Environmental Health Perspectives
Vol. 27, pp. 11–20, 1978

Energy-Related Pollutants in the Environment: Use of Short-Term Tests for Mutagenicity in the Isolation and Identification of Biohazards

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In an effort to gather information on the potential genetic hazards of existing or proposed energy-generating or -conversion systems, we have begun a correlated analytical and genetic analysis of a number of technologies. The work is divided into two phases: one deals with known compounds expected to occur in the environment through energy production, conversion, or use; the other deals with actual samples from existing or experimental processes. To approach the problems of coping with and testing large numbers of compounds, we set up a form of the "tier system." Operating units utilizing *Salmonella*, *Escherichia coli*, yeast, human leukocytes, mammalian cells, and *Drosophila* have been initiated. Various liquid-liquid extraction methods and column chromatographic separations have been applied to crude products and effluents from oil-shale, coal-liquefaction, and coal-gasification processes. Mutagenicity of the various fractions is assayed by means of reversion of histidine-requiring auxotrophs of *Salmonella typhimurium;* comparative studies are carried out with the other genetic systems. In order to incorporate metabolic activation of these fractions and compounds, rat liver homogenates (S-9) are used in the various assays. Results implicate chemicals occurring in the basic (ether-soluble) and the neutral fractions as potential genetic hazards. Chemical constituents of these fractions (identified or predicted) were tested individually for their mutagenic activity.

Introduction

Industrial activity in the modern world has created a large number of chemical pollutants. In addition to obvious toxic effects, chemical pollutants may also produce carcinogenic, mutagenic, or teratogenic effects whose expression may be divorced in time from the actual exposure(s). However, this chemical exposure does not originate from only industrial processes. Table 1 lists a wide variety of human exposure to chemicals that may conceivably have an impact on human health. Thus, thousands, perhaps tens of thousands, of chemicals have the potential for affecting the population. Although the health effects of a small list of chemicals in the environment are being studied extensively, it has become obvious that methods to cut down the research time and expense are necessary to permit evaluation of the large number of potentially hazardous substances.

To investigate the potential genetic (and carcinogenic) hazards associated with the developing synthetic fuel technologies, we initiated a coupled chemical and biological analysis of the products, process streams, and effluents of the existing or proposed energy-generating or -conversion systems. One phase of our investigation deals with known compounds expected to occur in the environment through energy production, conversion, or use; another phase deals with actual samples from existing or experimental processes. To approach the problems of coping with and testing large numbers of compounds, we set up a "tier system" of mutagenicity testing.

The research effort described here is specifically concerned with the question of genetic hazard; but, as has been pointed out recently (*1*), certain micro-

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Table 1. General classes of chemicals to which humans are exposed: potential environmental mutagens.

Drugs
Medicinal
Veterinary
Cosmetics
Pesticides
Stimulants
Food
Additives
Dyes
Preservatives
Sweeteners
Industrial products and effluents
Energy-related effluents
Energy production
Energy conversion
Energy use
Natural products

bial genetic assays (for example, the Ames test with *Salmonella)* show a high correlation between positive results in mutagenicity testing and the carcinogenicity of the compounds under test. The overall need to subject environmental chemicals to mutagenicity testing has been discussed in the Committee 17 Report on Environmental Mutagenic Hazards (*2*). Their key recommendation can be summarized as follows: "Screening should be initiated as rapidly and as extensively as possible." de Serres has discussed the utility of short-term tests for mutagenicity (*3*) and the prospect for their use in toxicological evaluation (*4*). He stresses that the data base of knowledge on untested environmental chemicals should be expanded, but cautions that we are not ready to extrapolate directly from data obtained in short-term tests for mutagenicity directly to man (*4*). Tests on other organisms must be performed to validate and reinforce results from short-term tests, which simply point out potential mutagenic and carcinogenic chemicals and serve to order priorities for further testing in higher organisms.

Battery of Tests: First Tier

Obviously, all of the chemical pollutants in question cannot be subjected to whole-animal testing. The expense in time and money would be overwhelming with genetic testing alone. We felt a scheme had to be developed which would reliably prescreen the genetically hazardous compounds and allow the investigator to select a smaller sample to be thoroughly tested in other systems. One example of such a battery of tests is shown in Table 2. A leveled or tiered system of testing [suggested by Bridges (*5, 6*)] would save considerable time and expense, while bringing the pertinent information to the industrial community and the general population in an organized and rapid manner. If the correlation of genetic damage with potential cancer danger is valid, additional information would be obtained, and conceivably, the choice of compounds to be subjected to extensive carcinogenic testing in the whole animal would be influenced.

Perhaps most important in a tiered system of screening is the initial level of testing. False negatives here presumably would terminate the testing of any particular compounds, while false positives would be clarified by further comparative testing. Thus, the initial test should be a high-resolution, sensitive assay, yet rapid and inexpensive. The *Salmonella* histidine reversion system (*7*) probably fits these criteria best of the currently available short-term assays.

Known mutagens have different mechanisms of action. The *Salmonella* tester strains detect and differentiate the various mechanisms by their spectrum of reversion. Mutagens like nitrous acid alter a single base in the DNA. Subsequent transcription of the altered codon leads to insertion of the wrong amino acid into a polypeptide chain—a missense mutation. Other mutagens (e.g., proflavin) have a mechanism of action that results in the addition or deletion of one or more base pairs in the DNA molecule; these are classified as frame-shift mutagens. The reading frame (one amino acid coded by a triplet of bases) is shifted by the base addition or deletion. Thus, the frame of reference is improperly chosen, and a chain of improper amino acids is coded and synthesized.

The *Salmonella* tester series (obtained through the courtesy of Bruce N. Ames and shown in Table 3) is composed of histidine mutants that revert after treatment with mutagens to the wild-type state (growth independent of histidine). Both missense mutants and frame-shift mutants comprise the set, and their reversion characteristics with a potential chemical mutagen imply the mechanism of action. In addition, the detection scheme yields the highest resolution possible by the inclusion of other mutations: the deep rough mutation, *rfa*, which affects the lipopolysaccharide coat, making the bacteria more permeable; and the deletion of the *uvrB* region, eliminating the excision-repair system (*8*). The procedures with the strains and their use in mutagenicity testing have been discussed in detail by Ames, McCann, and Yamasaki (*7*).

Generalized testing of compounds is accomplished by use of the three standard tester strains, TA1535, TA1537, and TA1538, in combination with the R-factor strains TA100 and TA98 (plasmid-carrying strains with increased sensitivity). Briefly, the compound to be tested is dissolved

Table 2. Batteries of mutagenicity tests arranged by tiers.[a]

Tier	Gene mutation	Chromosomal
Tier I		
	S. typhimurium	Yeast
	Histidine reversion	Gene conversion
	Forward mutation	Mitotic recombination
	E. coli	Plants(?)
	trp⁻ reversion (WP2)	*Allium*
	Mohn system	*Tradescantia*
	Maize (waxy), *B. subtilis*, *E. coli*, *S. typhimurium* } DNA repair assays	
	Yeast	
	Reverse mutation	
	Forward mutation	
Tier II		
	Drosophila	*Drosophila*
	Visibles	X-chromosome loss
	Sex-linked recessive lethals	Nondisjunction
	Mammalian cells (in culture)	Mammalian cells
	Mutagenesis	Chromatid aberrations
	DNA repair	Sister-chromatid exchange
	Mouse	Mouse
	The "spot test"	Dominant lethals(?)
		Heritable translocations
Higher Tiers		
	Mouse	Mouse
	Specific-locus test	Heritable translocations
		Chromosome loss
	New mammalian tests??	

[a] See text for references to various assays.

Table 3. Genotype of *Salmonella typhimurium* tester strains.

	Additional mutations	
Histidine mutation	*rfa*/Δ*uvr*B	*rfa*/Δ*uvr*B/⁺R
hisG46	TA1535	TA100
hisC3076	TA1537	—
hisD3052	TA1538	TA98

in dimethyl sulfoxide or buffer. Concentration is varied over a range of 1-500 μg added per plate except with highly toxic compounds. The various *Salmonella* strains are usually treated by use of the plate-incorporation assay of Ames (*7*), but other modified assays or assays for forward mutation can be used (*9*).

One other feature of the *Salmonella* system is the ease with which metabolic activation can be incorporated into the assay. Spot tests or quantitative plate tests can be performed in the presence of rat or other mammalian liver homogenates so that the mutagen can be metabolized to its ultimate, active form in the *in vitro* short-term test. In assays requiring activation, standard rat liver microsome preparations (*7*) from rats induced with Aroclor 1254 (Monsanto Corp.), sodium phenobarbital, or other inducers are used.

As an initial step in establishing testing procedures, we have investigated the use of the Ames *Salmonella* system with a large number of environmentally important chemicals and effluents, principally those known or predicted to occur in energy use, production, and/or conversion. However, other microbial tests should be used in comparison with the *Salmonella* results. Among these are bacterial systems, such as the *Escherichia coli* WP2 tryptophan reversion assay (*10*), the sensitive fluctuation test described by Green (*11*), and the multiple end point system developed by Mohn (*12*).

For example, the Mohn system, employing a well-characterized mutant of *E. coli* K-12 (343/113), has been utilized in our laboratory to test various compounds that are either identified or suspected to occur in various products and effluents of the synthetic fuel technologies. The mutation screening involves reversion of an auxotrophic marker for arginine requirement to prototrophy (Arg^+), induction of a forward mutation leading to 5-methyl tryptophan resistance (5 MTR), ability to utilize galactose as sole carbon source (gal^+), and a deletion in *gal* R region resulting in a new auxotrophic mutation for lysine requirement (gal^+, lys^-). Results can be compared and correlated with the Ames test results.

A number of bacterial assays detecting effects on strains either proficient or deficient in DNA repair are also widely used in this initial battery of tests. Among these are the "*rec*-assay" of Kada (*13*) in *Bacillus subtilis*, the assay using *E. coli* mutants deficient in DNA polymerase (*polA*$^-$) (*14*), and, again, the *Salmonella* system (*7*) using strains with DNA deficiencies in comparison with normal. The common hypothesis is that bacterial strains that are repair-deficient are much more sensitive to agents that alter cellular DNA than are the parent strain, wild-type or normal for DNA repair functions. Presumably, these same agents that preferentially alter DNA are potential mutagens and carcinogens.

Neurospora (*15*) and yeast (*16*) are also useful tools in mutagenicity screening. Many laboratories utilize the yeast strains developed by Zimmermann for detecting mitotic recombination and mitotic gene conversion along with assays for forward mutation and reverse mutation. We have chosen to develop a comprehensive gene mutation system (*17*) in *Saccharomyces cerevisiae*, somewhat analogous to the bacterial systems described.

A haploid yeast of genotype *a*; *rad2-1; CAN1, his1-7, hom3-10; lys1-1; trp5-48; ade2-1* is the basis of the reversion test system. This strain is defective in DNA excision-repair. General forward mutation can also be detected by selecting canavanine-resistant clones (*CAN1* → *can1*).

Base-pair substitution is monitored by observing the reversion of missense (*his1-7*) and nonsense (*lys1-1, trp5-48, ade2-1*) markers and by the generation of nonsense suppressors (various tRNA loci). Additions or deletions of base pairs are indicated by the reversion of a frame-shift (?) marker (*hom3-10*). Nonsense suppressors can also arise by addition/deletion. Metabolic activation of promutagens is provided by rat liver microsome preparations.

Thus, a battery of short-term microbial tests comprised of a selection of the above or other similar assays yields preliminary information on the question of whether or not the substance under test inflicts genetic damage at the highest level of resolution possible.

Tests with Higher Organisms: A Second Tier

At a second level of testing with selected compounds from the microbial screens (including some negatives) or with compounds of high importance or interest to man, the question becomes one of the mutagenicity of the compound in higher organisms. Because of the expense of these assays in both time and money, the probability of exposure and the actual intake by man may become deciding factors. The key objective of the approach is to gain sufficient information to enable the investigator to compare and eventually extrapolate from one system to another.

The *Drosophila* (insect) system can be utilized as a key assay at this level. The standard Basc, or Muller-5 (*18*), test for sex-linked recessive lethals can be used. Males are exposed to the test compound by feeding, injection, or inhalation of an aerosol, then mated to females, and finally removed after a mating period. Females are allowed to lay eggs for a period of days. F_1 heterozygous females are scored for sex-linked lethals.

The insect assay is a sensitive system, since loci throughout the length of the sex (X) chromosome can mutate to recessive lethals. Furthermore, the organism is diploid and possesses its own metabolizing system for mutagens/carcinogens. Additional genetic and cytogenetic end points can easily be observed and quantitated (e.g., chromosome loss, nondisjunction, translocations, and inversions). Additionally, by mating the treated male to a sequence of females, cells from various stages of spermatogenesis at the time of treatment can be assayed.

Mammalian somatic cell mutagenesis has progressed remarkably during recent years, although considerable development and validation is still necessary. The CHO/HGPRT system developed by Hsie et al. (*19*) appears to be the most reliable and promising. This system can be coupled to rat liver microsomal preparations prepared as for the microbial systems and used to detect the conversion of promutagens (and procarcinogens) to their active forms. DNA repair assays in mammalian cells (*20, 21*) are also highly sensitive and accurate predictors.

Mammalian cytogenetic assays such as the induction of aberrations in chromosomes of leukocytes from human peripheral blood or of mammalian cells *in vitro* are useful screens, as shown by Ishidate (*22*). The relatively new assay of sister-chromatid exchange (*23*) may be a most effective tool with chemical agents. Again, however, exogenous sources of metabolizing enzymes must be added to detect promutagens.

At a second level or tier of testing, preliminary assays with whole animals (the mouse) should be initiated in order to address the question of mutagenic potential for higher organisms. Although the work of the Comparative Mutagenesis Unit at Oak Ridge applies specifically to submammalian systems and cultured mammalian cells, the mouse assay will be briefly outlined here.

The dominant lethal assay (*24*) can be included as a potential screen. In this relatively rapid test for

chromosomal damage, different germ cell stages can be compared with respect to their sensitivity to the sample. This is done by studying uterine contents of females mated to exposed males at various intervals after the exposure. Another potentially useful assay for the whole animal involves the detection of somatic mutations *in vivo*, the so-called "spot test" designed by Russell (*25*). Embryos heterozygous for the same markers that are used in the specific-locus test (*26*) are exposed to the putative mutagen. Genetic changes that uncover the recessive at these loci are detected as spots of altered color in the fur. Since each animal scored represents several hundred cells in which the genetic changes could have occurred, the method combines the advantages of cell culture with an *in vivo* system. It can probably be used as a prescreen to select materials for use in the specific-locus test described below.

Higher Tiers: Risk Assessment (?)

Although only selected compounds would be tested in depth in the mammalian assays, these systems are the closest available bridge to actual risk assessment in human populations (*2*). Based on data accumulated in submammalian systems, gene mutation and cytogenetic data from cultured mammalian cells, and the preliminary whole-animal data from the "second tier," reasonable decisions to carry out more complex and expensive mammalian testing can be made. Data from, e.g., heritable translocations and the specific locus test in the mouse might prove most useful. The search for heritable genetic changes in mammalian germ cells may be the most critical and definitive one for the assessment of genetic risk to human beings.

The heritable translocation method (*24*) measures the frequency of chromosome breakage and rearrangement that is transmitted to the next generation. The test is a sensitive and reliable procedure for measuring breakage and exchange of parts between chromosomes induced in male germ cells. When a sperm carrying chromosome interchange is used in fertilization, the resulting progeny is heterozygous for the translocation and produces two types of gametes, balanced and unbalanced, in approximately equal proportions. Both types of gametes are capable of fertilization, but the unbalanced gametes result in embryonic lethality—i.e., translocation heterozygotes are only half as fertile as normal mice. In the heritable translocation procedure progeny of treated parents are simply tested for sterility and partial sterility. Confirmed sterile and partially sterile progeny are then verified cytologically for presence of a translocation. Thus, the heritable translocation procedure generates meaningful information for evaluating chromosome aberration hazards of test agents to the human population because it measures transmissible genetic damage.

In the specific-locus method, induced mutations can be objectively detected in the first generation by mating exposed mice to a stock carrying seven recessive markers in homozygous condition (*26*). This is the only practical method available to detect gene mutations in the germ cells of mammals and to determine their rate of induction by a given agent.

The use of a variety of tests on a series of selected compounds under standard conditions should allow a meaningful comparison between the less expensive and more rapid tests on microorganisms and the more definitive tests for effects in the mammal. Such a comparison will allow greater confidence to be placed in the tests on lower organisms, thus reducing the future effort required. Only such a comparative approach will allow rational decisions to be made between different testing and screening schemes.

The assays used here are discussed in detail in the series edited by Hollaender (*27*). Note that both gene mutation and chromosomal assays are included and that testing involves a move from the simple microbial tests (*Salmonella*, *E. coli*, and yeast), to tests with higher organisms (*Drosophila*, mammalian cells, human leukocytes), to tests with the whole mammal, presumably the mouse. Thus, if the testing priorities are established on the basis of use by or impact on man, complete testing may be reduced to groups of environmentally important compounds, e.g., selected polycyclic hydrocarbons or nitrosamines.

Screening Results

As an example of the type of data that can be derived from a comparative approach and the information that can be gained from such an approach, we list in Table 4 a group of substituted nitroso compounds (*N*-nitrosopiperidines) that have been assayed in both the *Salmonella* and yeast systems and then extended to the insect (*Drosophila*) sex-linked lethal system. The carcinogenicity (if known) is also listed. Note that all of the systems show a high correlation of mutagenic response with carcinogenicity. Furthermore, the importance of metabolic activation in the *in vitro* short-term assays should be stressed. The sensitivity of the *Drosophila* system for detection of this class of compounds appears to be high, with the fly supplying its own metabolism; however, the sensitivity breaks down when the oxygen-substituted com-

Table 4. Comparative mutagenicity of nitrosopiperidines.

Compound	*S. typhimurium* (plate assay), $his^- \rightarrow his^+$	*S. cerevisiae*, $CAN^S \rightarrow can^R$ or $his^- \rightarrow his^+$	*D. melanogaster*, X-linked recessive lethals	Carcinogenicity rats
N-Nitrosopiperidine (NP)	+	+	+	+
2-Methyl NP	+	+	+	+
3-Methyl NP	+	+	+	+
4-Methyl NP	+	+	+	+
3,5-Dimethyl NP	+	+	+	+
2,6-Dimethyl NP	−	−	−	−
3,4-Dehydro NP	+	+	+	+
3-Chloro NP	+	+	+	+
3,4-Dichloro NP	+	+	+	+
3,4-Dibromo NP	+	+	+	+
Nitroso-4-piperidinol	+	−	−	+
Nitroso-4-piperidone	+	−	−	+
Nitrosopipecolic acid	−	−	−	−
Nitrosoguvacoline	+	not tested	−	−

pounds are tested. Although positive for mutagenicity in the microbial systems, these carcinogenic compounds are negative in *Drosophila*. The usefulness of the assays from the view of structure-function relationships is also illustrated, i.e., nitroso-containing ring structures blocked in the positions alpha to the *N*-nitroso group show either a reduction or elimination of mutagenicity as well as carcinogenicity (*17, 28, 29*).

The potential usefulness of the Ames histidine reversion system as a prescreen is illustrated in Table 5. The large group of compounds either identified or suspected to occur in various energy-related effluents or products is listed with respect to mutagenicity in the *Salmonella* test. The unanswered question at this point is whether the testing of negative compounds can sensibly be terminated here (*30*).

Screening of Complex Mixtures

To determine rapidly the potential biohazards (mutagenicity/carcinogenicity?) of various crude and complex test materials derived from fossil fuel production or conversion activities, we have examined the feasibility of using short-term genetic assays to predict, isolate, and identify the chemical hazards. The biological screening assay was coupled to an analytical chemistry separation procedure so that the chemical work and priorities for identification could be determined by the bioassay. Again, the wide applicability of the bacterial test system developed by Ames can be demonstrated by the use of the assay as a prescreen for potential genetic hazards of complex environmental effluents or products [e.g., tobacco smoke condensates (*31*), hair dyes (*32*), soot from city air (*7*), fly ash (*33*), and (in our work) oils and aqueous wastes from synthetic fuel technologies (*30, 34*)].

Table 5. Mutagenic activity of coal fraction constituents (predicted or identified).[a]

	Strain	Mutagen
Anthracene	100	−
Benz[a]anthracene	100	+
Benz[b]anthracene	100	+
7,12-Dimethyl[a]benzanthracene	100	+
1,2,3,4-Dibenzanthracene	100	+
1,2,5,6-Dibenzanthracene	100	+
Pyrene	1537	±
Benzo[a]pyrene	98	+
Chrysene	100	+
Fluoranthene	98	±
Phenanthrene	98	±
Fluorene	100	−
9-Methylfluorene	100	−
2,3-Benzofluorene	100	+
9-Methylanthracene	98	±
2-Aminoanthracene	100	+
Biphenyl	100	−
Diphenylamine	100	−
Isoquinoline	100	−
Acridine[b]	1537	+
Carbazole	100	−
α-Naphthylamine	100	+
2,6-Dimethylquinoxaline	100	−
2,3-Dimethylquinoxaline	100	−
Hydroquinone	100	−
Methylpyridine (picoline)	98, 100	−
Ethylpyridine	98, 100	−
Dimethylpyridine (lutidine)	98, 100	−
Trimethylpyridine		
Bipyridine	98, 100	−
2,5-dimethylaniline	98, 100	+
Trimethylaniline	98, 100	−
Quinoline	98	+
7-Methylquinoline	100	+
8-Methylquinoline	100	+
2,6-Dimethylquinoline	98, 100	−
8-Hydroxyquinoline	100	+
8-Aminoquinoline	1537	+
8-Nitroquinoline	100	+
Dimethylpiperidines	98, 100	−

[a] Determined from plate assay, including metabolic activation with Aroclor-induced liver.
[b] Metabolic activation was not necessary.

To study the feasibility of applying mutagenicity testing to environmental effluents and crude products from the synthetic fuels technology, we attempted to perform screening with the highly sensitive Ames histidine reversion strains known to respond to a wide variety of proven 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 with carcinogenic assays. Finally, the procedures might show utility in monitoring plant processes, effluents, or personnel early in the formation of the engineering and environmental technology that will eventually evolve in the synthetic fuels industry. The approach and preliminary results showed that the coupled chemical-biological scheme is a feasible research mechanism and is applicable to the ascertainment of potential human health hazard of a wide variety of environmental exposures, either occupationally or to the population in general.

As an example of the screening of fractionated complex mixtures, we list in Table 6 the results from an aqueous sample containing a variety of organic contaminants. The sample consists of the condensate from an experimental coal gasification process (supplied by the Pittsburgh Energy Research Center). A large quantity of the aqueous sample was extracted and subjected to fractionation by the Stedman procedure (*35*). Previous publications have shown the usefulness of this technique with crude petroleum oils, synthetic crude oils, shale oils, and aqueous wastes (*30, 34, 36*).

Primary fractions were obtained as listed in Table 6. The table shows the analytical weight analysis of each fraction and lists the mutagenicity test results both as a specific activity of each fraction and as a weighted activity. 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 mutagenic fraction 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 a 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 (specific activity). Positive or questionable results were retested with a narrow 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 5-9 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 nonmutagenic in the bacterial test system. Additivity of the individual weighted

Table 6. Distribution of mutagenic activity in fractions of gasifier condensate.[a]

Fraction No.	Fraction[b]	Relative weight, (% of total)	Activity[c] Specific activity, rev/mg	Activity[c] Weighted activity, rev/mg[d]
1	$NaOH_I$	Not collected	—	—
2	WA_I	8.3	750	62
3	WA_E	34.5	25	9
4	SA_I	14.1	500	70
5	SA_E	9.9	600	60
6	SA_W	30.8	0	0
7	BI_a	0.03	0	0
8	BI_b	0.04	150	0
9	B_E	0.5	4000	18
10	B_W	0.1	Not tested	—
	Neutral	1.9	100	2
Total				221

[a] All assays carried out in the presence of crude liver S-9 from rats induced with Aroclor 1254; 0.9% (w/v) of solids from 6 liters extracted.

[b] Code: I = insoluble (fractions a and b), E = ether-soluble, W = water-soluble, WA = weak acid, SA = strong acid, B = base.

[c] Rev/mg = revertants/mg, the number of histidine revertants from *Salmonella* strain TA 98 determined 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.

[d] Weighted activity of each fraction relative to the starting material is the product of relative weight and specific activity. The sum of these products is given as a measure of the total mutagenic potential of the material.

Table 7. Comparative mutagenicity of Synfuel fractions.[a]

Test material	Test system[b]						
	Salmonella typhimurium, $his^- \rightarrow his^+$	*E. coli* $arg^- \rightarrow arg^+$/or $gal^- \rightarrow gal^+$	*Saccharomyces cerevisiae*, $his^- \rightarrow his^+$ or $CAN^S \rightarrow can^R$	*Drosophila melanogaster*, sex-linked recessive lethals	CHO cells, 6-thioguanine resistance[c]	Human leukocytes, chromosome aberrations	*Mus musculus*, dominant lethals[d]
Crude Synfuel[e]	+	NT	NT	NT	NT	NT	+
Basic fraction	+	+	+	+	+	NT	−
Neutral fraction	+	+	+	−	NT	+?	NT

[a] Liquid petroleum crude Synfuels of Pittsburgh Energy Research Center or FMC Corp.
[b] Code: (+) mutagenic; (−) nonmutagenic; (NT) not tested.
[c] A. W. Hsie, personal communication.
[d] W. M. Generoso, personal communication.
[e] Crude Synfuels are generally too toxic to test in most systems.

values was assumed, and a final total mutagenic potential was calculated.

The values given in Table 6 represent the determinations with strain TA98 metabolically activated with an Aroclor-induced rat liver enzyme preparation. The total extracted 6 liters contained 0.9 w/v% of solids yielding the various weight percent fractions shown. Although in most cases crude samples are too toxic to the bacterial assay to test in an appropriate concentration range, the crude aqueous condensate did show a positive effect with the Ames system—a value of 60 revertants/100 μl of condensate with strain TA98 and 75 revertants/100μl with strain TA100. The extracted concentrate values are shown in Table 6. We assume that the most accurate measure of the total mutagenic potential of the test material is the sum of the tested fractions, 221 revertants/mg of starting material (solids). Acidic and basic components in the aqueous sample appear to contain the major mutagenic components, in contrast to work with crude oils where the neutral fractions, presumbly polyaromatic hydrocarbons, are also major contributors (*34*).

In this initial feasibility study, however, the point in question is not whether these results reflect a relative biohazard for comparison with other materials or processes. The results simply show that biological testing—the Ames histidine reversion assay in this study—can be carried out with the newly developed tester systems, but perhaps only when coupled with the appropriate analytical separation scheme.

Comparative Mutagenesis of Complex Mixtures

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 in the histidine reversion assay for further testing in the various assays described in the section on the tier approach to testing. Preliminary results have been published in the Proceedings of the Second International Conference on Environmental Mutagens, Edinburgh, 1977 (*37*). Qualitative comparisons may be seen in Table 7. 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 by Epler et al. (*34*)]. In *Drosophila* (*38*) and in the mammalian cell gene mutation assay (*39*), detection has been a function of newly developed fractionation schemes (e.g., the use of LH-20) (*40, 41*) 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 they 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.

Conclusions—Precautions

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 distort the results obtained, as could 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 dis-

crepancies could also enter into the determinations. For example, concomitant bacterial toxicity could nullify any genetic damage assay that might be carried out; the choice of inducer for the liver enzymes involved could be wrong for selected compounds; or the choice of strain could be inappropriate to selected compounds. Furthermore, the applicability of the *Salmonella* test results to other genetic assays and the validation of the apparent correlation between mutagenicity and carcinogenicity still needs validation through sufficient fundamental research. Furthermore, the short-term assays chronically show negative results with, e.g., heavy metals. Similarly, compounds involved in or requiring cocarcinogenic phenomena would presumably go undetected. However, as a prescreen to aid investigators in ordering their priorities, the short-term tests appear to be a valid approach to testing the large number of hazardous compounds and complex mixtures that man encounters in his environment.

This research was jointly sponsored by the Environmental Protection Agency (IAG-D5-E681) and the Department of Energy under contract with the Union Carbide Corporation.

The authors thank the staff at the Pittsburgh Energy Research Center for their cooperation in providing samples. We are indebted to the staff of the Bio/Organic Analysis Section of the Analytical Chemistry Division of the Oak Ridge National Laboratory for the fractionated materials described in this study.

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