Genotoxicity of Complex Mixtures: CHO Cell Mutagenicity Assay

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February 1985

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EXECUTIVE SUMMARY

A Chinese hamster ovary (CHO) mammalian cell assay that measures forward mutation at the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus was used to evaluate the genotoxicity of complex mixtures (synthetic fuels). The genotoxicity (mutagenic potency) of the mixtures increased as the temperature of their boiling range increased. For example, mutagenic activity of low-boiling synfuels was comparable to that of a naturally derived crude petroleum. Based on an examination of data from chemically fractionated synthetic fuels, mutagenic activity does not appear to be associated with any single chemical class. Most fractions were genetically active, and the degree of activity of each chemical class varied not only with boiling point (bp) but also with synfuel process. Fractionation also revealed that antagonism between chemical fractions may inhibit expression of their full mutagenic potential; these antagonistic effects decreased as bp increased.

Most of the genotoxicity in the 750°F+ boiling-range materials was associated with the neutral polycyclic aromatic hydrocarbon (PAH) fractions. Chemical analysis data indicate that the PAH fractions of high-boiling coal liquids contain a number of known chemical carcinogens, including five- and six-ring polyaromatics (e.g., benzo[a]pyrene) as well as four- and five-ring alkyl-substituted PAH (e.g., methylchrysene and dimethylbenzanthracenes); concentrations are a function of bp. In vitro genotoxicity was also detected in fractions of nitrogen-containing polyaromatic compounds, as well as in those with aliphatics or hydroxy-containing PAH.

Mutagenic activity of some fractions was detectable in the CHO assay in the absence of an exogenous metabolic activation system; in some instances, addition of exogenous enzymes and cofactors inhibited expression of the "direct-acting" mutagenic potential of the fraction.

These data indicate that the organic matrix of the chemical fraction determines whether, and to what degree, various mutagens are expressed in the CHO assay. Therefore, the results of biological assays of these mixtures must be correlated with chemical analyses for proper interpretation of these data.

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ABBREVIATIONS

AH	= Aliphatic hydrocarbon(s)
APAH	= Amino polycyclic aromatic hydrocarbons
BaP	= Benzo[a]pyrene
bp	= Boiling point
CHO	= Chinese hamster ovary
DiMac	= 7,9-dimethylbenz(c)acridine
DMSO	= Dimethylsulfoxide
FBS	= Fetal bovine serum
HGPRT	= Hypoxanthine-guanine phosphoribosyltransferase
HPAH	= Hydroxy polycyclic aromatic hydrocarbon(s)
HPLC	= High-pressure liquid chromatography
Hx F12	= Hypoxanthine-free F12
I/P	= Initiation/promotion
ITSL	= Integrated two-stage liquefaction
NPAC	= Nitrogen-containing polycyclic aromatic compound(s)
PAA	= Primary aromatic amine(s)
PAH	= Polycyclic aromatic hydrocarbon(s)
PDT	= Population doubling time
PDU	= Process development unit
PNL	= Pacific Northwest Laboratory
SCE	= Sister chromatid exchange
SRC	= Solvent refined coal
2-AA	= 2-aminoanthracene
6-AC	= 6-aminochrysene
6-Th	= 6-thioguanine

INTRODUCTION

The initial problem in assessing the genetic hazard of complex mixtures from environmental and/or industrial sources is to evaluate their potential for causing somatic cell mutations (related to cancer initiation) and germinal cell mutation (hereditary damage). To detect and identify carcinogens and/or mutagens in the large numbers of materials which require testing, it is most effective to use *in vitro*, short-term screening assays.

The first step, or tier, in our protocol is the Ames assay, which measures mutation frequency in bacterial cells, and is fast and inexpensive. Results of Ames assays of synfuels are reported elsewhere (Pelroy et al., 1981, 1983; Pelroy and Gandolfi, 1980; Pelroy and Petersen, 1981; Pelroy and Stewart, 1981; Pelroy and Wilson, 1981a). However, results from previous studies with mammalian cells and *in vivo* skin-painting assays indicate that there was carcinogenic activity that was not detected by the Ames assay (Frazier and Mahlum, 1984; Mahlum, 1983b; Mahlum et al., 1983; Pelroy et al., 1984; Renne et al., *in press*).

Mammalian cells, however, have a different arrangement of genetic materials from that of bacteria. For example, mammalian cells have nuclear membranes, and bacterial cells do not; in addition, the organization of chromatin and the genetic information of eukaryotes is different from that of the prokaryotes (e.g., bacteria). Also, many types of genetic phenomena occur in mammalian cells (e.g., segregation, sister chromatid exchange [SCE], etc.) that do not occur in bacterial cells. Furthermore, the way in which mammalian-cell DNA repair mechanisms metabolize mutagens and promutagens, as well as a number of cellular constituents (e.g., cell wall versus cell membrane), differs from those in bacterial cells. The different sensitivities of the two assay systems probably reflect these contrasts in genetic make-up.

The recent development of reliable procedures for quantifying mutation in cultured mammalian somatic cells enhances our ability to assess the risk to man from environmental hazards and provides insights into the mechanisms of mutagenesis in eukaryotic cells. The data obtained to date do not always show the precision characteristic of studies with microorganisms. This lack of

precision, we believe, primarily reflects methodological difficulties. The methods we have used to overcome those problems are reported here in Appendix A. Mammalian-cell assays are now an essential component of our mutagen/carcinogen screening procedure.

Chinese Hamster Ovary (CHO) Cell Assay

We used a mammalian-cell mutation assay in which CHO cells serve as the indicator. The endpoint assessed was (forward) mutation of the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus, which is the most widely used and best understood for mutation studies with mammalian cells (Carver et al., 1980; Hsie et al., 1977, 1978, 1980; O'Neill et al., 1977; O'Neill and Hsie, 1979; Thompson et al., 1980). The HGPRT gene codes for an enzyme in the purine salvage pathway that converts hypoxanthine or guanine to the nucleotide form. Since the HGPRT gene is on the X chromosome, any mutation in an active X chromosome is expressed. For selecting mutants, we cultured exposed cells in medium containing the guanine analog 6-thioguanine (6-Th), which, when ribophosphorylated (by an HGPRT⁺ cell) and incorporated into DNA or RNA, rapidly killed nonmutated cells. Mutant cells (HGPRT⁻) that had greatly reduced (or zero) enzyme activity were spared, since they did not convert the precursor into its toxic form.

Synthetic Fuel Sources

This report examines the genotoxic activity of synthetic fuels from several sources. We compared the genotoxicity of crude fuels (not fractionated by any method), including a crude petroleum, as well as liquids from the H-Coal, integrated two-stage liquefaction (ITSL), solvent refined coal (SRC)-I, and -II processes, to that of known pure carcinogens.

Prior studies indicated a possible relationship between distillation temperature of a process stream and its genotoxicity (Frazier and Andrews, 1983; Pelroy and Wilson, 1981b). Thus, distillate cuts from various SRC-I and -II liquids were assayed to relate in vitro genetic activity to specific boiling-point (bp) ranges and, thus, to definable molecular-weight species within the distillate cuts.

METHODS

Cells and Medium

The derivation and biochemical and genetic characterization of the CHO cell subline used are described in detail by Kao and Puck (1967). Strain CHO-K1-BH₄ was originally cloned in the presence of the folic acid antagonist aminopterin to ensure that the cells had a gene coding for the enzyme HGPRT (Hsie et al., 1977, 1978). Cells were grown in 150-cm² T-flasks (Corning) in Ham's medium F12 (K. C. Biological, Inc.) supplemented with 10% dialyzed and heat-inactivated fetal bovine serum (FBS). Stock cell cultures were grown in an incubator humidified to near 100% at 37°C in an atmosphere of 95% air and 5% CO₂. Cells were routinely tested and found to be consistently free of mycoplasma. Under standard growth conditions, the population doubling time (PDT) of the CHO-K1-BH₄ cell line was between 13 and 14 hours.

CHO/HGPRT Mutation Assay

Procedures used for cytotoxicity and mutagenicity assays are outlined schematically in Figure 1. A well-characterized assay system for measuring mutation frequency in these cells has been described elsewhere (Carver et al., 1980; Hsie et al., 1977, 1978, 1980; O'Neill et al., 1977; O'Neill and Hsie, 1979; Thompson et al., 1980). Modifications of those assay procedures were necessary to conduct the experiments described in this report. These changes included the use of activating enzyme prepared from rat liver homogenates to detect compounds that required metabolic activation before they became mutagens. (See also Appendix A.) Another modification involved culturing the cells in six-well Linbro plates for expression. Other experimental details are included in Appendix A.

Mutagenicity assays were performed with CHO cells that had been selected for HGPRT⁺ activity, then frozen in aliquots. An aliquot was pre-screened to ensure that the cells contained a low background of spontaneous HGPRT⁻ mutants ($<2 \times 10^{-6}$). Cells were removed from liquid nitrogen ~3 days before the experiment and kept in log-phase growth. Cells were plated at $\sim 1 \times 10^6$ cells/T-25 flask (Corning) about 18 hours before challenge with chemicals.

FIGURE 1. Flow Chart of CHO/HGPRT Assay

Culture medium was removed from the culture flasks, the cells were rinsed with saline G (O'Neill and Hsie, 1979) to remove any remaining medium, and they were then exposed to graded concentrations of test materials (potential mutagens) with or without activating enzymes. The cells were incubated at 37°C for 4 hours. A typical experiment consisted of controls (positive and negative solvent) as well as three concentration levels of the test material in triplicate.

Following exposure, cultures were rinsed and allowed a 1-hour recovery period in F12-FBS10. Cells were trypsinized, washed, and centrifuged to remove trypsin; counted; and 150 or 300 cells/well (six-well Linbro chambers) were plated to determine cell viability. Only those concentrations of test compound showing $\geq 75\%$ survival (relative to controls) were used to determine mutation frequencies. Cells remaining after determining initial survival ($\sim 1 \times 10^6$) were carried forward in monolayer culture for mutant selection. These exposed cultures were trypsinized, and diluted to 1×10^6 cells at 2-day intervals. After a 7-day expression period, the cells were again trypsinized, washed, and counted. The cloning efficiencies and mutation frequencies were then determined.

In order to determine cloning efficiencies at the time of mutant selection, cells in each flask were trypsinized, washed, and diluted; a six-well plate was seeded with 150 cells/well, and another plate was seeded with 300 cells/well, each in 4 ml of hypoxanthine-free F12 (Hx⁻ F12).

For mutant selection, each well (of the six-well culture dish) was seeded with 50,000 cells in 3 ml of selection medium (containing Hx⁻ F12 and 6 $\mu\text{g}/\text{ml}$ of 6-Th). A total of 2.4×10^6 cells (48 wells) were screened for each data point. These cultured cells were incubated at 37°C in a 100% humidified incubator with a 5% CO₂, 95% air atmosphere. Cells were fixed and stained, with dilute crystal violet, after 7 days of culture. Cloning efficiency was counted using a Biotran III colony counter (New Brunswick Scientific Co.). A colony is defined as a group of 50 or more cells. Mutant colonies were scored by hand. All experiments were "blind": technicians who counted cells, exposed cells, or counted colonies were unaware of which samples or sample con-

centrations were being tested. Mutation frequencies were calculated by dividing the total number of mutant colonies by the number of viable cells plated.

Data Analysis

Each chemically treated or control culture was an independent data point. The mutation frequency was computed by correcting the absolute number of mutants in the sample for plating efficiency (number of colony-forming cells). This value, mutants/ 10^6 surviving cells, was statistically analyzed using two methods. First, average response to each dose was compared independently to that of the negative-control, using an unpaired Student's *t*-test. The presence of a linear dose-response relationship was then ascertained by linear regression analysis using the least-squares method. For most synfuels or synfuel fractions, dose responses in the CHO assay were approximately linear over at least a portion of the dose-response curve. Where dose response was non-linear, mutagenic potency was estimated by subtracting background from response and dividing by the lowest concentration of test material causing a statistically significant difference.

A mean value for the mutation frequencies was obtained from the slope of the dose-response curve, which was derived by linear regression analysis. In all cases, the "spontaneous" or background mutation frequency has been subtracted from the data reported.

Chemicals and Samples

Reagent grade 2-aminoanthracene (2-AA) and 6-aminochrysene (6-AC) were purchased from Aldrich Chemical Company. The 7,9-dimethylbenz(c)acridine (DiMac) was supplied by the chemical repository at ITT Research Institute. Benzo[a]pyrene (BaP) was obtained from Sigma Biochemical Company. Stock solutions in dimethylsulfoxide (DMSO) were stored at -20°C in tightly capped test tubes until used.

Wilmington (California) crude petroleum was obtained from the Pacific Northwest Laboratory (PNL; Richland, WA) repository.

SRC-I process solvent was obtained from the Wilsonville, AL pilot plant. This material had a boiling range between 450 and 850°F (plus a small amount of 850°F+ heavy ends). The process solvent was subsequently distilled, and sequential 50°F bp cuts were obtained. We analyzed the crude process solvent and four of the bp cuts (300-700, 700-750, 750-800°F and 800-850°F+) in the CHO assay. For a detailed description of the distillation procedure, and chemical fractionation and characterization of the resultant samples, see Wilson et al. (1982).

The SRC-II coal liquid used in these mutagenesis studies was produced at the Harmarville, PA P-99 process development unit (PDU), operated by Gulf Science and Technology Co. This material had a boiling range of 300 to 850°F, plus bottoms.

In addition to the wide-boiling-range SRC-II crude, we assayed five distillate cuts: 300-700, 700-750, 850-800, 800-850°F, and 850°F+ (bottoms). The preparation of these samples and their chemical fractionation and characterization were described by Pelroy and Wilson (1981b).

The ITSL samples were obtained from a PDU operated by the C. E. Lummus Co., in New Brunswick, NJ. A description of the process, including distillation procedure, chemical analyses, and run conditions for the samples assayed, is given in detail in Wilson et al. (Wilson, Buhl and Moroni, 1983; Wilson, Pelroy et al., 1984) and in the C. E. Lummus Quarterly Report to the U.S. Department of Energy.

Chemical Separation

Two methods of separating major compound classes in these synthetic fuels were used to provide fractions for in vitro assays. Alumina column chromatography (Later et al., 1981) yields four chemical subfractions: aliphatic hydrocarbons (AH), neutral polycyclic aromatic hydrocarbons (PAH), nitrogen-containing polycyclic aromatic compounds (NPAC), and hydroxy polycyclic aromatic hydrocarbons (HPAH). Semi-preparative high-pressure liquid chromatography (HPLC) separates chemical fractions by polarity and, to a lesser extent, by molecular weight (Toste et al., 1982). By coupling chemical data

with the mammalian-cell mutation assay data, we were able to draw some important conclusions about the genotoxicity of synfuels.

Qualitative and quantitative analyses of individual compounds in the complex mixtures were achieved by gas chromatography/mass spectrometry. Detailed procedures have been given elsewhere (Wilson et al., 1984).

RESULTS

General Features of CHO Response

After testing several known carcinogens in the system, we selected three specific compounds, one from each of the chemical classes of potential concern in synfuels (azaarenes, primary aromatic amines [PAA] and PAH); the latter served as positive controls. The PAA used most frequently, 6-AC, repeatedly showed a dose-response relationship of ~ 2 to 2.5 mutants/ μg and was relatively nontoxic (Figure 2). BaP, the neutral PAH, produced ~ 11 mutants/ μg (Figure 3). The azaarene positive control, DiMac, yielded between 10 and 11 mutants/ μg (Figure 4).

We obtained linear dose-response relationships with most complex mixtures (Figures 5 and 6). Data for a wide-boiling-range SRC-II liquid (Figure 6) illustrate two common findings with the assay: 1) linear dose-response relationships were observed only over a very narrow dose range (in this instance, between 0 and 15 $\mu\text{g}/\text{ml}$), and 2) as relative survival decreased, the variability in the system increased, and the response became nonlinear.

Figure 7 shows the nonlinear response of the 850°F+ HPLC Fraction 5, determined by taking the mean mutation frequency at 25 $\mu\text{g}/\text{ml}$ (6.5 mutants) and dividing by 25, which gives 0.26 mutants/ μg .

Use of CHO Assay for Comparing Synfuel Mutagenicity

Figure 8 compares the mutagenicity of certain synthetic fuels, a crude petroleum, and selected known chemical carcinogens. Pure compounds, such as BaP and DiMac, are nearly an order of magnitude more mutagenic than synfuels; SRC-II heavy distillate is about twice as mutagenic as the other synthetic fuels; Wilmington crude petroleum is low in mutagenic potency but caused a significant increase in the mutation frequency of CHO cells.

FIGURE 2. Induced Mutagenicity and Cytotoxicity as a Function of the Concentration of 6-AC; Exposure Duration was 4 Hours. Experiments were conducted in the presence of liver-activating enzymes from Aroclor-pretreated rats, an NADPH generating system and cofactors. A) Mutagenicity was determined by the frequency of drug-resistant colonies/10⁶ cells, taking into account the number of cells plated and the plating efficiency. Data are expressed as a mean value; error bars represent 1 SEM. Linear regression analyses of the data indicates a Y intercept of 2.1, a slope of 2.2 and a correlation coefficient of 0.97. B) Cytotoxicity was determined by comparing survival of CHO-K1-BH₄ cells treated with 6-AC to survival of DMSO-treated controls.

FIGURE 3. Mutation Frequency (A) and Percent Survival (B) of CHO Cells Exposed to varying Concentrations of BaP in the Presence of a Metabolic Activation System. Mutation frequency is expressed as number of thioguanine-resistant colonies/10⁶ viable cells; survival, expressed as percent, is relative to that of solvent controls, which is set at 100%. Absolute plating efficiency was $\leq 80\%$. Based on a linear regression analysis, the Y intercept is 8.6, the slope is 11, and the correlation coefficient is 0.96. The vertical lines represent 1 SEM.

FIGURE 4. Increase in Induced Frequency of Thioguanine-Resistant Mutants and Decreased Relative Survival with Increasing Exposure Dose of DiMac. Cultures of CHO cells were incubated for 4 hours with a range of concentrations, expressed as $\mu\text{g/ml}$, of the test compound in serum-free F12 medium and in the presence of a metabolic activation system. The mutation frequency (A) and percent survival (B) were determined as described in Methods. Linear regression analysis gave a Y intercept of 0.97, a slope of 10.9, and a correlation coefficient of 0.83.

FIGURE 5. Induced Mutation Frequency (A) and Relative Survival (B) of Cells Exposed for 4 Hours to an HPLC Fraction of an SRC-II 850°F+ Distillate in the Presence of a Metabolic Activating System. Following exposure, counts of initial survival (relative to the solvent controls), subculture for 7 days, and selection of mutants in 6-Th-containing medium were performed as described in Methods. Mutagenic responses, expressed as mutants/10⁶ viable cells, were corrected by subtracting the background mutation frequency ($>2 \times 10^{-6}$), then plotting the remainder as individual data points. Linear regression analysis of these data indicated a slope of 2.4, with a correlation coefficient of 0.72.

FIGURE 6. Concentration Response Relationship for Mutagenesis (A) and Cytotoxicity (B) of a Full-Boiling-Range (300°-850°F+) SRC-II Material. Cells were exposed to the SRC-II blend in the presence of a metabolic activating system consisting of liver enzymes from Aroclor-induced rats, an NADPH generating system, and cofactors. Mutagenicity, determined as described in text, was expressed as mutants/ $\mu\text{g}/10^6$ viable cells. Based on linear regression analysis, the Y intercept was -0.3, the slope was 1.14, and the correlation coefficient 0.94. Cytotoxicity was determined by comparing the initial survival of the cells in varying concentrations of the SRC-II material to the cloning efficiency of the solvent controls.

FIGURE 7. Induced Mutation Frequency (A) and Cytotoxicity (B) of CHO Cells as a Function of the Concentration of an HPLC Fraction from an SRC-II 850°F+ Distillate. Cultures were incubated for 4 hours to various concentrations of HPLC Fraction 5 in the presence of a metabolic activating system. Mutagenic potency was determined by subtracting the background from the responses. The mean response at 25 $\mu\text{g/ml}$, the lowest concentration of the HPLC fraction yielding a statistically significant difference from background, was 6.3 mutants/ 10^6 viable cells. That mean was divided by 25 μg , to give 0.25 mutants/ μg . Cytotoxicity was determined by comparing the survival of cells at each exposure level to the cloning efficiency of the solvent control.

FIGURE 8. Comparison of Mutation Frequencies of Selected Chemicals, a Crude Petroleum and Several Synthetic Fuels in the CHO/HGPRT Forward Mutation Assay. Potency values were taken from slopes of the dose-response curves, estimated by simple linear regression analysis, using the method of least squares. The samples tested, from top to bottom, are Wilmington (California) crude petroleum, SRC-II full-boiling-range blend, SRC-I process solvent, total liquid product from the ITSL process, BaP, DiMac, 2-AA, LC finer feed from ITSL and H-Coal product.

Boiling Range Versus Mutagenicity

Pelroy and Wilson (1981b) indicated a possible relationship between the boiling range of a coal liquid and its genotoxicity. Heavy distillate was active in the transformation assay, while middle- and low-boiling-range materials caused no transformation (Frazier and Andrews, 1983). Therefore, distillate cuts from wide-boiling-range SRC-I and -II liquids and two ITSL fractions were assayed in the CHO system to determine if in vitro genotoxicity was correlated with boiling ranges. Because distillate cuts usually contain definable molecular species, such data might indicate compounds of interest.

BaP is about eight times more mutagenic, in the CHO assay, than the most active coal liquid material, the SRC-II 850°F+ bp cut. The highest-boiling distillate cuts of both SRC-II and their SRC-I counterparts were more active mutagens than the lower-boiling fractions. Further, in a comparison of process, the most active was SRC-II, followed by SRC-I and H-Coal, then by the ITSL material (Figure 9).

HPLC Fractionation

To determine which chemical classes were causing mutations, we separated the most active bp cut (SRC-II 850°F+), using HPLC. The HPLC procedure provided eight fractions, separated by polarity and, to some extent, molecular weight (Figure 10).

Fractions 1 and 2 were nonpolar and contained PAH and AH as well as some nonpolar heterocyclic compounds. Fractions 3 and 4 contain moderately polar compounds, including nitrogen heterocycles (PAA, azaarenes and carbazoles); some of the nitrogen-containing heterocycles were alkylated or partially hydrogenated. Fraction 5 was a transition region, the composition of which is poorly characterized. Fraction 6 contained some bifunctional groups with hydroxylated components. Highly polar compounds (e.g., phenols and hydroxylated and multifunctional PAH and heterocyclic compounds) appeared in Fractions 7 and 8.

FIGURE 9. Potency in the CHO Assay of Discrete bp Cuts Derived from Three Coal Liquefaction Processes. Mutation frequencies, expressed as: mutants/ $\mu\text{g}/10^6$ cells, were taken from slopes of dose-response curves estimated by linear regression analysis, using the method of least squares.

FIGURE 10. HPLC Separation of the SRC-II 800-850°F+ bp Cut. A semi-preparative column was composed of Ultrasil NH₂, and ~200 mg of sample was applied. Elution with a hexane, methylene chloride, isopropanol gradient separated the mixture into eight identifiable regions. Elution times for various regions are indicated. Detection was by ultraviolet absorption at 250 nm.

The compounds of greatest interest were in Regions 1-4. The concentrations of selected compounds, representing various chemical classes, are shown in Table 1. Fractions 1 and 2 contained relatively high concentrations of PAH and were virtually devoid of NPAC. Fraction 2 contained the higher-molecular-weight PAH. Fraction 3 contained mainly azaarenes, with trace levels of amino polycyclic aromatic hydrocarbons (APAH) and carbazole, but it did not contain any neutral PAH. Fraction 4 contained, essentially, APAH and carbazoles.

CHO Versus Ames Data for HPLC Fractions

Active fractions in the mammalian-cell (CHO) assay were different from those in the microbial-cell (Ames) assay (Figure 11). The most active CHO mutagens were present in the neutral PAH-rich fractions, Fractions 1 and 2. In contrast, Fractions 1 and 2 were negative in the standard Ames assay. The azaarene-containing Fraction 3 was active in the CHO assay and had some mutagenic activity in the Ames assay. Fraction 4, which contained most of the APAH, was strongly mutagenic in the Ames test (90% of the total Ames assay response) but was much less active in the CHO assay.

There appears to be considerably more mutagenic activity in some of the fractions than in the distillate from which they were derived. Table 2 shows mutagenic activities of the fractions, weighted according to their proportional presence in the total crude. The cumulative mutagenic activity of the eight HPLC fractions was more than twice that in the crude material, suggesting that composition of the total crude may suppress expression of mutagenic potential of the whole mixture.

Ames Versus CHO Data for Fractions Separated by Alumina Column Chromatography

Coal liquids were separated by alumina column chromatography into AH, PAH, NPAC or HPAH, based on their elution with various organic solvents. In vitro mutation assays of an SRC-II 850°F+ cut fractionated by this method (Figure 12) also showed a difference in sensitivity between the Ames and CHO assays (Figure 13). All four fractions were active in the CHO assay; the PAH-containing fraction was at least twice as mutagenic as the others. Conversely, Ames mutagenicity was not detected in the AH fraction, and mutagenic-

TABLE 1. Major Chemical Components of SRC-II 850+°F HPLC Fractions

Compound	HPLC Fractions			
	1	2	3	4
	Quantity in ppm			
Pyrene	385	253	ND ^a	ND
Benz[a]anthracene	308	120	ND	ND
Dimethylbenzanthracene (isomers)	394	269	ND	ND
BaP	3037	3,861	ND	ND
Benzo[g,h,i]perylene/ananthrene	2929	10,688	ND	ND
Azachrysenes/benzanthracene				
Azachrysenes/benzanthracene	ND	ND	1039	t ^b
Azabenzopyrenes/azabenzofluoranthenes	ND	ND	4431	t
Azabenzo[g,h,i]perylene/azaananthrenes	ND	ND	2396	t
Aminopyrene/fluoranthenes	ND	ND	t	25
Aminochrysene/benzoanthracene	ND	ND	t	53
Aminobenzopyrenes/perylene	ND	ND	t	32
Benzocarbazoles	ND	ND	257	t

^a ND = Not detectable

^b t = Trace amounts; i.e., <1 ppm

FIGURE 11. Relative Mutagenic Potency of HPLC Fractions of an SRC-II 850°F+ bp Cut in Ames and CHO/HGPRT Assays. Potency is expressed as mutants/μg/10⁶ viable cells (for the Ames assay, in revertants/colony) and estimated by linear regression analysis of dose-response data.

TABLE 2. Weighted Activity of HPLC Fractions

Test Material	Percent of Total by Weight	Mutants/ μ g	Total Mutants/100 μ g	Percent of Mutagenic Activity
SRC-II 850 ^o +F crude	100	1.4	140	100
HPLC Fraction				
1 PAH	38	3.6	136.8	97.7
2 PAH $\leq$ 5 rings	7	6.5	45.5	32.5
3 Azaarene	16	3.3	52.8	37.7
4 Carbazoles and APAH	10	2.1	21	15
5 (Transition)	10	0.3	3	2.1
6 HPAH, polar	14	0.74	10.4	7.4
7 HPAH, polar	4	0	0	0
8 Strongly polar	<u>1</u>	<u>1.1</u>	<u>1.1</u>	<u>0.8</u>
Totals	100	--	270.5	193.2

FIGURE 12. Alumina Column Separation; Synthetic Fuels were Fractionated into Four Chemical Subclasses, Based on their Elution from the Column with Various Organic Solvents (Later et al., 1983)

FIGURE 13. Mutagenic Responses in the CHO/HGPRT and Ames Assays of AH, Neutral PAH, NPAC and HPAH Fractions from the SRC-II 850°F+ Cut. Unfractionated distillate is referred to as "crude."

ity in the PAH fraction was detectable only by modifying the Ames technique. The NPAC region was the strongest bacterial mutagen; the next most active class was the HPAH fraction.

Tables 3 and 4 show the concentrations of selected compounds present in the neutral PAH and NPAC fractions of SRC-II bp cuts. Average molecular weight of compounds in the distillate cuts increased with bp. The SRC-II 850°F cut contained mainly five- and six-ring PAH (Table 3). High levels of BaP, a potent carcinogen in mouse skin-painting experiments (Mahlum, 1983a,b; Renne and Smith, 1981; Renne et al., in press; Wilson et al., 1984), were found in both the 800-850°F and 850°F+ cuts; only a relatively small amount was present in the 750-800°F cut. Several four-ring PAH, which are known carcinogens, were present in the 750-800 and 800-850°F cuts, including 7,12-dimethylbenzanthracene, benzo(a)anthracene and 5-methylchrysene. These compounds probably constitute only a portion of the total carcinogens present.

The NPAC fraction contained three chemical subclasses of interest: azaarenes, carbazoles, and APAH (Table 4). The APAH compounds were minor constituents compared to the azaarenes and carbazoles. As in the PAH fraction, three- and four-ring compounds were present in the 750-800 and 800-850°F cuts, and some four-ring APAH and carbazoles were present in the 850°F+ bottoms, although most were five- and six-ring compounds.

It is not clear whether the mutagenicity observed in CHO cells from the AH fraction is a false-positive due to contaminating hydrogenated PAH compounds, or whether it represents the true mutagenic potential of these AH fractions for mammalian cells.

Responses in CHO Assay in the Presence or Absence of Exogenous Enzymes

Three sequential 50°F cuts from an SRC-II liquid (750-800, 800-850°F and 850°F+) were fractionated using the alumina column technique. The resultant chemical fractions (Figure 12) were assayed in the CHO/HGPRT system in the presence or absence of an exogenous metabolic activation system (Figure 14).

TABLE 3. Concentration of Representative Neutral PAH in SRC-II bp Cuts

Boiling Range Cuts (°F): Compound	Quantity µg/g			Structure
	750-800	800-850	850+°	
Phenanthrene	424	211	58
Fluoranthene	2,373	732	30
Pyrene	32,769	2,375	235
Benz(a)anthracene	5,713	2,997	---
Chrysene	7,109	7,324	31
Methylchrysene	8,726	35,201	215
7,12-Dimethylbenzanthracene	---	4,532	---
Benzo(k)fluoranthene	---	3,972	306
Benzo[e]pyrene	---	5,755
Benzo[a]pyrene	101	3,530	3,637
Benzo[ghi]perylene	---	---	15,311

TABLE 4. Concentration of Representative NPAC in SRC-II bp Cuts

Boiling Range Cuts (°F): Compound	Quantity µg/g			Structure
	750-800	800-850	850+°	
Carbazole	34	---	---
Benzo[a]carbazole	18,482	20,544	---
Phenanthridine	69	---	---
1-Azapyrene	1,632	47	---
2-Azafluoranthene	29,365	3,745	---
Benz[c]acridine	1,237	2,357	---
Azabenzopyrenes/-benzo-fluoranthenes, etc.	---	4,431		
Azabenzo[ghi]perylene	---	2,396		
2-Aminofluorene	143	68	9
3-Aminophenanthrene	872	120	12
2-Aminoanthracene	371	76	4
2-Aminopyrene	475	771	16
8-Aminofluoranthene	1,092	2,132	54
3-Aminochrysene	119	1,032	120
7-Aminobenzo[a]pyrene	---	73	412

FIGURE 14. Potency in the CHO Assay of Several SRC-II bp Cuts and their Chemical Fractions, Obtained by Alumina Column Separation (Later et al., 1983). The mutagenic responses of CHO cells, in the presence or absence of a metabolic activation system, are compared. Unfractionated distillates are referred to as "crude." Chemical fractions are: 1 = AH; 2 = neutral PAH; 3 = NPAH; and 4 = HPAH.

The following observations are noteworthy: 1) SRC-II coal liquids contain some direct-acting mutagens, or they contain promutagens, which are "activated" (metabolized) by CHO cells without the addition of exogenous enzymes. 2) In most instances, mutagenic activities (both direct and indirect) of various chemical subclasses decrease with bp, as was seen for crude bp cuts. 3) Fraction 2, which contains the neutral PAH compounds, contains no direct-acting mutagens, as measured in our CHO assay. 4) Individual fractions of the 750-800°F cut showed considerable activity; however, this activity seemed to be masked by the milieu of the crude.

DISCUSSION

Using the CHO/HGPRT system, we obtained data for complex mixtures that are in good agreement with carcinogenic and tumorigenic responses observed in mouse skin-painting assays using these same materials (Mahlum et al. 1983; Pelroy et al., 1984; Renne et al., in press). The relationship among assays is shown in Figures 15 and 16. Both genotoxic and carcinogenic activities of SRC-II distillates increase monotonically with bp (Figure 15). SRC-II distillates were generally more active than comparable SRC-I cuts (Figure 16). The SRC-I <650°F cut was negative in all assays.

These data, as well as previously reported results (Mahlum et al. 1983; Pelroy et al., 1984; Renne et al., in press), serve to validate the CHO system as an in vitro predictor of carcinogenic skin activity in rodents. However, in several instances, we detected activity in fractions showing no activity in skin-painting assays. For example, hydrotreated synfuels showed mutagenic activity in our CHO cell assay even though these materials did not induce significant skin carcinogenesis (Frazier and Mahlum, 1984).

Likewise, the CHO assay indicates that HPAH fractions from several SRC-II distillate cuts contain strong mutagens, but they are only weakly tumorigenic in the initiation/promotion (I/P) assay. Cellular toxicity, the failure or lack of an appropriate enzyme-activating system, or the absence of a sensitive target in mouse skin may be explanations for these observations.

We cannot explain the mutagenic activity of AH fractions in the CHO assay. This fraction may be contaminated with hydrogenated PAH; however, these fractions did not induce skin tumors in the I/P assay (Mahlum et al., 1983). Again, CHO cells may be sensitive to the mutagenic potential of AH or hydrogenated PAH although skin cells are not. On the other hand, the CHO assay may detect compounds capable of causing tumors in organs other than mouse skin. We do not know, at this time, whether these observations represent false-positive results or whether they represent real carcinogenic potential. Even if the CHO response to AH compounds is a false-positive, the data can be used to determine which component(s) of that fraction are causing the observed re-

FIGURE 15. Mammalian-Cell (CHO) Mutagenesis, I/P and Chronic Skin-Painting Data for SRC-II bp Cuts. The in vitro test consisted of a mammalian-cell mutagenesis assay employing CHO cells. The skin-painting studies in mice included both an I/P skin carcinogenesis assay, which examined the initiation phase and used total tumors as the endpoint, and a chronic skin-painting assay. In the chronic assay, maximum latency to tumor appearance was used for comparison. N.T. = not tested.

*Relative to SRC-II 850 + °F

FIGURE 16. Comparison of In Vitro Mutagenesis and I/P Skin-Painting Carcinogenesis Data for SRC-I bp Cuts. Tests and endpoints are as described in Figure 15. N.T. = not tested.

sponse and to decide which in vitro assays should be used to screen similar fractions.

The CHO assay is the only test that has detected direct-acting mutagens in synfuel fractions. With it, we can determine whether the active components are converted (by CHO cells) from promutagens to mutagens by enzymes not present in rat skin cells (I/P) or whether they truly represent direct-acting mutagens that are not detected by the Ames assay because of the matrix of the complex mixtures.

Good correlation of results with those of other assays (e.g., that of CHO data with I/P data on skin carcinogenesis for the PAH and NPAC chemical classes) is only one of several criteria for determining the value of an assay system. Other advantages of the CHO/HGPRT assay for screening potential mutagenic/carcinogenic compounds include:

- 1) It is sensitive to most classes of chemical mutagens (even in complex mixtures), in contrast to the Ames assay, which detects only primary amine activity in these mixtures.

- 2) It provides a range of dose-response data that can be used to quantitatively compare the mutagenic potential of various test materials. In vivo assays, such as the I/P, usually examine only a single exposure level, making it difficult to make such comparisons.

The unique information from each type of assay adds to our basic understanding of the test compounds under study.

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APPENDIX A
DETAILED DESCRIPTION OF CHO CELL MUTAGENICITY ASSAY

Development of CHO Assay

We adapted a mammalian-cell forward mutation assay (Chinese hamster ovary/hypoxanthine-guanine phosphoribosyltransferase; CHO/HGPRT) for use in assessing the genotoxicity of complex organic mixtures. In these studies, synthetic fuels, their distillate cuts and chemical fractions have served as model materials. Using this system, we have been able to obtain dose-response relationships with and without exogenous metabolic activating enzyme systems.

We selected the purine salvage pathway enzyme, HGPRT locus, to measure mutation frequencies because it is not essential for growth of cultured cells under normal conditions. Thus, the assay is responsive to various genotoxic mechanisms, including deletions, frameshift mutations, base substitutions, and chromosomal breaks and rearrangements. These lesions all cause loss or inactivation of the enzyme (Gupta and Singh, 1982). Most other genetic markers commonly used to measure mutation frequencies of CHO cells detect only mutations resulting from base substitutions (Wilson et al., 1984).

Our recloning procedure and use of prescreened cells from frozen pools minimize variation in the frequency of spontaneous (background) mutants in the solvent (or negative) controls. Figure A1 shows a historical distribution of mutants/ 10^6 viable cells based on 124 control cultures from 62 experiments. These pooled negative control data yield a median value of less than two mutants/ 10^6 viable cells.

Before assaying complex organic mixtures, we determined whether the assay system was sensitive to the type (chemical class) of compounds expected in petroleum and synthetic fuels. Previous experience showed that CHO cells detected "direct-acting" mutagens (compounds not requiring an exogenous metabolic activation) in diesel exhausts. However, with complex mixtures we anticipated that genotoxicity would require conversion of promutagens to mutagens, via exogenous metabolic activation; therefore, we used an Aroclor-induced rat liver postmitochondrial homogenate, coupled with an NADPH generating system.

FIGURE A1. CHO/HGPRT Mutagenesis, Background Frequency. These data represent 124 independent observations from 62 experiments. Cultures of CHO cells were incubated with solvent (dimethylsulfoxide; DMSO) for 4 hours in the presence of a metabolic activation system. Mutation frequency was determined by counting the number of thioguanine-resistant colonies/10⁶ viable cells. The resultant data were pooled and expressed as histograms.

To obtain linear dose responses with pure compounds required 2 years of examining experimental variables. The most crucial feature of the assay appeared to be development of the metabolic activation system and use of a constant volume (15 μ l) of solvent test chemical for challenge. Cytotoxicity of the enzyme system is considerably reduced by using HEPES buffer instead of KCl; increasing calcium and magnesium concentrations; and carefully preparing the postmitochondrial fraction (activating enzyme).

Preparation of Activating Enzyme

Hepatic microsomal enzymes were induced in Sprague-Dawley rats with Aroclor[®] according to the procedure of Kuroki et al. (1979), a method for preparing activating enzymes that differs slightly from those used for microbial (Ames) assays. Rat livers were minced in the presence of HS buffer (0.2 M sucrose, 2 mM HEPES, pH 7.4), and the cells were gently disrupted using a dounce-type tissue homogenizer. The homogenate was checked, using a phase-contrast microscope, to ensure that cell cytoplasmic membranes were disrupted and that most nuclear membranes remained intact. The resultant material was centrifuged at 9000 x g for 10 minutes at 4°C, and the supernatant was recentrifuged for 1 hour at 105,000 x g. The 105,000 x g pellet was resuspended in HS buffer, assayed for protein, diluted to 20 mg/ml, divided into aliquots and frozen at -80°C until used. Because rat liver (activating enzyme) preparations can vary greatly in their properties, tests were run under a range of protein concentrations and with a number of known mutagens (belonging to different chemical classes) to optimize mutation frequencies.

The reaction mixture consisted of 3.5 ml of F12 medium, 0.5 ml of activating enzyme and cofactors, and 15 μ l of DMSO containing the appropriate concentration of test material. To expose the cells, fresh, serum-free F12 was added to each culture; a 0.5-ml mixture of liver enzymes and cofactors was then added. Cofactors included 3.3 mM glucose 6-phosphate, 1.5 mM NADP, 10 mM Mg^{++} , 10 mM Ca^{++} and 50 mM sodium phosphate, pH 7.5. The concentration of protein (rat liver activating enzymes) was dependent on the specific preparation but was usually 0.5 mg/ml. After gently mixing the test compound in DMSO, it was added to each culture, and the resultant mixture was agitated a second time before incubation.

Cell Culture Medium and Exposure Conditions

Experimental parameters of the assay procedure warrant careful consideration. Obviously, good equipment and trained personnel are required to obtain reproducible data. In addition, all procedures must be standardized, and they should be designed to minimize variations among cultures within, as well as between, experiments.

To obtain good dose-response data, we found it best to use volumes greater than 10 μ l when making dilutions, pipetting, or delivering samples to culture flasks. Many coal liquids have high viscosities, and errors in pipetting are significantly increased when pipetting <10- μ l volumes; therefore, we suggest using the largest volumes possible. Stock samples were warmed for 10 minutes at 37°C. Also, appropriate dilutions should be prepared so that all cultures receive the same challenge volume (~15 μ l). Incomplete or altered solubility of chemicals, or interference due to cytotoxicity, may still preclude good dose-response (mutation frequency) relationships.

All stocks of medium and fetal bovine serum (FBS) must be tested to ensure that they support colony formation efficiently in both standard and selective medium. Different lots of dialyzed FBS can be particularly cytotoxic; similarly, enhanced cytotoxicity may be specific to selective medium. Culture medium can be stored up to 1 month in the dark at 2°C if serum and/or drugs are not added until use.

Controlling Mutation Frequency and Cytotoxicity

Controlling background mutation frequency of the HGPRT locus is the major factor in determining the assay's sensitivity; therefore, for optimal results, the background mutation frequency should be less than two mutants/ 10^6 viable cells. Mutated cells tend to increase during subculture; therefore, experiments must be conducted with cultures regrown from small inocula (e.g., 100 cells). We routinely grow such cultures and freeze a large number of aliquots, usually 25 to 40 vials containing 5×10^6 cells each. When needed, an aliquot is removed from the freezer, and the background mutation frequency is

tested. Only cultures with less than two mutants/ 10^6 cells are used in assays, all from the same lot of cells.

Sufficient numbers of cells must be maintained throughout the assay procedures. For example, when the mutation frequency is 2×10^{-6} in a large population, 1×10^6 cells contain, on the average, two mutants. However, five percent of the time, the actual number would be less than one or more than four; so at low mutation rates, the frequency can vary by a factor of four without being statistically significant. Finally, if 90% of the initial culture ($\sim 2.0 \times 10^6$) is killed by the mutagen, the remaining population of viable cells would be $\sim 2.0 \times 10^5$ cells. Because background mutation frequency is only $\sim 2/10^6$ cells in a large population, use of a small sample is likely to result in its artifactual alteration, thereby increasing the sampling error, since the sample will contain either too few or too many spontaneous mutants.

By carefully observing these procedures and the cautionary suggestions, reproducible results have been achieved with the CHO assay.

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