet are mutagenic. The mutagenicity of an artificial sweetener, saccharin, appears to be variable; its determination is complicated by the requirement of high concentrations to yield any biological effect (O'Neill and Hsie, unpublished).

Correlation of Mutagenicity in the CHO/HGPRT Assay with Reported Carcinogenicity in Animal Tests

Among a total of 109 chemical and physical agents studied, at different stages of completion, 56 have been reported to be either carcinogenic or noncarcinogenic in animal studies. Mutagenicity in the CHO/HGPRT assay of 54 of these agents correlated with documented animal carcinogenicity. The concurrence (i.e., known carcinogens are mutagenic and noncarcinogens are nonmutagenic in CHO/HGPRT assays) of each class of agents so far tested is 100% except for nitrosamines and relatives (93%) and ICR compounds (83%) (Table 4). The existence of a high correlation [54/56 (96.43%)] between mutagenicity and carcinogenicity speaks favorably for the utility of this assay in prescreening the carcinogenicity of chemical and physical agents. However, this result should be viewed with caution, since so far only limited classes of chemicals have been tested and some of the preliminary results remained to be confirmed.

A possible false negative was formaldehyde, which has been shown to be either carcinogenic or noncarcinogenic depending on the way test animals are exposed to it. An apparent false positive was ICR-191, a potent mutagen for microorganisms and CHO and other mammalian cells, which has been shown to be noncarcinogenic in a recent study.

A Study of EMS Exposure Dose: Differential Effects on Cellular Lethality and Mutagenesis

Earlier, we found that EMS-induced mutation frequency to TG resistance in cells treated for a fixed period of 16 h is a linear function over a large range of mutagen concentrations (0.013-0.8 mg/ml), including both

Table 4.
Correlation of Mutagenicity^a in the CHO/HGPRT Assay with
Reported Carcinogenicity^b in Animal Tests^c

Agent ^d	Total no. studied	Concurrence ^e	False negatives ^f	False positives ^g
<u>Energy-technology-related substances</u>				
Polycyclic hydrocarbons	27	6/6 (100%)	0	0
Metallic compounds	15	4/4 (100%)	0	0
Nitrosamines and relatives	16	14/15 (93.33%)	1/15 (6.67%)	0
Quinolines	5	2/2 (100%)	0	0
Physical agents	7	3/3 (100%)	0	0
Subtotal	70	29/30 (96.67%)	1/30 (3.33%)	0
<u>Other Chemicals</u>				
Direct-acting alkylating agents and relatives	11	11/11 (100%)	0	0
ICR compounds	10	5/6 (83.33%)	0	1/6 (16.67%)
Aromatic amines	5	4/4 (100%)	0	0
Miscellaneous compounds	13	5/5 (100%)	0	0
Subtotal	39	25/26 (96.15%)	0	1/26 (3.85%)
All agents	109	54/56 (96.43%)	1/56 (1.79%)	1/56 (1.79%)

Table 4.

(Continued)

^aAgents studied are found to be either mutagenic (regardless of "mutagenic potency") or nonmutagenic. The mutagenicity is assayed either directly or coupled with a metabolic activation system *in vitro* in *in vivo*. In the S₉-coupled assay the microsome used was prepared from livers of Aroclor 1254-induced male Sprague-Dawley rats. The effects of other inducers or of conditions of the activation system have not been investigated extensively and are under study.

^bAgents studied are denoted as either carcinogenic, noncarcinogenic, or uncertain based primarily on published data from USPHS (44) and IARC (23, regardless of "carcinogenic potency". Carcinogenicity data on many compounds is not yet available. The search for such data is admittedly neither exhaustive nor updated.

^cIn part from Table VII of ref. 21.

^dThe data are compiled from all agents studies, excluding those whose carcinogenicity is either unknown or uncertain. Thus, only 56 out of 109 agents studied are compiled in this table.

^eKnown carcinogens are nonmutagenic in CHO/HGPRT assays, e.g. MNNG, ICR-292, Ni, B()P, hycanthone, UV.

^fKnown carcinogens are nonmutagenic in CHO/HGPRT assays, e.g. formaldehyde.

^gKnown noncarcinogens are mutagenic in CHO/HGPRT assays, e.g. ICR-191.

the shoulder region (0-0.1 mg/ml) and the exponentially killing portion (0.1-0.8 mg/ml). To investigate whether EMS-induced mutagenesis can be quantified further, cells were treated with several concentrations of EMS for intervals of 2-24 h. Mutation induction increased linearly with EMS concentrations of 0.05-0.4 mg/ml for incubation times of up to 12-14 h. However, cell survival decreased exponentially with time over the entire 24-h period. This difference in the time course of cellular lethality vs mutagenicity might be due to the formation of toxic, nonmutagenic breakdown products in the medium with longer incubation times, or it might reflect a difference in the mode of action of EMS in these two biological effects. Further studies using varying concentrations (0.05-3.2 mg/ml) of EMS for 2-12 h showed that the manifestation of cellular lethality and mutagenesis occurs as a function of EMS exposure does in that the biological effect is the same for different combinations of concentration multiplied by duration of treatment which yield the same product. From these studies the mutagenic potential of EMS can be described as 310×10^{-6} mutants (cell mg ml⁻¹ h)⁻¹. Thus, the CHO/HGPRT system appears to be suitable for dosimetry studies which are essential for our understanding of the molecular mechanisms involved in mammalian mutagenesis (31).

Screening for the Mutagenicity of Fractionated Synthetic Fuel

In addition to studying the mutagenicity of individual environmental agents such as polycyclic hydrocarbons, quinolines, nitrosamines, metallic compounds, etc., we have found that the CHO/HGPRT assay can detect the cytotoxicity and mutagenicity of a crude organic mixture, in this case 3 subfractions of a crude synthetic oil (fractionated by M. R. Guerin of the

Analytical Chemistry Division, ORNL) supplied by the Pittsburgh Energy Research Center. The acetone effluent (which contains tentatively identifiable heterocyclic nitrogen compounds) derived from the basic fraction is most mutagenic in the presence of a metabolic activation system (Table 5) (Hsie and Brimer, unpublished). Earlier, it appeared that the extreme toxicity of the unfractionated crude fuel prevented meaningful mutagenicity studies in the CHO/HGPRT system (Hsie and Brimer, unpublished). The chemistry (14), mutagenicity in microbial systems (12), environmental testing (13) of the Synfuel are presented elsewhere in this Symposium.

Preliminary Development and Validation of the CHO Genetic Toxicity Assay for the Simultaneous Determination of Cytotoxicity, Mutagenicity, Chromosome Aberrations, and Sister-Chromatid Exchanges

We have so far shown that CHO cells are useful for studying the cytotoxicity and mutagenicity of various individual physical and chemical agents and a crude organic mixture. The CHO cells and other hamster cells in culture were also found to be suitable for studying carcinogen-induced chromosome and chromatid aberrations (1, 15, 24, 28) and sister-chromatid exchanges (1, 34, 41). In our preliminary studies, we have found that these assays are useful in evaluating the cytogenic effects of B(a)P and DMN when CHO cells are coupled with the standard microsome preparation described earlier (San Sebastian and Hsie, unpublished).

The successful development and validation of the multiplex CHO cell genetic toxicity system will be extremely valuable from both the scientific and economic points of view in genetic toxicology, because this system will allow the simultaneous determination of 4 distinct biological effects:

Table 5.

Cytotoxicity and Mutagenicity of Subfractions of Synfuel A Basic Fractions^a

Subfraction ^b	Concentration ($\mu\text{g/ml}$)	Relative cloning efficiency (%)		Observed mutation frequency (TG mutants/ 10^6 cells)	
		With S_9	Without S_9	With S_9	Without S_9
Benzene	0.25		92		4
	1		102		<1
	2.5		117		1
	10	109	91	<1	1
	25		90		16
	50		71		13
	100	<0.2	0.2	-	25
Isopropanol	0.25		95		1
	1		94		1
	2.5		103		6
	10	108	95	<1	4
	25		102		16
	50		82		7
	100	<0.2	58	-	2
Acetone	0.25		89		6
	1		101		5
	2.5		93		<1
	5		100		9
	10	58	96	13	22
	25	22	56	6	46
	50	<0.3	4	15	49
	100	<0.2	<0.2	-	135

Table 5 (cont'd).

Subfraction ^b	Concentration (μg/ml)	Relative cloning efficiency (%)		Observed mutation frequency (TG mutants/10 ⁶ cells)	
		With S ₉	Without S ₉	With S ₉	Without S ₉
Controls					
EMS	200			279	-
B(a)P	.8			-	557
Solvent		100	100	4	9

^aUnpublished data of Hsie and Brimer.

^bSee ref. 14 for details about the chemical separation of Synfuel A.

cytotoxicity or *cloning efficiency* measures the reproductive capacity of a single cell to develop into a colony; *single gene mutagenesis* involves changes in the nucleotide sequence of DNA of a specific gene resulting in the acquisition of a novel or altered phenotype; *chromosome aberrations* involve microscopically identifiable changes in the number and/or structure of the chromosome; and *sister-chromatid exchange* measures the extent of double-strand exchange in the DNA duplex after breaks and rejoining of subunits of chromatids each of which consists of one DNA duplex.

SUMMARY AND CONCLUSIONS

Conditions necessary for quantifying mutation induction to TG resistance, which selects for >98% mutants deficient in the activity of HGPRT in a near-diploid CHO cell line, have been defined. Employing this mutation assay, we have determined the mutagenicity of diversified agents, including 11 direct-acting alkylating agents, 16 nitrosamines, 10 heterocyclic nitrogen mustards, 15 metallic compounds, 5 quinolines, 5 aromatic amines, 27 polycyclic hydrocarbons, 13 miscellaneous compounds, and 7 ionizing and nonionizing physical agents. The direct-acting carcinogen MNNG is mutagenic, while its noncarcinogenic analogue *N*-methyl-*N'*-nitroguanidine is not. Coupled with the rat liver S₉ activation system, procarcinogens such as nitrosopyrrolidine, B(α)P and 2-acetylaminofluorene are mutagenic while their analogues 2,5-dimethylnitrosopyrrolidine, pyrene, and fluorene are not. The mutagenicity of the 56 agents documented to be either carcinogenic or noncarcinogenic correlated well [54/56 (96.43%)] with the reported animal carcinogenicity. A possible false negative was formaldehyde and a false positive was ICR-191. Preliminary studies on a synthetic crude oil show that the acetone effluent

(tentatively identifiable as heterocyclic nitrogen compounds) derived from the basic fraction of Synfuel A is the most mutagenic fraction. Thus the assay appears to be applicable for monitoring the genetic toxicity of crude organic mixtures in addition to diverse individual chemical and physical agents. The quantitative nature of the assay enables a study of EMS exposure dose: the mutagenic potential of EMS can be described as 310×10^{-6} mutants (cell mg ml⁻¹ h)⁻¹. It is also feasible to expand the CHO/HGPRT system for quantifying cytotoxicity and mutagenicity to determination of chromosomal aberrations and sister-chromatid exchanges in cells treated under identical conditions. Thus it is possible to study simultaneously these four distinctive biological effects.

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SHORT-TERM BIOASSAY OF COMPLEX ORGANIC MIXTURES:
PART III. ENVIRONMENTAL TESTING

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INTRODUCTION

Environmental Toxicology is a term that conveys different images to different people. In its broadest sense it encompasses all of the research necessary to evaluate the potential ecological effects, to determine the ultimate fate in the environment, and to identify critical pathways to man that might occur as the result of release of a particular material (Table 1). Evaluation of the potential toxicity of the material can be accomplished through three types of testing: short-term bioassays (environmental screening); subacute organismic, population, community, and ecosystem evaluation; and mechanistic studies. The latter two types are resource intense in that they generally require substantial manpower and time commitments. However, they are also essential for developing predictive capabilities regarding the potential for long-term chronic environmental effects resulting from the release of a complex effluent stream. Environmental screening, on the other hand, requires less manpower with results often obtained in less than a week's time. Unfortunately, most environmental screening does not provide the types of data necessary either for predicting potential ecological effects or for estimating potential environmental risks. This paper is limited to a discussion of short-term environmental bioassays and of potential uses of information obtained from this type of testing. The integration of chemical characterization and fractionation into the testing protocol is discussed, as well as the

Table 1.
Description of Various Segments of
Environmental Toxicology Research

^aLC₅₀, the concentration of original test material that will result in mortality of 50% of test organisms in certain time (usually 48 to 96 hours).

^bGR₅₀, the concentration of original test material that will reduce the growth of the test organisms during a certain time (usually 48 hours).

rationale for selecting appropriate test systems. Two of the systems currently employed are described.

Short-term bioassays have the potential for providing toxicity data for three sets of users. They can (1) provide guidance to control technologists and waste management personnel regarding which components of a complex mixture are biologically active and, hence, may require removal before effluent discharge; (2) provide guidance to the environmental scientist in determining which components of a complex mixture require further evaluation in subacute and mechanistic studies; and (3) provide semi-quantitative hazard assessment data when comparisons are made to standard reference compounds or toxicity data from other complex mixtures.

USE OF A BATTERY OF TESTS

A major consideration in the environmental testing of complex chemical mixtures is that such mixtures contain chemicals of a wide variety of classes and an equally wide range of concentrations potentially capable of causing additive, antagonistic, or synergistic responses in test organisms or at specific receptor sites. Such interactions may be a function of the combined dose of toxicants or of the inherent genetic susceptibility of a particular target organism. The genetic susceptibility of an organism may be due to (1) the presence or absence of effective barriers to absorption or translocation of a chemical; (2) the selective accumulation of the chemical in a bound or inactive form, or in tissues remote from a receptor site; (3) the presence or absence of the ability to detoxify the substance through biotransformation; (4) the existence or absence of specific target or receptor systems in exposed cell systems;

or (5) some combination of the above factors (1). In addition, since a specific compound may influence different biologic receptor sites within an organism through a variety of pathways, the problem of environmental hazard assessment is extremely complex.

To establish with absolute certainty the degree of hazard posed by a particular material would require testing of all potential target organisms for their various responses. In the light of the large number of complex mixtures entering the environment each year which require evaluation for their potential hazard, it is readily apparent that the costs, both of time and money, for such an option prevent the making of assessments in a realistic time frame. At the other end of the spectrum, however, predictions concerning potential hazards based on the response of a single species to a chemical mixture have an unacceptably low level of certainty or dependability (because of differential species, responses, etc.).

The task then is to arrive at some intermediate point in cost and effort which permits an acceptable estimate of hazard potential. One method of increasing the level of confidence is to use a series of test systems and develop a hazard estimate based on pooled results from these systems. This group of systems would ideally attempt to represent as broad a spectrum as possible of taxa of organisms, tissue ages, growth forms, routes of exposure, and environmental variables. In this manner, we anticipate being able to obtain estimates of potential hazard which will better reflect the range of response variability expected from a normal population of organisms.

CRITERIA FOR TEST SYSTEM SELECTION

For a specific system to be functional in environmental screening it must be of short duration, require minimal quantities of material, and be a standard test system; that is, it must employ an organism for which a large base of toxicological data is available.

The test system must be of short duration not only to minimize manpower costs but also to prevent that confounding interpretation of results might occur from chemical changes in the aqueous media. Chemical separation to provide relatively discrete fractions is costly and time consuming and produces only small amounts of material (2). It is impractical to utilize this approach, for example, for fish requiring several gallons of water per replicate, or plants requiring similar quantities of test media. The final criterion, using a test system for which a large data base already exists, is essential if even a semiquantitative assessment is to be made.

DESCRIPTION OF TEST SYSTEMS EMPLOYED

Bioassays of varying types have been used successfully over a wide range of applications. Prominent among these applications are tests with herbicides, insecticides, and plant hormones that have dealt essentially with effects of specific compounds. When employing a bioassay test in the screening of complex mixtures of chemicals, there are two basic assumptions which are made: (1) the species used will show an injury response in proportion to the concentration of the biologically active chemical species, and (2) the response obtained are reproducible (3).

The two species used in the experiments discussed in this paper are the zooplankter, *Daphnia magna*, and the radish, *Raphanus sativus*. The

parameter used in the zooplankton system is the 48-hr LC_{50} . Although these aquatic organisms are easily cultured in the laboratory, they have been found to be sensitive to aquatic pollutants. Four *D. magna* are placed in 80 ml of each toxicant test solution in 100-ml beakers covered with watch glasses. The small numbers of test organisms and the small volumes of media used are necessitated by the small quantities of test materials (often less than one gram) that are derived from the chemical fractionation procedure. Water temperature is maintained at $22 \pm 0.5^{\circ}C$ by placing the beakers in an environmental chamber. Photoperiod is maintained under a 12-hr light/dark regime. Toxicant solutions are prepared with filtered spring water (pH 7.8, alkalinity 119 mg/liter, hardness 140 mg/liter). All tests are run in triplicate. Serial dilutions with each concentration being 60% of the previous one are made for each test material. Controls of spring water without added toxicant are included. The range of dilutions are selected to bracket the 48-hr LC_{50} , which is obtained by computerized PROBIT analytical procedures (4). The presence of toxic interactions between fractions is determined using the additivity index of Marking and Dawson (5).

Radish seed responses to chemical mixtures are expressed in terms similar to the LC_{50} value used in the *Daphnia* studies. The value obtained represents the concentration of the original mixture (or concentration of specific components in original mixture) which reduced the yield of a specific species by 50% (3, 6, 7), and is expressed as GR_{50} (concentration for 50% growth reduction). Measurement parameters used as estimates of yield include percent germination time to root emergence, fresh weight, and root elongation. A series of seed germination tests (according to

the procedure discussed below) with water blank control solutions adjusted to a range of pH's are performed at different pH levels and serve as controls. The pH of each test material is determined when the material is received. Seed germination tests are then performed on each test material which consists of seeds in petri dishes containing filter paper moistened with the toxicant. Germination percentages are determined, and length, fresh weight, and time to root emergence are measured and compared with control seeds. Toxicants showing inhibitory effects on germination percentages or root growth are diluted and tested as above to determine a GR_{50} . Toxicants showing no effects in the original test are not tested further. Specific fractions of material are also tested to determine GR_{50} .

ISOLATION OF TOXIC COMPONENTS

The sequential procedure employed to isolate and identify the toxic components of complex mixtures is shown in Fig. 1. In the first step the mixtures are screened for acute toxicity (i.e., the Bodean test). Mixtures not found to be acutely toxic do not undergo further testing. This does not mean that potential problems do not necessarily exist with those materials, but identification of such potential problems would need to await chemical screening. Those mixtures found to be acutely toxic are separated by chemical extraction into their organic and inorganic components and tested for acute toxicity. If acute toxicity is found in the organic components (the component is fractionated into acid, base, and neutral fractions). At this time the relative amount of each fraction in the total organic component is also determined. The toxicity of each fraction is determined and its contribution to the toxicity of the original mixture calculated by relating the relative toxicity of the fraction to its concentration in the original mixture. If

Fig. 1. System for the isolation and identification of toxic compounds of complex mixtures.

further isolation of the actual toxic compounds of the organic fractions is desired, subfractionation and testing can be performed. Up to 14 such subfractions have been separated from synthetic fuel process effluents for use in mutagenesis testing, and these same subfractions could be produced for acute toxicity testing (8). Ultimately, tests with specific compounds can be used to evaluate the toxicity of individual chemical components of the mixture.

A different approach is used to identify the toxic components of the inorganic fraction of the complex mixture. If the inorganic fraction is found to be acutely toxic, it is chemically characterized to determine its composition. The toxicities of the individual components are ascertained and the contribution of each component to the toxicity of the original mixture assessed by the same method used for the organic fraction (Fig. 1).

In the last step of this environmental screening an effort is made to determine whether the testing procedure has accounted for all of the toxicity of the original complex mixture. The individually identified inorganic components present in the original mixture are combined to produce a "reconstituted" mixture. The acute toxicity of the reconstituted mixture is tested and compared to the toxicity of the original mixture.

The procedure outlined above has been used to identify the toxic components of several types of synthetic fuel aqueous effluents. The data from two are presented in this paper as examples. They include one in which inorganic components were the most toxic and one in which organic components were the most toxic. Only data from the *Daphnia* system are presented. Both of the effluents tested are complex mixtures of hundreds

of individual organic and inorganic compounds which are by-products of coal conversion processes. The first material tested was an untreated process-water effluent from the Solvent Refined Coal (SRC) pilot plant in Ft. Lewis, Washington. The second material tested consisted of two effluents, untreated and biologically treated Hydrocarbonization (HCZ) process-water from the process development unit (PDU) at the Oak Ridge National Laboratory.

The results of the application of the acute toxicity testing protocol to the SRC effluent revealed the total effluent to be acutely toxic to *Daphnia*, with a 48-hr LC_{50} estimated to be at a dilution of 15.7% (9). The inorganic portion of the effluents was found to be non toxic; that is, concentration was necessary before an LC_{50} could be produced. Further testing required chemical separation. Of the three fractions extracted from the organic portion of the effluent, the neutral fraction had the highest toxicity with a 48-hr LC_{50} of 9 mg/liter (Table 2). The 48-hr LC_{50} 's of the acid and base fractions were 29.5 mg/liter and 45.8 mg/liter, respectively. Based on the concentrations of the individual fractions in the SRC effluent, the toxicity contribution of each fraction to the toxicity of the whole SRC effluent was calculated (Table 2). These toxicity contributions ranged from 53% for the acid fraction to 3.6% for the base fraction.

One of the values of being able to test the whole effluent as well as the various fractions is that toxic interactions between fractions can be detected and quantified. Testing of whole effluents (before fractionation) and reconstituted effluents enables gross evaluation of the role of the fractionation scheme in producing artifacts in the effluent com-

Table 2.

Acute Toxicity of Organic Components
of Untreated SRC Effluents

Components	Acid	Neutral	Base
Concentration in effluent (mg/l)	99.5	24.8	10.6
Composition of total effluent (%)	28.5	7.1	3.0
<i>Daphnia</i> (48-hr LC ₅₀ in mg/l)	29.5	9.0	45.8
Relative contribution to effluent toxicity (%)	53.0	43.4	3.6

ponents. The results of an experiment using effluents from an SRC facility (Table 3) suggest that neither toxic interactions or artifact formation occurred in the SRC effluents. The 48-hr LC_{50} 's of the whole effluent and the reconstituted effluent were found to be 15.7% and 15.5%, respectively, calculated as a percent dilution of the effluent (Table 3). These values were not significantly different (t , $P = 0.05$), and indicated both that (1) the chemical fractionation procedure did not alter the toxicity of the fractions and (2) all of the toxicity of the effluents was accounted for in the four fractions. Using the method of Marking and Dawson (5), the additivity index was 0.03 for the whole SRC effluent and 0.00 for the reconstituted effluent (Table 3). These values were not significantly different (t , $P = 0.05$), indicating that the toxicities of the individual fractions were directly additive within the effluents. As a comparison, if each fraction acted independently, an additivity index of 0.95 would be predicted.

The second example of the environmental screening approach deals with effluents from the ORNL Hydrocarbonization Unit (HCZ). In the first step of the toxicity screening of the HCZ effluent, the toxicities of the untreated and treated effluents were compared. The treatment process was determined to have reduced the toxicity of the effluent by 99% in the *Daphnia* system. Further testing showed that the inorganic portion of the effluent contributed 99.5% of the toxicity (Table 4), with the remaining 0.5% contributed by phenols. Of the inorganic constituents, ammonia was the principle toxic agent, contributing 96% of the toxicity.

The determination of the interactions between the HCZ effluent components demonstrated a less than additive or antagonistic behavior.

Table 3.

Contributions of SRC Fractions to Total Toxicity of SRC Effluent

Fraction	48-hr LC ₅₀	Fraction concentration (mg/l) at 48-hr LC ₅₀ concentration		Contribution to whole ₃ effluent toxicity (%)		Additive index	
		Reconstituted effluent	SRC effluent	Reconstituted effluent	SRC effluent	Reconstituted effluent	SRC effluent
Acid	29.5	15.2	15.6	51.5	51.3	0.00 ²	0.03 ²
Base	45.8	1.6	1.7	3.5	3.5		
Neutral	9.0	3.8	3.9	42.2	41.9		
Inorganic	1000 ¹	32.7	33.6	3.0	3.3		

¹ No mortality occurred at highest concentration in test series. 1000 mg/l is assumed to be a very conservative estimate of the 48-hr LC₅₀.

² Additive index > 0 = more than additive toxicity (synergism)
 Additive index = 0 = simple additive toxicity
 Additive index < 0 = less than additive toxicity (antagonism)

³ The contribution of a fraction to the toxicity of the whole effluent is given by the expression:

$$\frac{\frac{Am}{Ai}}{\frac{Am}{Ai} + \frac{Bm}{Bi} + \dots + \frac{Zm}{Zi}} \quad \text{where A, B --- Z, are the fractions, i the toxicities (LC}_{50}\text{'s) of the individual}$$

fractions, and m the fraction concentration at the LC₅₀ concentration of the whole effluent.

Table 4.
Acute Toxicity Data for Hydrocarbonization

	Organics	Inorganics
Concentration in effluent (mg/l)	110	2846
Relative quantity (%)	3.7	96.3
<i>Daphnia</i> (48-hr LC ₅₀ in mg/l)	774	31.7
Relative contribution to effluent toxicity (%)	0.5	99.5

This indicated that the sum of the toxicities of the effluent components calculated individually was greater than when they were present together in the effluent.

SUMMARY AND CONCLUSION

The previous discussion has shown how coupling of chemical separation and fractionation with environmental testing is able to identify those materials most biologically active, at least, in acute toxicity. In the case of the SRC effluent the primary activity was attributed to the acidic organic fraction, where the phenolics are located. Because of the relative removal efficiency of phenolics through chemical stripping and biological treatment (10), near-field acute toxicity from effluent releases would not be expected from aqueous effluents released from a facility similar to the SRC pilot plant. The ultimate conclusion concerning potential effluents from the HCZ Process Development Unit is the same, although the biologically active component was the inorganic NH_3 .

Environmental testing can help the control technologist and waste management engineer identify materials of potential environmental consequence. These screening activities are not intended to, and should not, replace subacute toxicity and mechanistic toxicity studies for developing data for predictive purposes with respect to chronic low level effects in the far-field environment.

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THE ROLE OF *DROSOPHILA* IN CHEMICAL MUTAGENESIS TESTING*

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INTRODUCTION

An important question facing our society is the impact of numerous chemical insults on the health of man and his environment. Faced with a staggering array of chemicals and enormous testing costs, only a few chemicals can be tested for possible carcinogenic effects. Recent results with the *Salmonella*/mammalian microsome mutagenesis assay developed by Ames (2) demonstrating a striking correlation between carcinogenicity and mutagenicity of many chemical compounds offer the possibility that mutagenesis assay systems can provide a quick identification of potential carcinogens. Results from microbial assays can serve as a guideline for further mutagenesis testing as well as identify those compounds requiring more extensive analysis in mammalian systems.

Unquestionably, man is more closely related to other mammals than bacteria and information regarding pharmacokinetics can only be obtained from mammals. Detection of point mutations and small deletions in mammals, however, requires considerable costs, time, and labor; thus the number of chemicals that can be investigated is restricted. Other mammalian assay systems which rely solely on chromosome breakage do not suffer from these disadvantages, but their utility as diagnostic tests are questionable in light of recent results obtained in *Drosophila*. Vogel (10) has shown that many chemicals are very effective in producing point mutations and small deletions but do not produce chromosome breakage effects at all, while others produce chromosome breakage, but only at concentrations much higher than that required to produce point mutations. Such compounds would appear safe in any assay which measured only chromosome breakage.

Reliance on the results from a single mutagenic assay system is rather risky. It seems preferable, in our opinion, to use a battery of tests (the tier approach) which would include the rapid microbial assays as well as mammalian systems. Also the use of *Drosophila* as a bridge between the microbial and mammalian assays has many desirable features as will be discussed in the following paragraphs.

ADVANTAGES OF *DROSOPHILA* AS A TEST ORGANISM

As a mutagenesis test organism, *Drosophila* is not as economical nor as rapid a screen as the microbial assays but few *higher organisms* offer the economy and short generation time that can be achieved with *Drosophila*. *Drosophila* mutagenic assays can be used in pre-screening tests but perhaps the most useful approach is to use them as a confirmation of results obtained in microbial assays and extension of the analysis to include genetic end-points which are unattainable in the microbial systems.

Due to the availability of a wealth of tester strains, the assessment of a variety of induced genetic changes is readily obtainable in *Drosophila*. Genetic end-points easily scored cover a wide spectrum including point mutations and small deletions, translocations, chromosome loss, non-disjunction and genetic recombination. Thus mutagenic assays in *Drosophila* can detect genetic damage due to both point mutation and chromosome breakage.

In many mutagenesis screening programs, method of exposure is often an important parameter. In those cases, the advantage of using *Drosophila* again becomes apparent as the chemical compound may be administered via feeding, injection, inhalation or direct treatment of sperm. Feeding and injection are the most commonly used methods but inhalation of a gas or aerosol is also very effective. A serious disadvantage of the aerosol

method is that a considerable volume of the chemical agent is required; a disadvantage which is overcome by the injection technique where only microliter quantities are needed. For a more thorough discussion of the advantages and disadvantages, see the review by Lee (8). Whatever method is chosen it should be kept in mind that a negative result may be due to the particular method of exposure (17), and in these cases an alternate route of administration should be used.

Chemical mutagens often show a cellular specificity (3) and failure to detect mutagenic activity may result from a stage-specific response. In *Drosophila* the mutational response to a chemical insult in different germ cell stages may be studied by the brood pattern analysis (a technique whereby the mutation frequency of successive mating is obtained). Though somewhat more time consuming the additional information gained can give a more detailed picture of the mutagenic activity of a chemical. The method of brood pattern analysis developed for use in radiation genetic works equally well with chemicals except that with chemicals one has to consider the lingering effect of chemicals which remain in the body and which results in exposure of germ cells over a longer period of time.

Another feature which adds to the utility of *Drosophila* is the presence of a mixed function oxidase system which is similar to that of the mammalian liver in its ability to activate indirect mutagens. In recent years considerable attention has been focused on the metabolism of certain drugs and pesticides by insects and it has been shown that the crucial step in such metabolism is a oxidative attack by mixed function oxidases which can be isolated as a microsomal fraction (1, 5). *Drosophila* are insects and the evidence that they also possess microsomal activities

similar to the mammalian liver was indirect. It was based largely on the fact that some forty-fifty compounds that require metabolic activation are, when tested in *Drosophila*, effective in inducing recessive lethals (11). These compounds fall into several different groups with widely differing structures. From these studies one can conclude that the *Drosophila* enzyme systems are similar to the mammalian systems in the versatility and lack of substrate specificity. Recently, Baar *et al.* (4) have presented evidence that isolated *Drosophila* microsomes possess cytochrome P450 and aryl hydrocarbon hydroxylase activity and Nix *et al.* (9) have shown that *Drosophila* microsomes are capable of activating numerous promutagens when they are substituted for rat liver microsomes in the *Salmonella* histidine reversion assay.

THE USE OF DROSOPHILA IN THE MUTAGENIC ANALYSIS OF COMPLEX MIXTURES

Potential health effects of existing, as well as new, fuel technologies have become of increasing concern. Epler *et al.* (6, 7) have used the *Salmonella*/mammalian-microsome test system to assay environmental effluents and crude products from the synthetic fuels technology. Complex mixtures were fractionated, and each fraction was tested for possible mutagenic activity. Such procedures identified several fractions as mutagenic and as candidates for further biological testing. Experiments described here represent an attempt to extend their observations to a eucaryotic organism and to identify other genetic effects. In addition we describe the isolation of a crude *Drosophila* microsome fraction and the use of such fractions in the *Salmonella* mutagenicity test system.

Oregon-R wild-type males and Muller-5 [In (1) $sc^{S1L}sc^{8R}+S$, $sc^{S1}sc^{8a}B$] males and females were collected as needed from the Oak Ridge

stock collection. The *Salmonella* strain used was TA98 (*hisD3052*, *uvrB*, *rfa*, frameshift plus R factor), obtained from Dr. Bruce Ames, Berkeley, California.

Synthetic fuel fractions were dissolved in DMSO and then diluted with a sterile sucrose solution to a final concentration of 2% sucrose and 4% DMSO. A glass-fiber filter paper was placed into an empty glass vial and then saturated with 175 microliters of the appropriate test solution. Wild-type (Oregon-R) males, 1-2 days old, were starved for 5 h, placed in the vials containing the test solution (25 males per vial), removed after 24-48 h and mated to virgin Muller-5 females. In the brood-pattern analysis treated males were mated for five successive 3-day broods. F₁ females were mated and progeny scored for the presence of X-linked recessive lethals.

In the *Salmonella*/microsome mutagenicity tests the standard procedures given by Ames *et al.* (2) were employed except that *Drosophila* microsomes were substituted for rat-liver microsomes. Concentrations of buffer and cofactors were as previously described by Ames.

For the isolation of *Drosophila* microsomes, wild-type (Oregon-R) flies were grown on standard media which contained no live yeast. Adults were collected 7-10 days after emergence, etherized, and placed on ice. Two volumes (w/v) of ice-cold potassium phosphate buffer (pH 7.5) was added and flies were homogenized by gently pounding in a mortar until a smooth brei was formed (approximately 120-150 strokes with the pestle). The homogenate was filtered through four layers of cheesecloth and the filtrate was spun at 750 *g*. The resulting supernatant was spun two times at 10,000 *g* and after the final spin the supernatant was immediately tested in the *Salmonella* system; the remainder was frozen at -70°C.

Our primary concern in the assay of the mutagenic effects of the synthetic fuels was to confirm the results in a higher organism and then if possible to extend the analysis to include other genetic effects. For this purpose we selected the X-linked recessive lethal assay as it has been shown to be the most sensitive in *Drosophila*. Vogel (10) has carried out a comparative study of the frequency of induction of recessive lethals, dominant lethals, and chromosome loss by various concentrations of different mutagens. For all mutagens studied the recessive lethal assay was the most sensitive. We find a similar result for a series of cyclic nitrosamines as shown in Table 1. In addition we find a very close correlation between mutagenicity as measured by the X-linked recessive lethal assay and carcinogenicity in rats.

Since the crude synthetic fuel is toxic to *Drosophila*, only selected fractions could be tested. The results of a brood pattern analysis is shown in Table 2. Fractions 7 and 9 are ineffective in inducing X-linked recessive lethals in broods 1-3, although fraction 9 seems to be slightly mutagenic for spermatogonial cells. Using fraction E, the acetone soluble portion of a more highly purified subfraction of the combined basic fractions from the Stedman fractionation scheme, (6,7), we find a significant increase in the frequency of lethals in broods 1 and 2 but not brood 3. This suggests that fraction E is an effective mutagen for mature sperm and spermatids but not meiotic cells. With this in mind, we then fed fractions 7, 9, and 14 at several different concentrations and monitored the production of X-linked recessive lethals in mature sperm and spermatids. Inspection of Table 3 reveals that fraction 7 is ineffective at all concentrations tested. Fractions 9 and 14, at the two lower concentrations

Table 1.

Induction of X-linked Recessive Lethals and Sex Chromosome
Loss in *Drosophila* by a Series of Cyclic Nitrosoamines

Compound	Mutagenicity in <i>Drosophila</i>		Carcino- genicity in rats*
	X-linked recessive lethals	Chromosome loss	
Nitrosopiperidine (NP)	+	-	+
2,6-Dimethyl NP	-	-	-
2-Methyl NP	+	-	+
4-Methyl NP	+	-	+
3,4-Dichloro NP	+	-	+
Nitrosopipericolic acid	-	NT	-
Dinitrosopiperazine	+	NT	+
2,3,5,6-Tetramethyl- dinitrosopiperazine	-	NT	-
Nitrosomorpholine	+	NT	+

*The carcinogenicity data was kindly provided by Dr. W. Lijinsky
NT = Not tested.

Table 2.

Brood Pattern Analysis of X-linked Recessive Lethals
Induced in *Drosophila melanogaster* by Synthetic Fuels

Fraction	Conc. fed ($\mu\text{g/ml}$)	Brood	Chromosomes tested	Lethals	% Lethals
Control	---	1	1334	3	0.22
		2	1839	4	0.22
		3	1318	1	0.08
		4	803	2	0.25
7. BI _a *	994	1	1071	0	0.00
		2	1039	3	0.29
		3	984	3	0.30
9. B _E *	1059	1	1083	4	0.37
		2	1197	1	0.08
		3	1230	2	0.16
		4	1295	7	0.54
E ⁺	500	1	1661	11	0.66
		2	1686	13	0.77
		3	1780	3	0.17

*Basic fractions isolated from a crude synthetic fuel product by the Stedman fractionation procedure.

⁺Acetone subfraction of Stedman basic fraction which is further fractionated by LH-20 (Epler *et al.*, these proceedings).

Table 3.

Induction of X-linked Recessive Lethals in Mature Sperm
and Spermatids of *Drosophila melanogaster* by
Subfractions of Synthetic Fuel

Fraction*	Conc. fed ($\mu\text{g/ml}$)	Chromosomes tested	Lethals	% Lethals
Control	--	1753	4	0.23
6. SA _w	15.02	1214	2	0.16
7. BI _a	994	1097	0	0.0
	397	1067	3	0.28
	199	1036	1	0.10
9. B _E	1059	1346	0	0.20
	423	702	3	0.42
	212	861	4	0.46
14. Neutral	870	1012	2	0.20
	435	1185	6	0.51
	218	1065	5	0.47

*For explanation of fractions see Epler, *et al.*, these proceedings.

tested, increase the frequency of lethals 2-fold over the spontaneous level but this is not statistically significant. In order to show a significant doubling with a critical region of 0.05 one would need to test 12,000-15,000 chromosomes. From these results we can conclude that the basic fractions (7, 9), which are mutagenic in the "Ames" assay, induce at the most only a 2-fold increase in the frequency of X-linked recessive lethals in *Drosophila melanogaster*. Further purification of these fractions results in a subfraction which shows a slight mutagenic activity in *Drosophila*; it induces an increase of 3-4x over the spontaneous level. Thus, we confirm the mutagenic activity of fractionated products of synthetic fuels in a eucaryotic organism but the activity is very low compared to that obtained in the microbial assay.

One of the advantages of *Drosophila* as a mutagenesis test organism is the presence of metabolic activation system. By substituting isolated *Drosophila* microsomes for rat liver microsomes in the *Salmonella*/histidine reversion assay one can correlate mutagenic activity of a chemical compound *in vivo* with the ability of isolated microsomes to activate the chemicals. Results of experiments in which we tested the ability of *Drosophila* microsomes to activate fractions 7, 9, and E are shown in Figure 1 and Table 4. Instead of Aroclor-induced rat liver fractions, 400 microliters of *Drosophila* 10,000 g supernatant was used; all other procedures were as described by Epler *et al.* (6, 7).

In light of the *in vivo* activity, these results are rather surprising. Figure 1 shows the number of revertants/plate plotted versus concentration. For all three fractions we obtained a linear dose-response curve over the concentrations tested. The slope of each induction curve was

Fig. 1. Effect of increasing concentration of synthetic fuel fractions on *his*⁺ reversion in *Salmonella* strain TA98. All reagents were as described by Epler *et al.* (6,7) except that 400 microliters of *Drosophila* S-9 was substituted for rat liver S-9.

●, 7_B, Δ, 9_B, ○, E

Table 4.

Comparison of Mutagenic Activity of Synthetic Oils
Activated by *Drosophila* and Aroclor-Induced Rat Liver Microsomes

Fraction*	Specific activity (rev/mg)	
	Rat liver	<i>Drosophila</i>
7. BI _a *	45,000	30,000
9. B _E	28,900	85,000
E (acetone)	222,000	1,300,000

*See footnotes to Table 2.

determined and these results along with those obtained using Aroclor-induced rat liver are shown in Table 4. It is of interest that *Drosophila* microsomes are just as effective as Aroclor-induced rat liver microsomes in the activation of all three fractions and is even more effective with fractions 9 and E. We have tested several pure compounds in the *Salmonella*/*Drosophila* microsome assay. Of these, 2-acetylaminofluorene and aflatoxin B₁ showed the highest mutagenic activity, 144,000 and 180,000 revertants/mg respectively; thus Fraction E gives almost a 10-fold increase in mutagenic activity over any compound we have thus far tested. In these instances, results with uninduced *Drosophila* microsomes compare very well with those of induced rat liver. Comparisons based on the number of histidine revertants per milligram of S-9 protein are even more striking in favor of *Drosophila* as typical *Drosophila* microsomes preparations contain about one-fourth the protein of induced rat liver microsomes.

The discrepancy between the *in vivo* and *in vitro* mutagenic activity of fractionated complex mixtures is interesting but at this point we have no explanation. One must keep in mind that the metabolism of foreign compounds involve enzymatic pathways which result in toxification as well as detoxification. The balance between the two will often determine whether a generated active metabolite will remain in the cell and exert genetic damage or be detoxified before any damage can be done.

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