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General Approach to the Biological Analysis of Complex Mixtures

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The study of potential health effects of combustion effluents involves identifying the substances present and estimating the probable health hazards of each. Unfortunately, this second step cannot be done by using present techniques.

Approximations of health hazards by bacterial and human cell assays are being used to set priorities for further biological studies and to suggest needs for modifications of combustion systems. The assumptions underlying this approximation are discussed, and several examples of combustion effluents are reviewed.

The study of potential health effects of combustion effluents involves identifying the substances present and estimating the probable health hazards of each. Unfortunately, there are no methods which permit sufficiently precise or sensitive estimates of health hazards. We are presently using techniques for which we have no means of independently assessing accuracy.

In our programs at MIT, we have chosen bacterial and human cell mutation assays as a means of measuring genetic change and as an indication of the inherent hazard associated with a chemical or complex mixtures. We will not attempt to defend this choice here, beyond pointing out that humans do indeed suffer from a myriad of diseases of demonstrable genetic origin; this is accompanied by an assumption that environmental chemicals cause an appreciable amount of these diseases. If the assumption is correct, it thus follows that human exposure to chemicals which do, in fact, cause significant genetic damage should be reduced (*1*).

Our approach to complex mixtures depends on the interaction and cooperation of a number of scientists and engineers (*1–10*). In this paper, we will draw illustrative data from an ongoing study of emissions from fossil fuels combustion. Close collaboration involving soot production and chemical and biological analysis has led us to the hypothesis that, at low concentrations, such as those to which people are expected to be exposed, there will not be significant interactions among the chemicals affecting the amount of genetic damage induced by each. Thilly and Kaden have tested this hypothesis fairly vigorously by combusting kerosene in a diffusion flame (*2*). Thus, our expectation for burner emissions can be given by Eq. (1),

$$M = \sum_i c_i A_i \qquad (1)$$

in which M is the fraction of bacterial cells mutated by a known amount of a complex combustion mixture and C_i is the concentration (molar) of each chemical present in the test mixture and A_i is the specific mutagenic activity of each chemical (mutants × liter/survivor × mole) at the concentration tested.

Complex mixtures we have examined in efforts to understand fossil fuel combustion exhausts include light duty diesel exhaust, home oil burner exhaust, airplane turbine exhaust, air particulates and experimental samples of a turbulent diffusion flame (kerosene) and flat flames (hexane, toluene).

The extracts of a turbulent diffusion flame and a light duty diesel engine exhaust will be utilized to demonstrate our general approach to the analysis of complex combustion mixtures and our theory of additivity.

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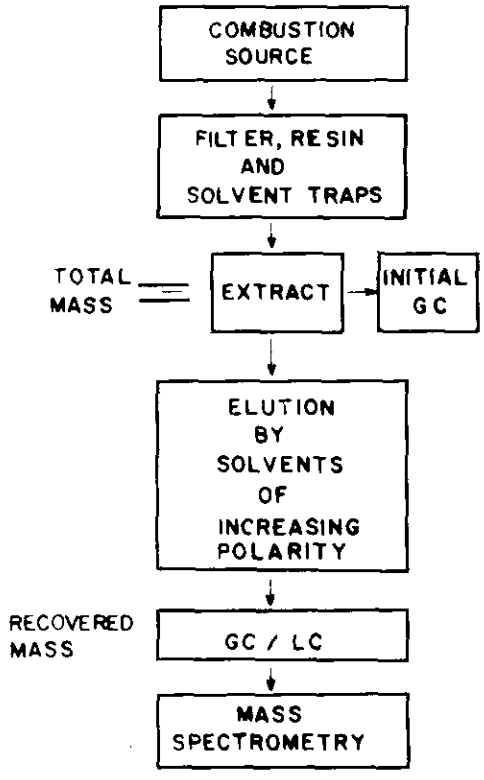

FIGURE 1. General scheme for the chemical analysis of complex combustion mixtures.

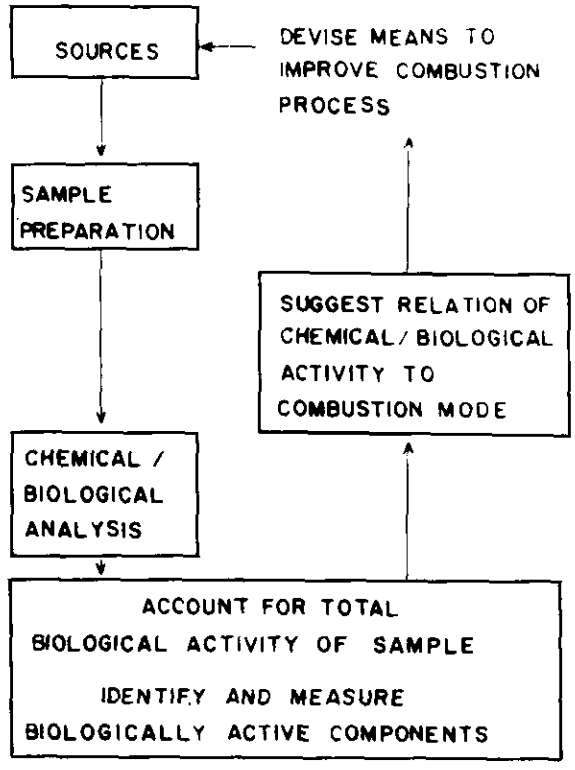

FIGURE 2. Cooperative effort of biologist, chemist and engineer to produce less hazardous high-efficiency combustion systems.

Figure 1 shows our scheme for chemical analysis. We should emphasize that each step requires critical analysis to assure that chemical reactions do not occur solely as a result of the means of collection and separation.

Good examples of these problems are that large masses of biologically active materials in the form of combustion gases are not captured by filters which are very efficient at collecting particles, and that polycyclic aromatic hydrocarbons trapped on filter surfaces achieve a chemical activity of solids and could readily react with vapor-phase materials, such as nitrous oxides, yielding analytical findings consistent with actual exhaust content.

Central to this area is the developing understanding that large amounts of polycyclic aromatic hydrocarbons (PAHs) can be produced from systems such as home oil burners under operating conditions which produce little or no particulate material (soot).

Figure 2 outlines a cooperative effort across disciplines to produce less hazardous high-efficiency combustion systems. Information on the chemical analysis and mutagenic potential of soot extracts generated under a variety of combustion modes is reported to the engineers who then devise the means for improving combustion processes.

In our first example of soot analysis (*2*), Ron Hites' analytical lab extracted the particulates resulting from the combustion of kerosene in a turbulent diffusion flame. Using GC/MS, he was able to determine that virtually all of the components passing through the GC column were PAHs. The chemicals were identified, and their relative abundance was measured. In this way he accounted for some 80% of the whole particulate extract. The gas chromatogram is shown in Figure 3.

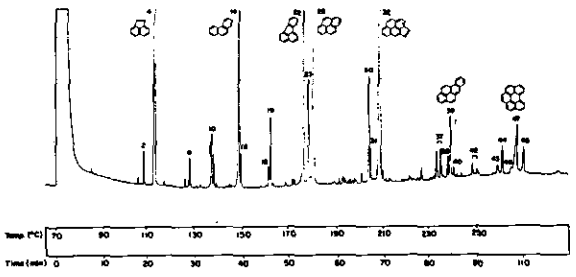

FIGURE 3. Capillary column gas chromatogram of the PAH fraction of kerosene combustion products.

Mutagenic activity was measured by forward mutation to 8-azaguanine resistance in *S. typhimurium* TM677 in the presence of a rat liver post mitochondrial supernatant (4). The assay protocol is outlined in Figure 4.

Figure 5 summarizes the biological fact that, of the more abundant chemicals in this extract, three—cyclopenta(c,d)pyrene, fluoranthene and perylene—were found to have specific mutagenic activities at concentrations which were approximately equal to that of benzo(a)pyrene.

Table 1 gives the order of abundance of the major particulate extractables, which were acenaphthylene (23% w/w) and cyclopenta(c,d)pyrene (15% w/w) with a series of compounds of lesser abundance, including the benzo(a) and (c) pyrenes, which together constitute about 1% of the extract.

In Table 1 we have also summarized the result of calculating the expected mutagenicity for each compound identified in the soot extract using the abundance measurements and the concentration dependence of mutation for each compond, thus, defining the terms A_iC_i given in Eq. (1). The comparison of the Σ of mutagens expected calculations and the actual observed mutation differed by a factor of two. Knowledge of the variance of a single assay has led us to believe this is a fair measure of equality between the calculated and observed mutagenicities. As a further test, the fourteen most abundant compounds were mixed to approximate a "pure soot" and compared in several simultaneous experiments to the raw soot extract.

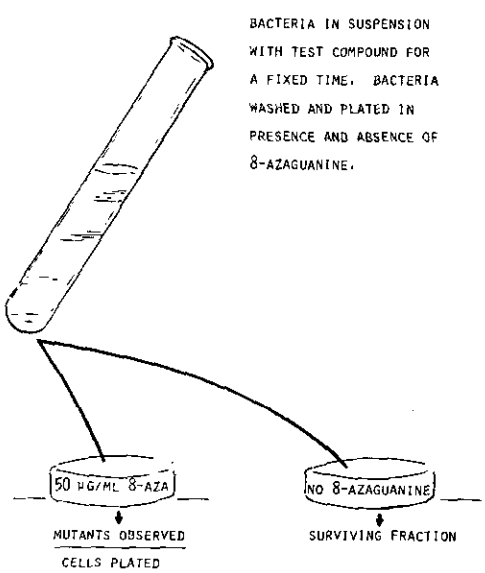

FIGURE 4. Protocol for a quantitative forward mutation assay in *S. typhimurium* using 8-azaguanine as a genetic marker.

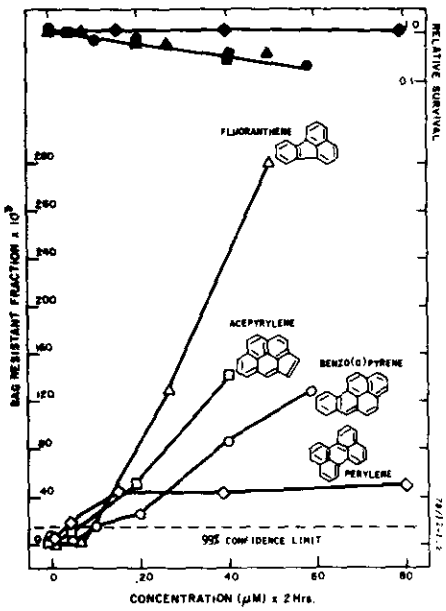

FIGURE 5. Concentration-dependent mutagenicity (○,□,Δ,◇) and toxicity (●,■,▲,◆) of (□,■) acepyrelene, (▲,Δ) fluoranthene, (○,●) benzo(a)pyrene, and (◇,◆) perylene to *S. typhimurium*.

Table 1. Mutagenic contribution of individual PAH to kerosene soot extract.

Compound	Amount present, µg/mL	Mutagenic contribution, induced mutant fraction × 10^5
Acenaphthylene	23	0
Cyclopenta(cd)pyrene	15	165
Pyrene	8	1.7
Benzo(ghi)perylene + anthanthrene	8	3.4
Coronene	5	0
Fluoranthene	4	105
Naphthalene	3	0
Benzo(ghi)fluoranthene	3	—[a]
Phenanthrene + anthracene	2	0
Benzacenaphthalene	2	—
Benzofluoranthene	2	—
Perylene	2	34
Acenaphthalene	1	0
Indeno(1,2,3-cd)pyrene	1	—
Benzo(a)pyrene + benzo(e)pyrene	1	3.4
4H-Cyclopenta(def)phenanthrene	1	—
Benzofluorene	0.4	0
Fluorene	0.3	0
Uncharacterized material	18.3[b]	—
Σ component contributions		312.5
Methylene chloride extract	100	150

[a]Component not available for testing.

[b]Material lost in the characterization process, plus those compounds which could not be identified by gas chromatography-mass spectrometry.

As can be seen in Figure 6, these experiments demonstrated nearly identical concentration dependence of mutation and led us to the hypothesis of simple additivity.

Figure 7 shows that fluoranthene and cyclopenta (cd)pyrene were found to be the most active soot mutagens for human cells. Perylene, a potent mutagen for *S. typhimurium,* did not induce significant mutation under identical conditions of exposure to diploid human lymphoblasts.

Interestingly, Hites (7) has found fluoranthene in abundance in urban air samples, while cyclopenta-(c,d)pyrene has not been found, presumably due to its photoreactivity.

Busby at MIT is evaluating the carcinogenic activity of fluoranthene. Preliminary results show a 35% incidence of lung adenomas, which is far above negative control values. This response is expected to increase once all animals have been sacrificed and examination of tissue sections is performed. Also, J. Babson, performing DNA-binding studies, has tentatively identified a fluoranthene adduct which binds to guanine.

The next example is of an extract provided by Exxon Research and Engineering Co. They presented us with 2 g of a methylene chloride extract of soot particulates collected on filters in a dilution tunnel from an Oldsmobile 350 diesel exhaust. This material was mutagenic for both bacterial and human cells in the presence of a rat liver enzyme preparation (Fig. 8). Although this extract was a potent mutagen for *S. typhimurium,* it did not mutate human cells in the absence of the metabolic surrogate (Fig. 9). This observation is in accordance with the ability of urban air particulate extracts (Fig. 10) to mutate human lymphoblasts only in the presence of an exogenous metabolizing system and not in its absence. These findings have led the MIT groups to a series of critical assumptions. We decided that the identification of the complex mixtures and compounds mutating both human and bacterial cells should have a higher priority than those compounds which mutated *S. typhimurium* only. This is supported by the fact that time-dependence experiments (data not shown) have indicated that rat liver extracts rapidly metab-

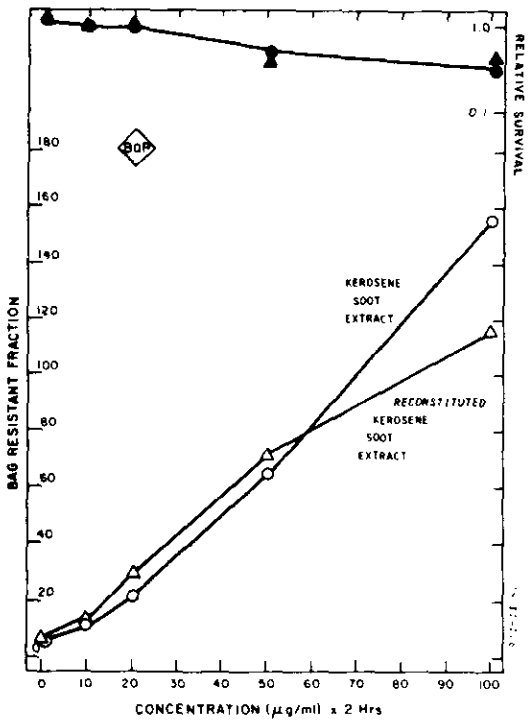

FIGURE 6. Concentration-dependent mutagenicity (○,Δ) and toxicity (●,▲) of a (○,●) methylene chloride kerosene soot extract and (Δ,▲) a reconstituted kerosene soot extract to *S. typhimurium* in the presence of Aroclor-induced PMS. The reconstitution mixture to which the cells were exposed contained acenaphthylene (2,3 mg/mL), acepyrelene (1.5 mg/mL), pyrene (800 μg/mL), coronene (500 μg/mL), benzo(ghi)perylene (400 μg/mL), anthanthrene (400 μg/mL), fluoranthene (400 μg/mL), naphthalene (300 μg/mL), perylene (200 μg/mL), phenanthrene (100 μg/mL), anthracene (100 μg/mL), acenaphthalene (100 μg/mL), benzo(a)pyrene (50 μg/mL) and benzo(e)-pyrene (50 μg/mL). Each point represents the average of two independent determinations. BaP = benzo(a)pyrene (80 μM). 8AG = 8-azaguanine.

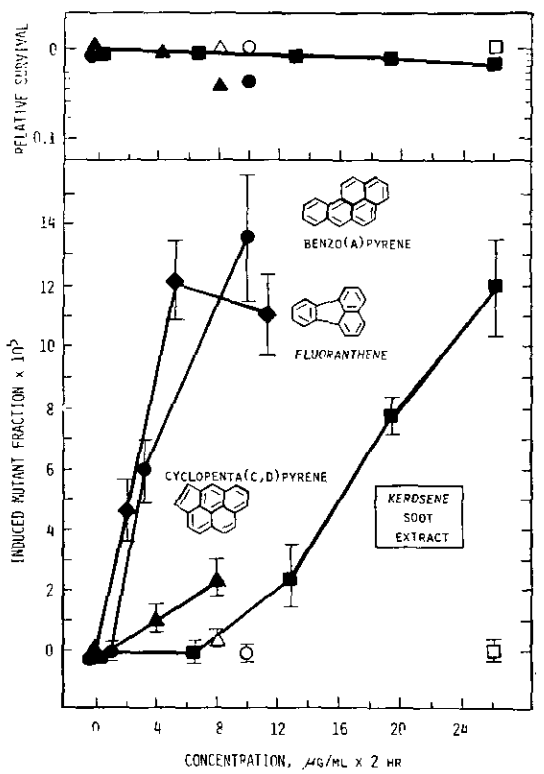

FIGURE 7. Concentration-dependent mutagenicity and toxicity of mutagenic soot components to human lymphoblasts.

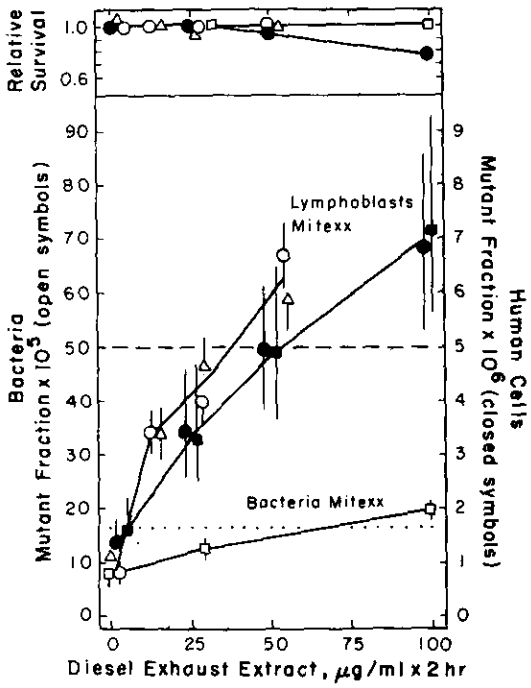

FIGURE 8. Concentration-dependent mutagenicity and toxicity of a diesel soot extract to *S. typhimurium* and human lymphoblasts in the presence of metabolic activation.

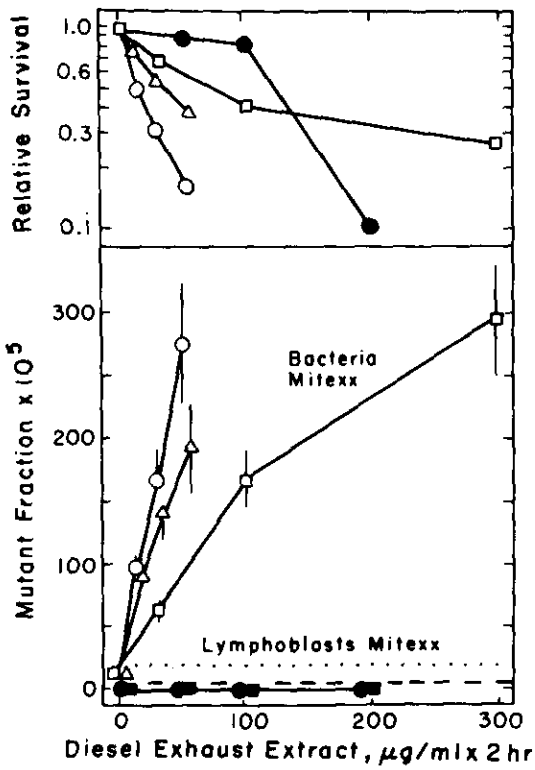

FIGURE 9. Concentration-dependent mutagenicity and toxicity of a diesel soot extract to *S. typhimurium* and human lymphoblasts in the absence of metabolic activation.

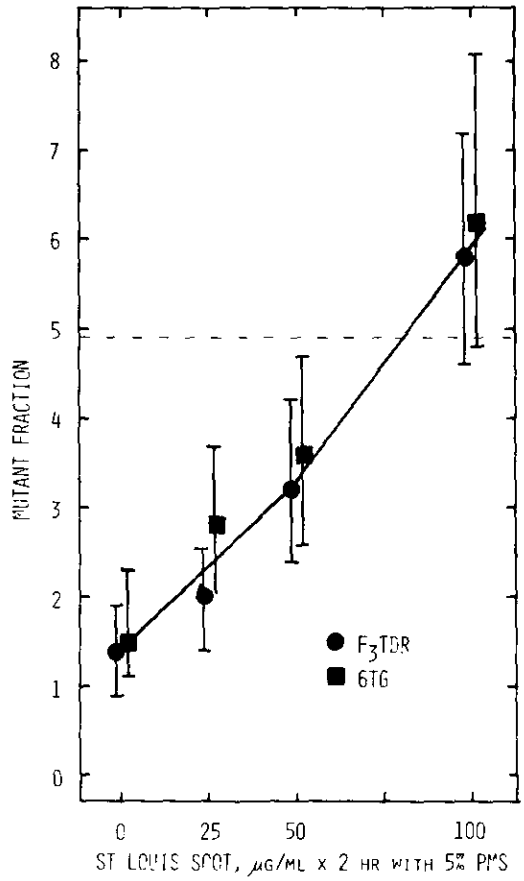

FIGURE 10. Concentration-dependent mutagenicity and toxicity of an urban air particulate extract to human lymphoblasts in the presence of an exogenous metabolizing system.

olize these compounds mutagenic to bacteria to inactive species. We then subjected these compounds to an iterative procedure of comparing bacterial responses to human cell responses when the rat liver metabolizing system was included. These results further demonstrated that the set of compounds mutating human and bacterial cells were similar.

As a means of facilitating chemical analysis, Ron Hites employed a silicic acid column which, when eluted successively with solvents of increasing polarity, yielded 70–80% of the original mass of the sample. Of the loaded material, 20–30% seems to be accounted for in the form of a brown, nonmutagenic band which did not move down the column and is hypothesized to be high molecular weight material soluble in warm, but not room-temperature, methylene chloride. As Figure 11 shows, the fractions demonstrating the most mutagenicity were those eluted by a hexane:toluene 1:1 mixture and by toluene. One should note that the extract was not treated with acidic or basic mixtures, as is a

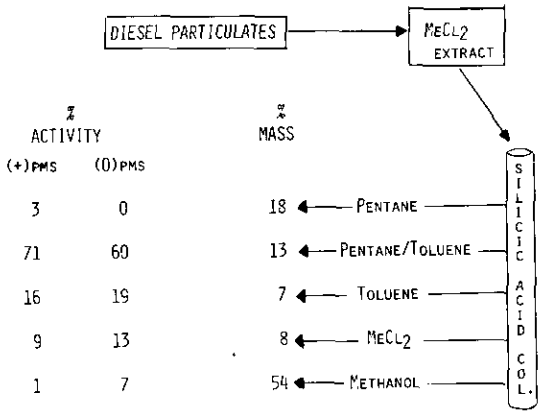

FIGURE 11. Percent mutagenicity associated with various fractions produced by fractionating a diesel particulate extract on a silicic acid column with solvents of increasing polarity when assayed with *S. typhimurium*.

common practice. We feel that the capability of this method to produce chemically and biologically unaltered fractions has not been satisfactorily evaluated. Silicic acid column separation results in no loss of biological activity of reconstituted fractions when compared to equivalent weights of the unfractionated extract. One should further note that such comparisons absolutely require mutagenic potency assessments of the various fractions, both with and without the metabolizing enzymes. In these cases the more polar fractions and the crude extract were markedly toxic in the absence, but not in the presence, of postmitochondrial supernatant.

The hexane:toluene fraction was analyzed by GC/MS, as shown in Figure 12, and the principal findings are summarized in Table 2. In short, fluoranthene (3%), the alkyl phenanthrenes (25%) and alkyl fluorenes (11%) were found in the greatest abundance; benzo(a)pyrene was estimated to occur at no greater than 0.2% of the fraction.

The synthesis and testing of individual alkyl phenanthrenes and fluorenes is in progress. Already, the mutagenicity of 40-50% of this fraction for both human and bacterial cells can be accounted for by fluoranthene and specific monomethyl phenanthrenes. Human cell studies under the direction of Thilly indicate that the 1-methyl and 9-methyl derivatives

Table 2. Diesel soot extract, hexane/toluene fraction (approximate composition).

	Recovered from GC,%	
Fluoranthene	3.3	
C_1	1.6	
Phenanthrene	3.3	
1-ME	2.0	
2-ME	3.3	
3-ME	2.4	
4+9-ME	2.0	25
C_2	8.0	
C_3	4.6	
C_4	2.7	
Fluorene	0	
C_1	0.4	
C_2	2.7	
C_3	1.6	11.1
C_4	3.3	
Benzopyrenes	0.4	
Nitropyrene	0.4	
Perylene	0.04	
Other identified compounds	28.0	
Unidentified compounds	40.0	

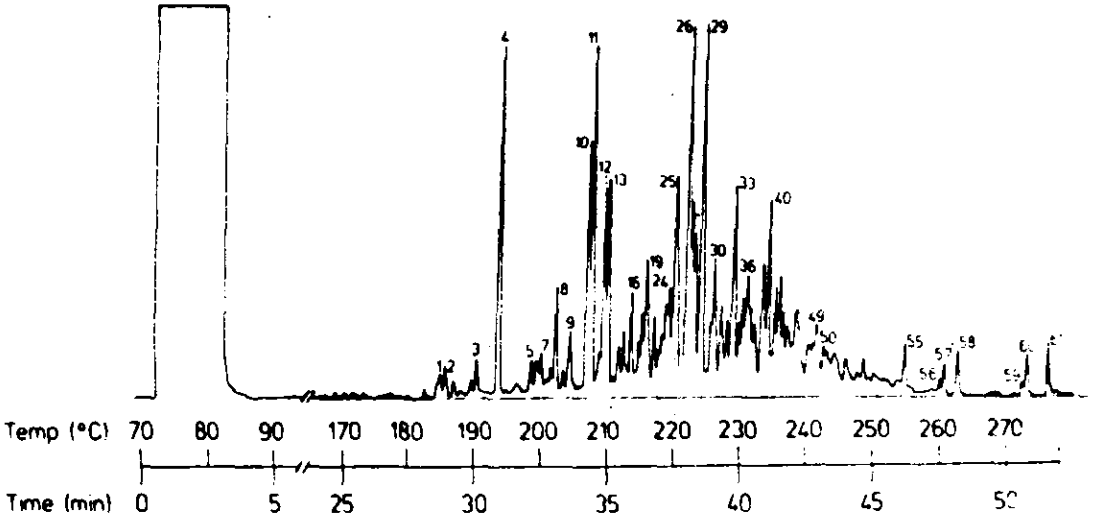

FIGURE 12. Capillary column gas chromatogram of the hexane/toluene fraction of diesel particulate extracts.

of phenanthrene approach the specific activity of benzo(a)pyrene (Fig. 13). However, 2-methylphenanthrene is the most mutagenic of the series when assayed in bacteria (data not shown). W. Roush of MIT's Department of Chemistry has set out to synthesize a selected subset of C_2, C_3 and C_4 derivatives, and we anticipate future research identifying principal metabolites, DNA adducts and premutagenic reaction products with our collaborators, Marletta, Wogan and Essigmann.

The last example is given in Figure 14, where we see the result of injecting 13 μg of a combined extract of the soot particulates and combustion gases from a residential oil burner directly onto a liquid chromatograph. We hope to achieve a one-step approach for identifying the mutagenic chemicals of a complex mixture while separating the mixture under nondestructive, nonreactive conditions.

Knowing the amount of mutation and toxicity produced by each fraction of the HPLC output, we were able to account for 90–100% of the original activity in the unfractionated sample. Note that four major peaks accord with very minor peaks from the absorbance detector of the HPLC. We

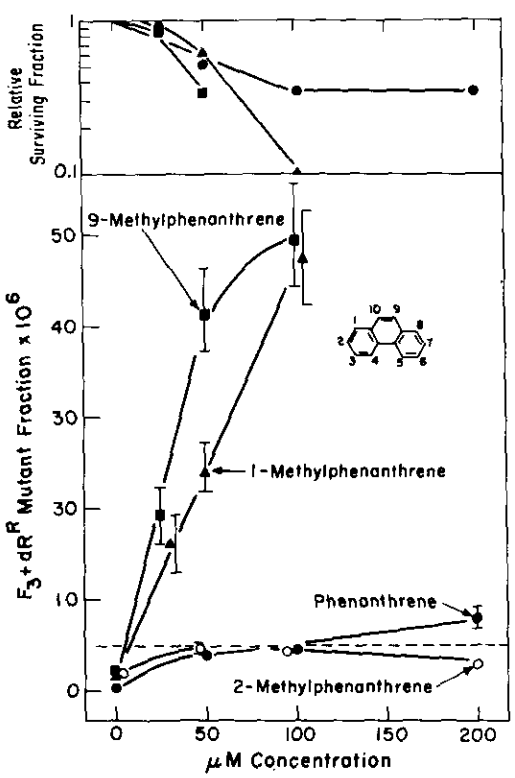

FIGURE 13. Concentration-dependent mutagenicity and toxicity of phenanthrene and its methylated derivatives to human lymphoblasts in the presence of postmitochondrial supernatant.

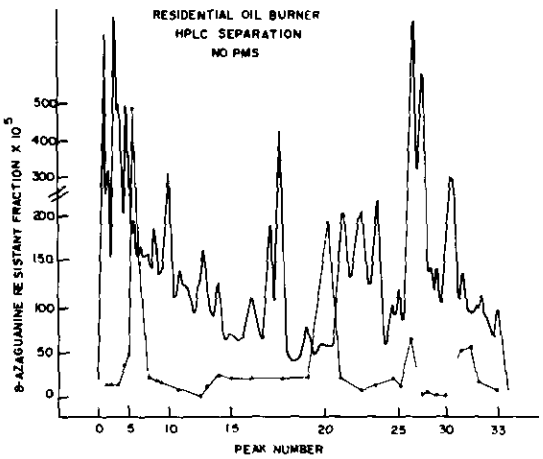

FIGURE 14. High pressure liquid chromatographic evaluation of mutagenicity to *S. typhimurium* of a residential oil burner extraction in the absence of postmitochondrial supernatant.

believe this method constitutes an efficient step toward the identification of the mutagenic compounds in this particular complex mixture which contains a variety of compounds of varying polarity. To evaluate the mutagenic activity of the HPLC fractions, it was necessary to scale down our forward mutation assay for bacteria so it could be performed in volumes of 10–100 μL, capable of detecting the equivalent of 10–100 ng of benzo(a)-pyrene (unpublished development).

This paper outlines the general approach to the analysis of complex combustion mixtures. The identification of those components of complex mixtures which are the principal biologically active agents with respect to their concentration is an important first step. Mutagenic analysis can be performed initially in bacterial assay, and, when warranted, in more expensive human cell systems. In general, however, we urge you to consider the approach by which we are studying our complex mixtures, criticize it for its shortcomings and use it when it is appropriate.

The work discussed herein has been primarily supported by the National Institute of Environmental Health Sciences (at MIT) and the United States Department of Energy (at MIT and Indiana University). This paper was prepared as an oral review for this conference and draws frequently on previously published work for examples.

Engineers studying combustion (J. Longwell, J. Howard, A. Sarofim and J. Beer) in the MIT Department of Chemical Engineering were responsible for setting up, operating burners under reproducible conditions and devising means to assure collection of all the effluent material. Extraction and preliminary chemical identifications were followed by capillary column gas chromatography separations and identifications by mass spectrometry in the MIT Department of Chemistry (J. Leary and K.

Biemann) and in the Department of Chemistry at Indiana University (R. Hites). Bacterial mutation assays were performed in MIT's toxicology testing laboratory (D. Kaden). In the MIT Department of Nutrition and Food Science, human lymphoblast cell studies (W. G. Thilly) were performed when required (H. Liber, T. Skopek and T. Barfknecht). Also, in the MIT Department of Nutrition and Food Science, the DNA adduct of the potent soot mutagen fluoranthene has been tentatively identified (G. N. Wogan and J. Babson), and preliminary studies on fluoranthene's carcinogenic potency (W. Busby) are in progress.

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