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UC-90d

Chemical Characterization and Genotoxic Potential Related to Boiling Point for Fractionally Distilled SRC-I Coal Liquids

B. W. Wilson
R. A. Pelroy
D. D. Mahlum

July 1982

Prepared for the U.S. Department of Energy
under Contract DE-AC06-76RLO 1830

Pacific Northwest Laboratory
Operated for the U.S. Department of Energy
by Battelle Memorial Institute

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CHEMICAL CHARACTERIZATION AND GENOTOXIC POTENTIAL RELATED TO BOILING POINT FOR FRACTIONALLY DISTILLED SRC-I COAL LIQUIDS

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Pacific Northwest Laboratory
Richland, Washington 99352

FOREWORD

This document reports on an initial attempt to establish correlations between results of in vivo and in vitro biological assays on a set of boiling point cuts of a coal liquid, and to relate bioassay results to chemical composition. Understanding the interrelationships among various biological test results and the chemical constituency of a coal liquid will be of value in identifying ameliorative strategies to be applied to any coal conversion process. The work was conducted by investigators in the Biology and Physical Sciences Departments at Pacific Northwest Laboratory (PNL), and the Department of Chemistry at Brigham Young University, under subcontract to PNL.

EXECUTIVE SUMMARY

This report summarizes selected research efforts at the Pacific Northwest Laboratory that are oriented toward ameliorating the genotoxic potential of direct coal liquefaction materials through modification or optimization of process conditions. Optimizing the distillation step in direct liquefaction to isolate genotoxins in heavy-end materials has been proposed as one way to produce a coal liquid distillate that is substantially less genotoxic than coal liquids produced to date from pilot plant operations of the SRC-I and -II processes. Studies described in this report were conducted to evaluate the utility of optimized distillation for coal liquids from the SRC-I process.

SRC-I process solvent (PS) obtained from the Wilsonville, Alabama pilot plant were distilled into 50°F-range boiling point (bp) cuts at the Air Products research facility in Linwood, Pennsylvania. Chemical analysis of the fractions showed that aliphatic content decreased, while nitrogen-containing polyaromatic compound content increased with increasing bp. Polyaromatic hydrocarbon (PAH) and hydroxylated PAH content also increased with bp. Analysis of amino-PAH (APAH) showed that mutagenic APAHs containing 3 or more rings were found primarily in fractions boiling above 750°F. Previous studies had indicated these as the most mutagenic APAH (Pelroy and Wilson 1981.

Three microbial tester strains were used to screen for genetically active agents in the SRC-I distillate bp cuts. Reverse mutation with the Ames tester strain TA98 demonstrated that mutagens were concentrated in the bp cuts boiling above 700°F. For this tester strain most of the genetic activity in these

distillates was attributable to chemical fractions enriched in APAH having 3 or more rings. Mutagenicity data obtained with TA98 was in good agreement with skin carcinogenesis results from the mouse-skin initiation/promotion (in vivo) test system. Forward mutation with TM677 was bimodal as a function of boiling point (as described in section 2) with most of the genetic activity associated with components boiling between 600° and 700°F. The strongest response in the forward mutation assay did not occur in the most carcinogenically active fractions. Low levels of lambda prophage induction in Escherichia coli K12 (strain 8177) were observed for most of the bp cuts. Prophage induction turned out to be poorly related to carcinogenesis results.

Results of initiation/promotion experiments used to measure the relative potency of bp cuts as initiators of mouse skin carcinogenesis again showed that fractions boiling above 750°F were substantially more potent than those boiling below 750°F. PAH with boiling points at or above that of the dimethyl benzanthracenes were concentrated in the most active bp cut. Compounds reaching their nighest concentrations in the highest boiling and most carcinogenically active cut included known carcinogens such as benzo[a]pyrene and dimethyl benzanthracene.

Thus, all biomedical test results indicate that consideration should be given to conducting distillation so as to minimize, in the distillate product, the concentrations of those biologically active compounds found in cuts boiling above 700°C (as defined in this study). Studies have been initiated to assay chemical fractions of these bp cuts to determine which specific compounds or groups of compounds are responsible for tumor initiating activity of SRC-I coal liquids.

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1.0 INTRODUCTION

Modifications such as two stage liquefaction (TSL) and redistillation of blended liquids from various process streams are currently being evaluated for use in the SRC-I process. It is anticipated that these process improvements will reduce the mutagenic and carcinogenic potential of resultant coal liquid products. For example, catalytic hydrotreatment, such as occurs in the second stage of TSL, substantially reduced the mutagenicity of an SRC-II fuel oil blend (PNL-3464). Hydrotreatment was achieved using conventional petroleum catalysts. The effect appears to result from deamination of amino polycyclic aromatic hydrocarbons (APAH) present in the source material, although substantial denitrification and desulfurization of heterocyclic polyaromatic compounds also occur.

During hydrogenation there occurs a concomittant reduction in average molecular weight and aromaticity owing to ring cleavage. Both effects probably contribute to reducing the genotoxicity and/or carcinogenicity reported for several hydrogenated coal liquids (Wilson et al. 1980). Additional effects of hydrogenation, include an overall decrease in heteroatom content which results in fewer polar constituents; the most notable reduction being in the concentrations of phenolic compounds. Most evidence indicates that hydrotreated coal liquids are more benign from environmental and health effect standpoints than are their pre-hydrotreated counterparts. However, increased renal toxicity has been noted during skin painting of hydrotreated fossil fuels in rodents (Personal communication, M. Holland, 1981).

A characteristic of SRC-II coal liquefaction materials studied in our laboratory is that their mutagenic and carcinogenic activity is confined almost exclusively to higher-boiling constituents (PNL-3189, PNL-3787). Analysis of boiling point (bp) cuts from bench-scale distillations and distillations carried out under pilot-plant conditions showed that nearly all the mutagenicity and carcinogenicity exhibited by these materials were confined to components boiling at temperatures above 700°F (PNL-3787). This suggests that one strategy for removing active components from SRC materials might be to fractionally distill the coal liquid and separate the high-boiling or "heavy-end" portions from the remainder of the SRC full-boiling-range material.

Studies conducted within the last year under the DOE Fossil Energy Program have evaluated the effectiveness of hydrotreatment and fractional distillation, or a combination of the two in reducing the mutagenicity and carcinogenicity of coal liquids.

This status report describes results of chemical analyses, in vitro tests, and initiation/promotion mouse skin carcinogenesis assays on 50°F distillate bp cuts of SRC-I process solvent with a bp range from about 450°F to 850°F; the 800°F to 850°F^{+} fraction included the distillation bottoms.

Battelle
Pacific Northwest Laboratories
P.O. Box 999
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Telephone (509)
Telex 15-2874

August 12, 1982

Recipients of PNL-4277

Dear Recipients:

RE: Chemical Characterization and Genotoxic Potential Related to Boiling Point for Fractionally Distilled SRC-I Coal Liquids, Pacific Northwest Laboratory, Richland, WA. July 1982

Please replace page 2.1 with the attached.

Sincerely,

B W Wilson

B. W. Wilson
Staff Scientist

BWW:vmg

BWW 12/9/82
Not re-ficked

2.0 CHEMICAL ANALYSIS

The process solvent (PS) used in this study was produced at the Wilsonville, Alabama pilot plant and had a bp range of 450°F to 850°F plus a small amount of 850°F$^+$ heavy-ends. The material was distilled into 50°F bp cuts at the Air Products, Inc., research facility in Linwood, Pennsylvania. Distillation run conditions and inspection data are given in Table 2.1. The distilled coal liquid cuts and the source SRC-I PS should not necessarily be considered representative of coal-derived fuels which may eventually be produced commercially.

TABLE 2.1. Distillation Run Conditions and Inspection Data SRC-I Boiling Point Cuts

Wilsonville Run 220	Wilsonville Run 220
C5-450°F	450-850°F
Int-200°F	Int-450°F
200-250°F	450-500°F
250-300°F	500-550°F
300-350°F	550-600°F
350-400°F	600-650°F
400-450°F (BTMS)	650-700°F
	750-800°F
	800-850°F (BTMS)

Distillation Run Conditions:
Efficiency: 20-25 Theoretical Plates at Total Reflux.
Reflux Ratio: 12-10-5-2:1
Run Pressure: Atmospheric--with a N_2 Blanket on the Still.
Observations: Material Foams--probably because of high N_2 content. This condition reduced the boil-up rate considerably in an extended running time. Indication of some cracking or polymerization during an ASTM D-86 determination on the original sample.

Cuts were kept at a maximum temperature of 4°C under a N_2 blanket.

2.0 CHEMICAL ANALYSIS

The process solvent (PS) used in this study was produced at the Wilsonville, Alabama pilot plant and had a bp range of 450°F to 850°F plus a small amount of 850°F$^+$ heavy-ends. The material was distilled into 50°F bp cuts at the Air Products, Inc., research facility in Linwood, Pennsylvania. Distillation run conditions and inspection data are given in Table 2.1. The distilled coal liquid cuts and the source SRC-I PS should not necessarily be considered representative of coal-derived fuels which may eventually be produced commercially.

TABLE 2.1. Distillation Run Conditions and Inspection Data SRC-I Boiling Point Cuts

Temperature °F	API Gravity 60°F	Specific Gravity 60/60°F	Liquid Temperature °F	Pressure Wt% MM	Chg
IBP-350	37.4	0.8378	403	100	1.9
350-400	29.2	0.8805	425	100	1.9
400-450	230.0	0.9159	464	100	2.8
450-500	22.1	0.9212	491	100	3.1
500-550	18.6	0.9427	516	100	4.8
550-600	13.2	0.9779	480	0.8	10.2
600-650	9.4	1.0050	512	0.8	7.8
650-700	4.2	1.0427	567	0.8	8.4
700-750	2.0	1.0599	619	1.0	6.7
750-800	1.4	1.0647	640	1.2	3.9
800-850	-0.7	1.0724	700	3.5	5.5
850-BTMS	--	1.214			41.4

1956CB Orig Feed Stock (Lummus 2LCF-29)

Distillation Run Conditions:
Efficiency: 20-25 Theoretical Plates at Total Reflux.
Reflux Ratio: 12-10-5-2:1
Observations: There were no signs of significant cracking.
The maximum Pot Temperature at 800°F head was 700°F.

Estimates were made of the relative aliphatic, PAH, nitrogen-containing polyaromatic and hydroxylated PAH content for each cut boiling above 600°F. Major compounds present were characterized with respect to molecular weight and probable ring structure. The cut containing amino polycyclic aromatic hydrocarbons was derivatized according to the method of Later et al. (1982) and analyzed specifically for APAH. PAH fractions were analyzed quantitatively for several different compounds including phenanthrene, pyrene, chrysene and benzo[a]pyrene. Semi-quantitative estimates of concentration were obtained for a number of other PAHs. Analyses were restricted to the 600°F or higher boiling materials, because previous work on coal-derived liquids showed that middle distillates exhibited essentially no mutagenic or carcinogenic activity (PNL-3189, PNL-3787).

CHEMICAL CLASS FRACTIONATION

Details of the column chromatographic separation method used to isolate chemical class fractions were previously described (Later et al. 1981). A schematic diagram of the modified separation scheme is shown in Figure 2.1. Figures 2.2 and 2.3 show the relative weight percentages of the four major compound classes found in the 50°F cuts boiling above 600°F. The classes isolated by alumina column chromatography included aliphatic hydrocarbons, neutral PAH nitrogen-containing polycyclic aromatic compounds (N-PAC), and hydroxy polycyclic aromatic hydrocarbons (HPAH). The N-PAC fraction was further separated into amino polycyclic aromatic hydrocarbons (APAH) and two separate subfractions of polycyclic aromatic nitrogen heterocycles (PANH). Figures 2.2 and 2.3 show that increasing bp is associated with changes in the

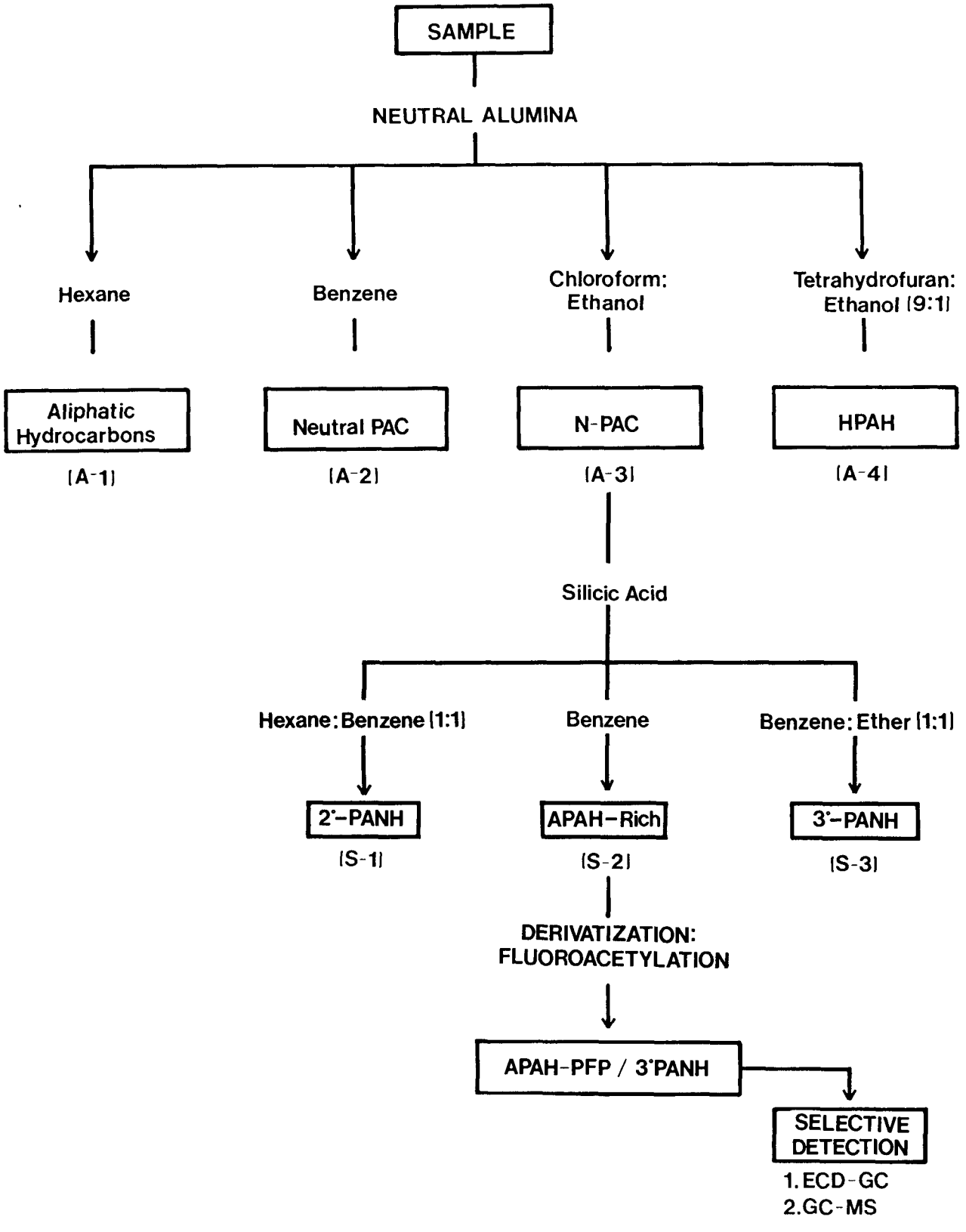


FIGURE 2.1. Schematic Separation Method Used to Obtain Chemical Class Fractions.

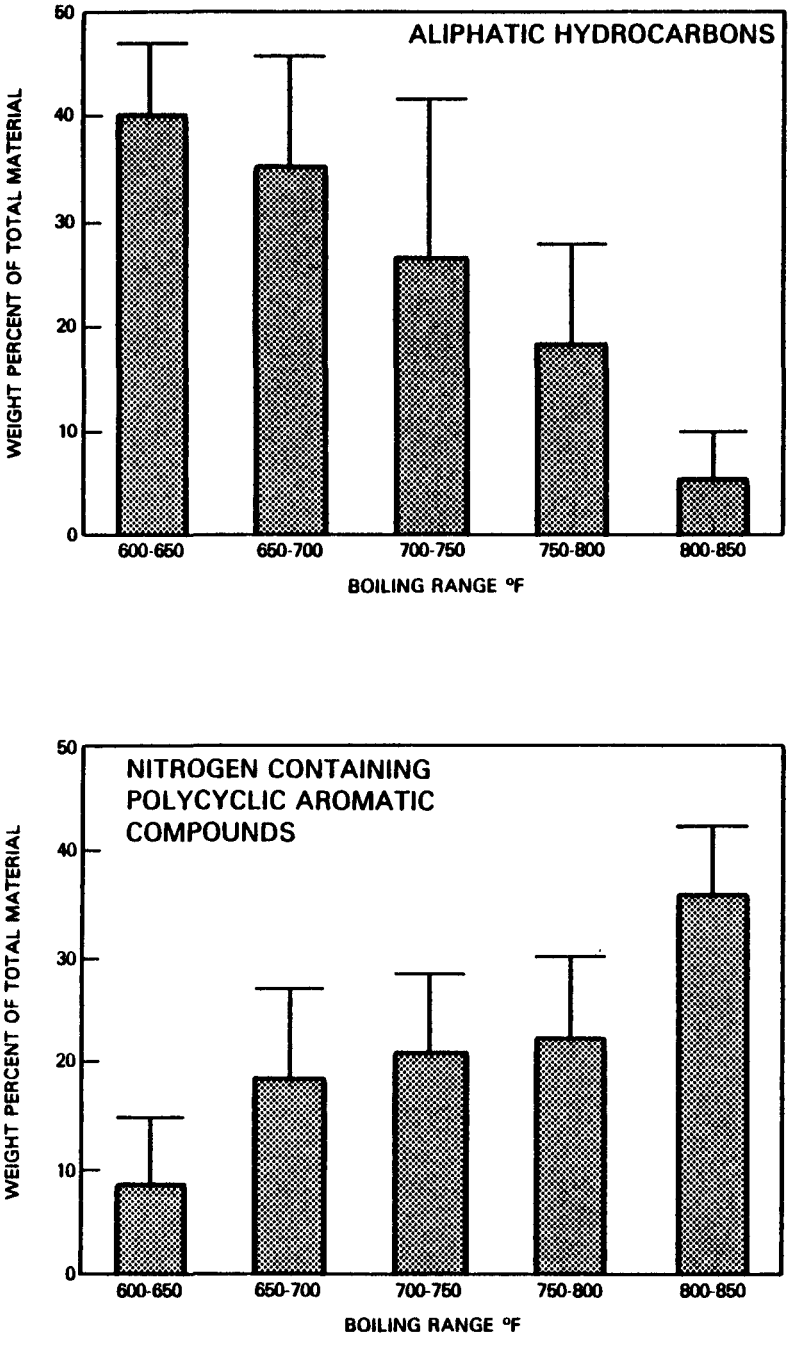


FIGURE 2.2. Comparison of Nitrogen-Containing Polyaromatic Hydrocarbon and Aliphatic Hydrocarbon Content as a Function of Boiling Point as Estimated by the Method of Later et al. (1981). The error bars represent the range obtained from 3 or more determinations.

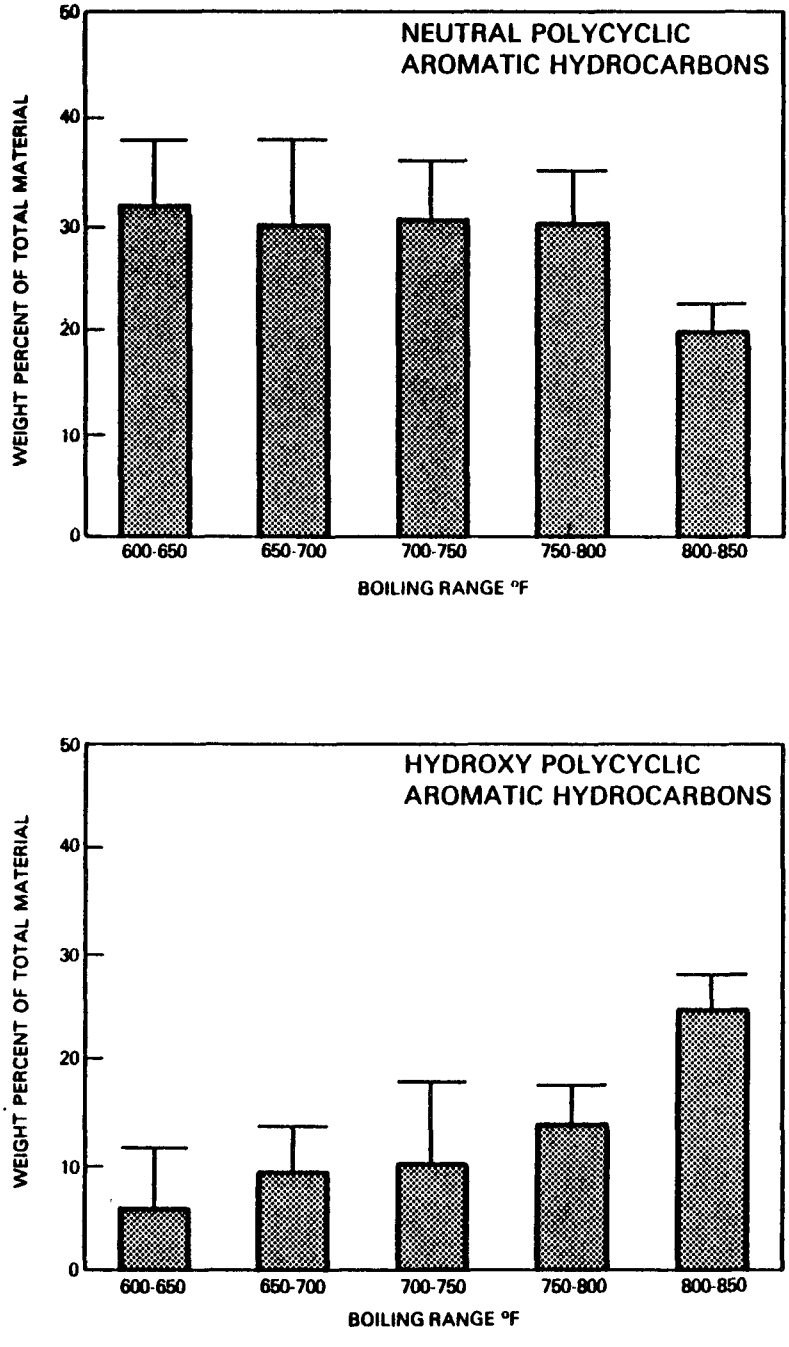


Figure 2.3. Comparison of Neutral Polycyclic Aromatic Hydrocarbons and Hydroxy Polycyclic Aromatic Hydrocarbons Content as a Function of Boiling Point as Estimated by the Method of Later et al. (1981). The error bars represent the range of values obtained from 3 or more determinations.

relative concentrations of at least two types of compounds; the concentrations of aliphatic hydrocarbons decrease, and the N-PAC concentrations increase, with boiling temperature.

DETERMINATION OF PAH

PAH fractions obtained from each of the SRC-I bp cuts were analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GCMS) to determine those compounds present. The fractions were also analyzed by simultaneous nitrogen-specific and flame ionization GC detectors to check for the presence of nitrogen-containing contaminants in the neutral PAH fractions.

Estimated concentrations for the compounds monitored in the cuts are plotted in Figures 2.4 through 2.7. Estimates of PAH concentration by GCMS were made for several individual compounds by construction of standard curves related to isotopically labelled internal standards (d_{10} anthracene and d_{12} perylene) added in known concentrations to the mixtures prior to analysis. Samples were run in replicate on both GC and GCMS, and concentrations listed in most cases were averaged from two or more determinations.

Concentrations of some higher-molecular-weight PAHs were estimated by probe inlet mass spectrometry. The method descriminates, to some extent, against alkylated homologs, since these compounds fragment more readily, and it does not allow differentiation among isomers. However, the method is valuable in obtaining an overall estimate of the average molecular weights for the PAH fractions and in establishing an upper limit on the concentration of higher molecular weight compounds.

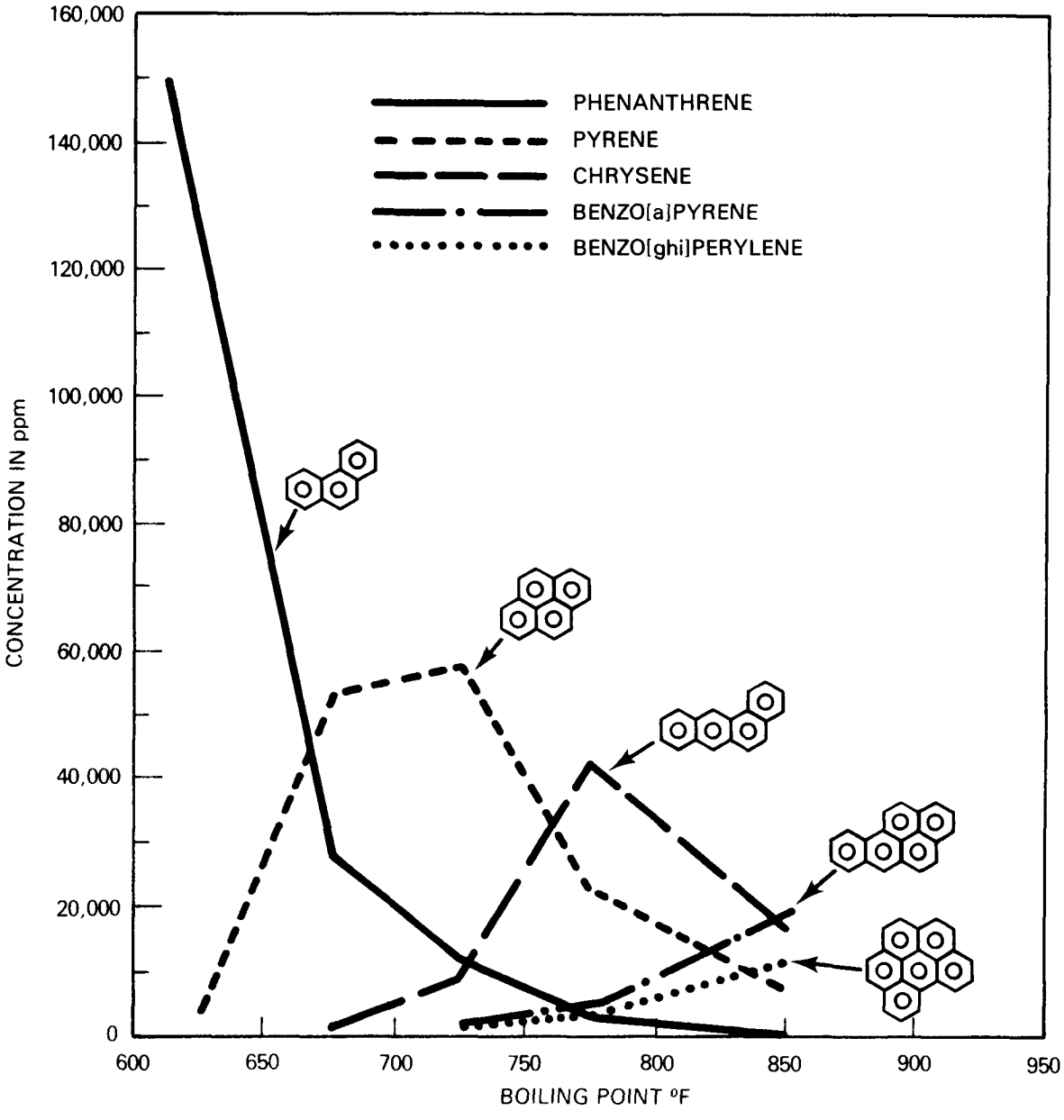


FIGURE 2.4. Estimated Concentration of all Isomers for Several Parent PAH structures in the A-2 Fraction from SRC-I Boiling Point Cuts. These values were obtained by the direct probe MS method.

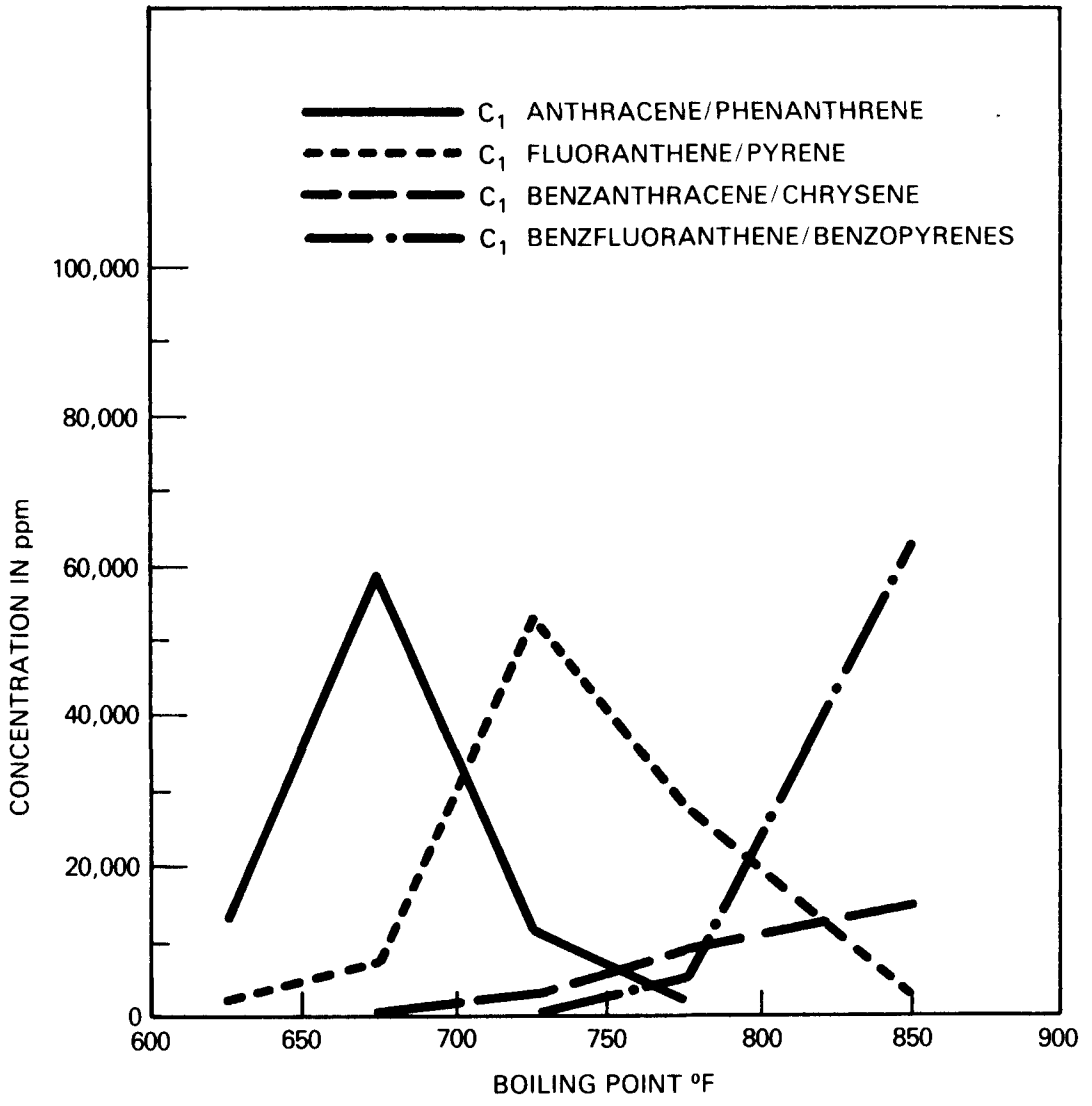


FIGURE 2.5. Estimated Concentration for C_1 Homologs of All Isomers for 3-, 4- and 5-ring PAH Structures in the A-2 Fraction From SRC-I Boiling Point Materials. Values were obtained by the direct probe MS method. Estimates for C_1 fluoranthrene/pyrene also include benzo fluorenes.

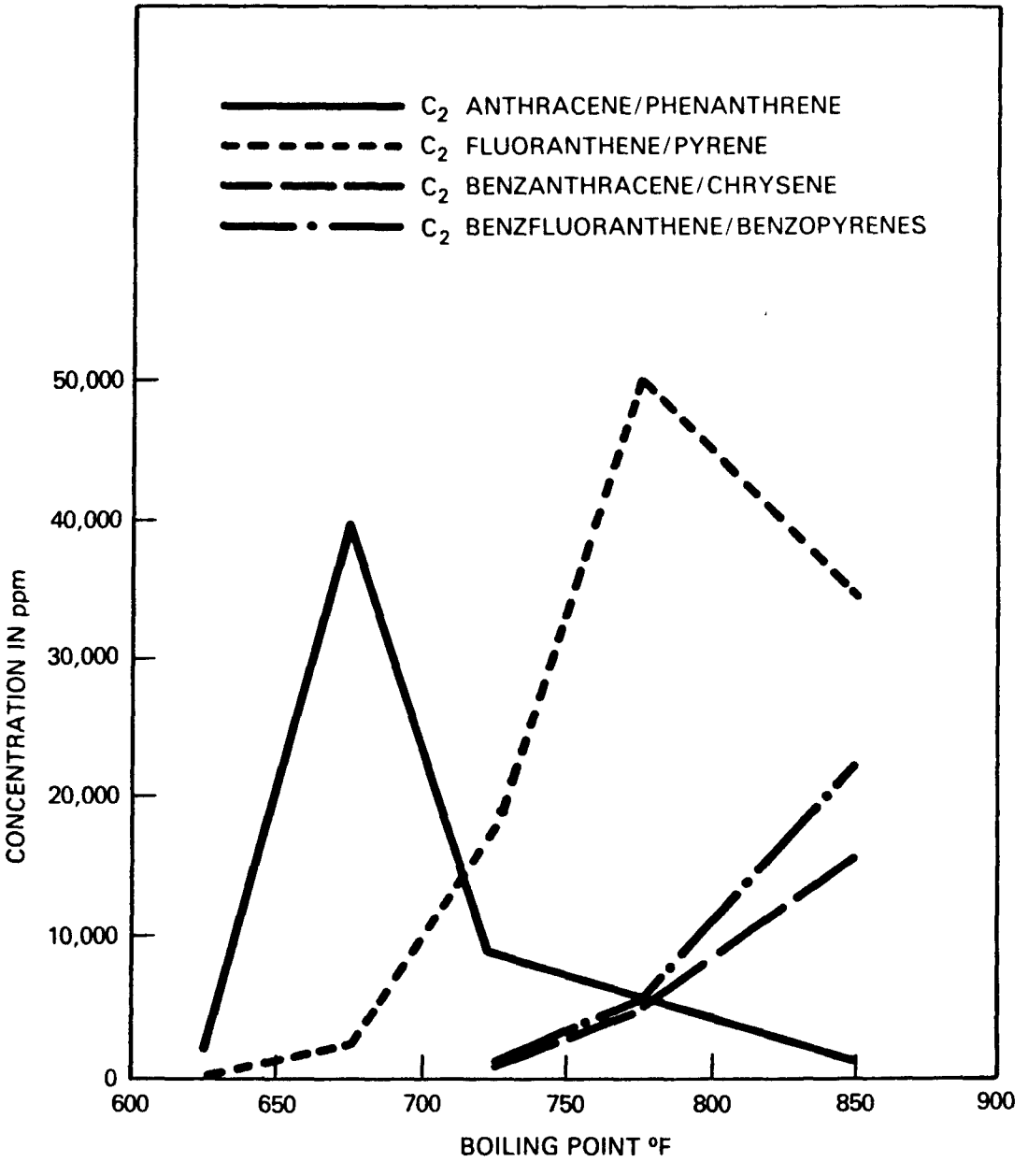


FIGURE 2.6. Estimated Concentration of C_2 Homologs for 4 PAH Structures in the A-2 Fraction of the SRC-I Boiling Point Cuts. Estimates include all isomers. C_2 fluoranthene/pyrene estimates also include C_1 benzo fluorenes.

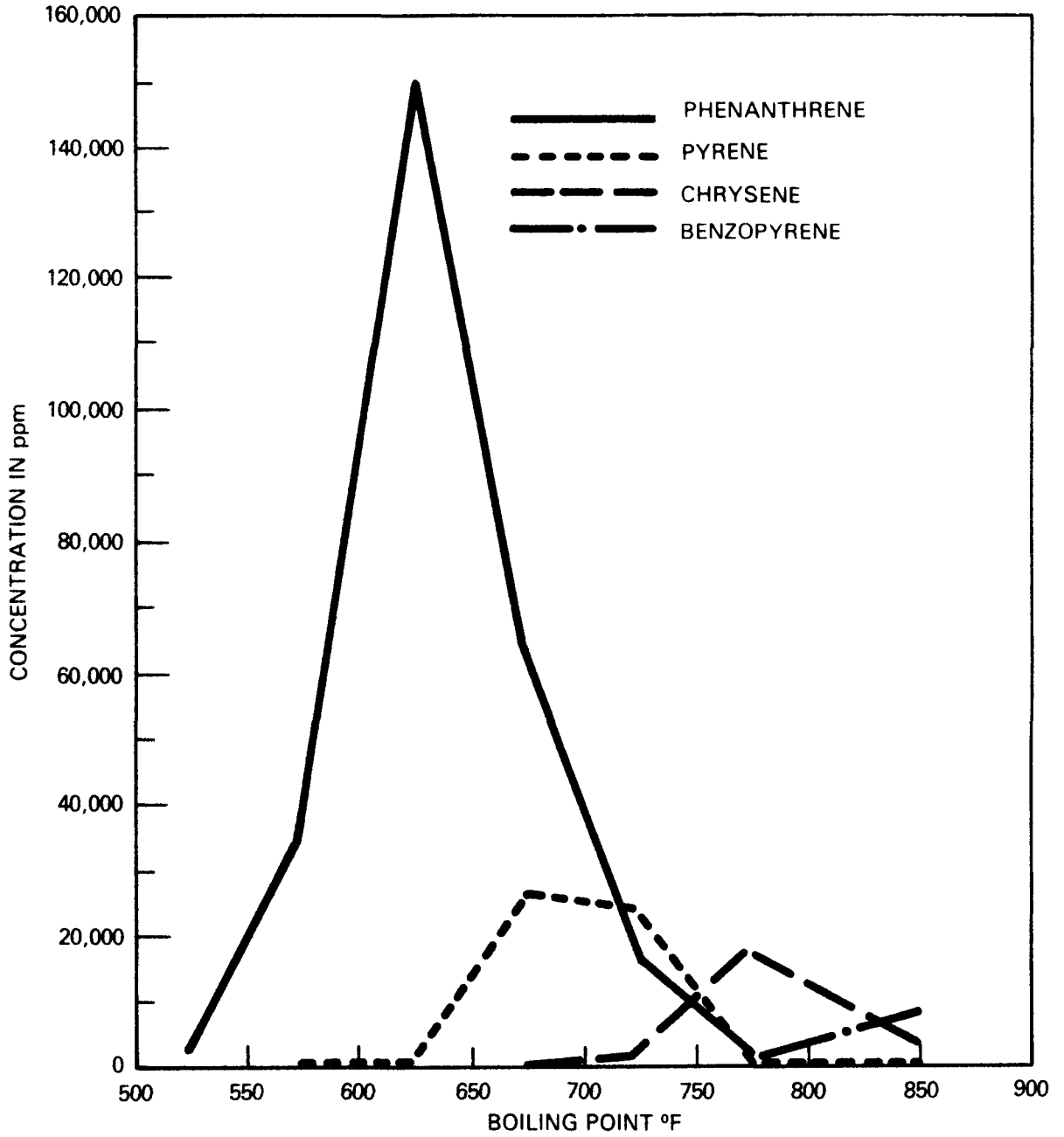


FIGURE 2.7. Measured Concentrations of Phenanthrene, Pyrene, Chrysene and Benzo[a]pyrene in the A-2 Fraction of SRC-I Boiling Point Cuts.

DETERMINATION OF APAH

The APAH-rich subfractions generally exhibited the greatest mutagenic activity of any SRC-II fraction (PNL-3787). Hence, it was of interest to determine specific APAH structures in various bp cuts of SRC-I coal liquids, since the number of aromatic rings for APAH in various SRC-II distillation cuts has been related to mutagenic activity.

After treating the APAH subfractions with pentafluoropropionic (PFP) anhydride to form their fluoramide derivatives, identification was achieved using electron capture detection (ECD), gas chromatography, direct probe mass spectrometry (MS) and gas chromatography/mass spectrometry. The development and application of these methods are described elsewhere (Later et al. 1982).

Figure 2.8 shows ECD gas chromatograms of the derivatized APAH subfractions from materials in bp cuts between 600°F and 850^{+}°F. The ECD detector is several orders of magnitude more sensitive to the fluoramide derivatives than to underivatized amines or azaarenes. Because carbazoles were removed from the primary amine subfraction by the chemical separation procedure described above, and the nitrogen heterocycles present did not react, the chromatograms represent derivatized APAH. The chromatograms of the distillate cut subfractions lack base-line resolution because of their chemical complexity. From MS analysis, these subfractions were estimated to contain about 20 to 30% APAH by weight, the remainder of the fraction weights consisted of azaarenes. Table 2.2 lists the mass-to-charge ratio of molecular ions in derivatized APAH fractions from the various bp cuts as detected by electron impact MS and GCMS. Where standards were available, retention time matches were also made from reconstructed selected ion chromatograms for the molecular ions of the PFP derivatives.

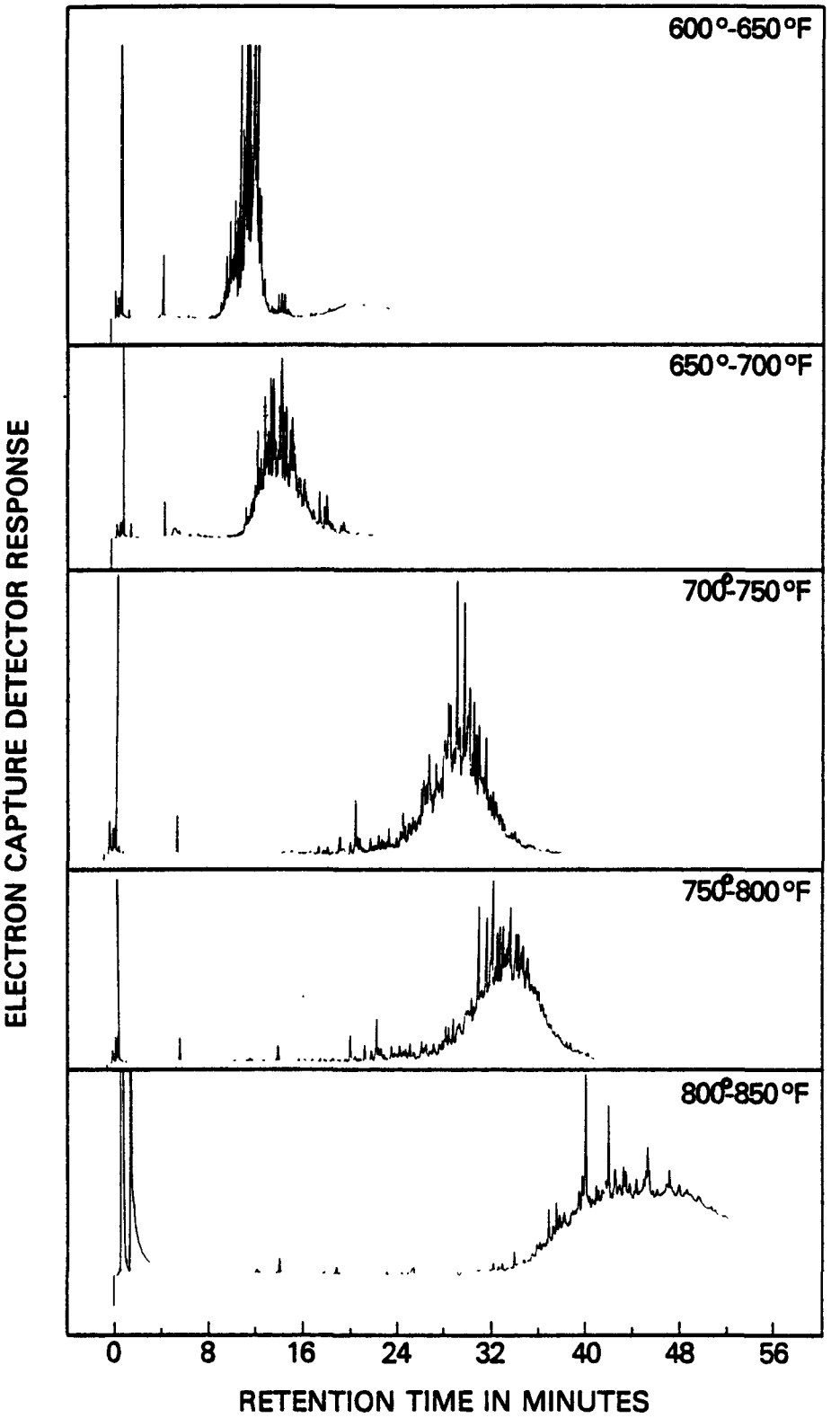
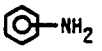
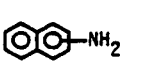
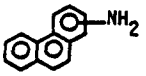
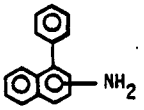
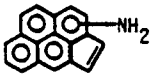
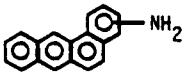
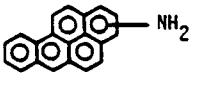


FIGURE 2.8. ECD Gas Chromatograms of the Derivatized APAH in the SRC-I Boiling Point Fractions

TABLE 2.2. Distribution of Amino PAH Among the Distillate Cuts

Compound	Molecular Weight Underivatized	Molecular Weight PFP	Boiling Range, °F 600–650	650–700	700–750	750–800	800–850+	Representative Structure
C_3 Aniline	135	281	X					
C_4 Aniline	149	295	X					
C_1 Aminoindan	145	291	X					
C_2 Aminoindan	159	305	X					
Aminonaphthalene	143	289	X					
C_1 Aminonaphthalene	157	303	X	X				
C_2 Aminonaphthalene	171	317	X	X				
C_3 Aminonaphthalene	185	331	X	X				
C_4 Aminonaphthalene	199	345		X				
Aminobiphenyl	169	315		X				
C_1 Aminobiphenyl	183	329		X				
C_2 Aminobiphenyl	197	343		X				
C_3 Aminobiphenyl	211	357		X	X			
C_4 Aminobiphenyl	225	371		X	X			
Aminofluorene	181	327		X				
C_1 Aminofluorene	195	341			X			
C_2 Aminofluorene	209	355			X	X		
C_3 Aminofluorene	223	369			X	X		
C_4 Aminofluorene	237	383				X		
Aminophenanthrene	193	339			X	X		
C_1 Aminophenanthrene	207	353			X	X		
C_2 Aminophenanthrene	221	367			X	X		
C_3 Aminophenanthrene	235	381			X	X		
C_4 Aminophenanthrene	249	395				X	X	
Aminophenylnaphthalene	219	365			X	X		
C_1 Aminophenylnaphthalene	233	379				X		
C_2 Aminophenylnaphthalene	247	393				X		
C_3 Aminophenylnaphthalene	261	407						
C_4 Aminophenylnaphthalene	275	421					X	
Aminopyrene	217	363				X		
C_1 Aminopyrene	231	377				X		
C_2 Aminopyrene	245	391				X		
C_3 Aminopyrene	259	405				X	X	
C_4 Aminopyrene	273	419					X	
Aminocyclopentapyrene	241	387				X	X	
C_1 Aminocyclopentapyrene	255	401				X	X	
C_2 Aminocyclopentapyrene	269	415					X	
Aminobenzanthracene	243	389				X	X	
C_1 Aminobenzanthracene	257	403				X	X	
C_2 Aminobenzanthracene	271	417				X	X	
C_3 Aminobenzanthracene	285	431					X	
C_4 Aminobenzanthracene	299	445					X	
C_5 Aminobenzanthracene	313	459					X	
C_6 Aminobenzanthracene	327	473					X	
Aminobinaphthyl	269	415					X	
C_1 Aminobinaphthyl	283	429					X	
C_2 Aminobinaphthyl	297	443					X	
C_3 Aminobinaphthyl	311	457					X	
C_4 Aminobinaphthyl	325	471					X	
Aminobenzopyrene	267	413					X	
C_1 Aminobenzopyrene	281	427					X	

Possible structures for the molecular ions are listed and the boiling range or ranges in which these ions were detected are also indicated. Previous work on SRC materials utilized high-resolution mass spectrometry to demonstrate that ions detected at these mass-to-charge ratios in the derivatized amine-rich subfractions had the proper elemental compositions for fluorinated acyl derivatives of APAH (Later et al. 1982). Adequate separations were achieved chromatographically to assign identifications to most derivatized species within the complex mixtures. Figure 2.9 shows a selected ion chromatogram for the m/z 289 ion in the derivitized S-2 fraction of SRC-I PS. Ion identifications are indicated. Figure 2.10 is a mass spectrum from the PFP derivative of 2 aminonaphthalene obtained at the scan number where the concentration of this compound was at its maximum in the chromatogram.

Derivitization with PFP was also a useful complement to high resolution mass spectrometry in the determination of polyaromatics with more than one nitrogen. Phenanthrolines and other tertiary di-nitrogen-compounds do not react with the PFPA derivitizing reagent and can be determined by analyzing for them after derivitization. Those di-nitrogen species that do react have at least one primary amine functionality, and several such compounds were detected in SRC-I coal liquids and SRC-II heavy distillate. These compounds are mainly aminoquinolines and aminocarbazoles.

Alkyl anilines, aminoindans, aminonaphthalenes and aminobiphenyls, major components in the cuts boiling below 700°F, do not exhibit appreciable mutagenic activity in microbial assays. For example, as pure compounds they are substantially less active than amino pyrene (Ho et al. 1981). The 3-ring compounds, such as aminoanthracene, aminophenanthrene, and aminofluorenes, are

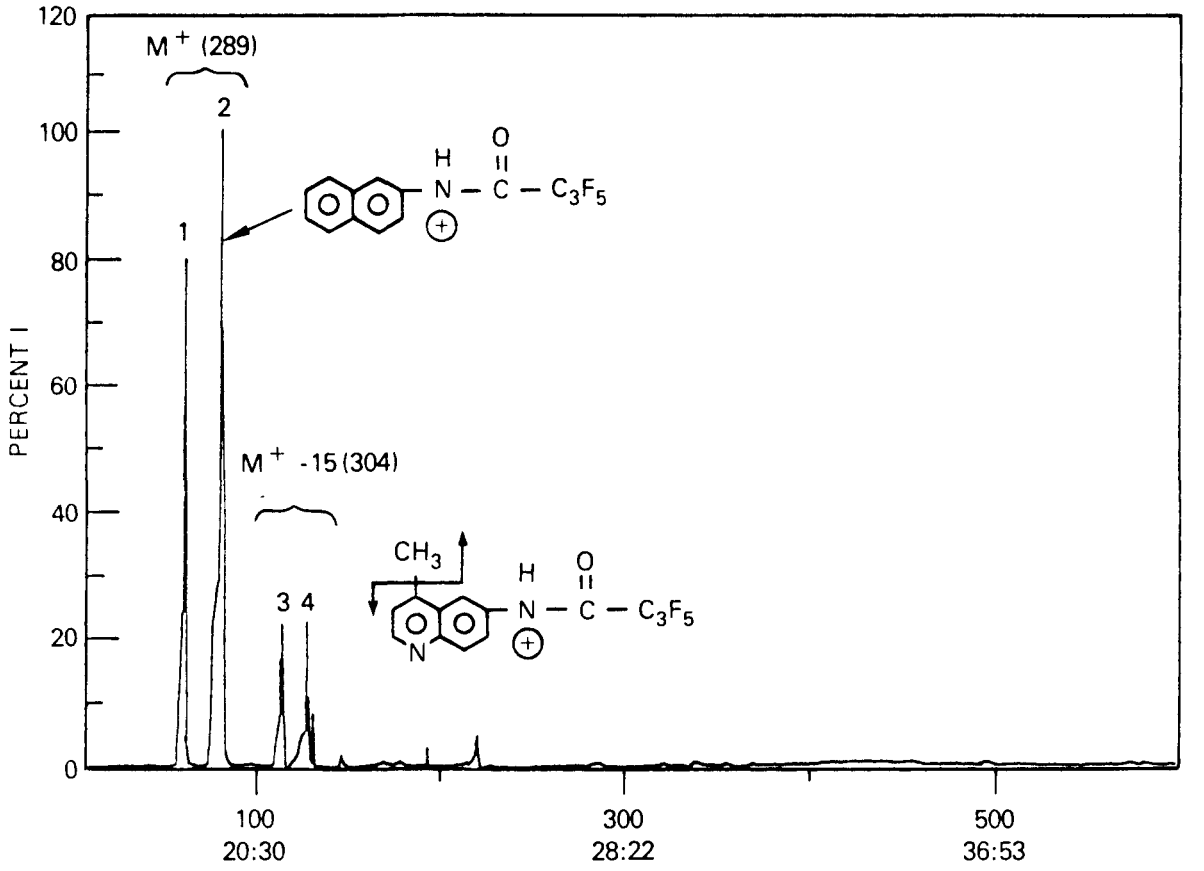


FIGURE 2.9. Reconstructed Single Ion Chromatogram of the Molecular Ion for the PFP Derivative of the Aminonaphthalenes (m/z 289) Peaks Number 1 and 2 are 1 and 2 Aminonaphthalene. Peaks number 3 and 4 are M-15 fragment ions from PFP derivatives of C_1 aminoquinolines (m/z 304). Arrows indicate mass spectral fragmentations which yield the m/z 304 ions.

moderately active as pure compounds and in complex coal liquids, with certain positional isomers exhibiting more potency than others. These more active constituents did not reach appreciable concentrations until boiling temperature exceeded 700°F. Four-ring compounds, such as aminopyrenes, aminochrysenes and/or aminobenzanthracenes, are extremely active mutagens in coal liquids (Wilson et al. 1980; Pelroy et al. 1981). Concentrations of these compounds, based on relative MS peak height, were highest in the SRC-I 750°F to 800°F bp

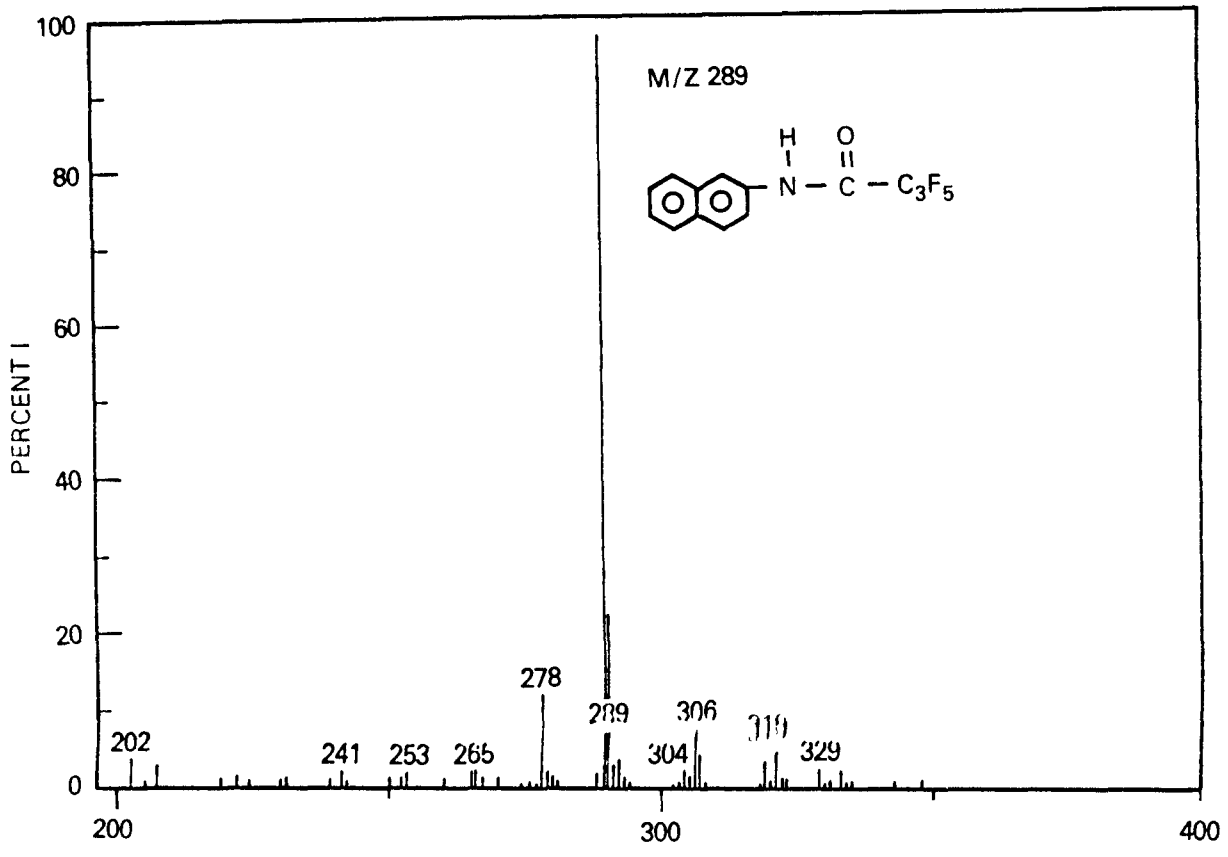


FIGURE 2.10. Mass Spectrum (from m/z 200 to m/z 400) of the 2-Aminonaphthalene, Showing the Intense Molecular Ion at m/z 289. Typically these spectra are complex below m/z 200 due to the presence of underivitized aza compounds which elute in the S-2 fraction

range and decreased slightly in the 800°F to 850^{+}°F cut, although the total amine content of the latter cut increased. Compounds with more than four rings were confined to the 800°F to 850^{+}°F bp cut. Thus, it would appear that APAHs that are expressed in complex mixtures, i.e., those with three or more aromatic rings, were essentially removed from cuts boiling below 700°F. (For a discussion of the relative potencies of APAH derived from various ring systems, see Ho et al. 1981.)

Analysis of the PAH fractions showed that known skin carcinogens such as the C_2 benzanthracenes and benzo[a]pyrene were concentrated in the material boiling above 800°F; although there were some 5-ring and C_2 4-ring compounds present in the 750°F to 800°F cut. The only nitrogen compounds detected in the PAH fractions were carbazoles whose concentrations did not exceed 0.05%.

Liquids boiling above 700°F constituted about 25% by weight of the SRC-I process solvent used in this study; those boiling above 750°F constituted about 18%. In a full-scale production facility, liquids boiling at these high temperatures would constitute a significantly smaller fraction of the total liquid product. The exact percentages would depend on the facility operating mode and the material balance.

3.0 IN VITRO ASSAY OF DISTILLED FRACTIONS

Distillate cuts discussed earlier were 50°F bp ranges between 350°F and 850°F and were tested in a battery of in vitro assays for their capacity to induce mutations and/or DNA damage in microbial systems.

AMES TEST

Agar plate mutagenicity assays utilized the Ames histidine reversion test (Ames et al. 1975) and Salmonella typhimurium strain TA98. Dimethylsulfoxide (DMSO) was used as a solvent for all test materials. Fixed concentrations of 2-aminoanthracene, benzo[a]pyrene and a complex (isooctane soluble) basic fraction from SRC-II heavy distillate were tested against Aroclor 1254 induced rat liver (S9) homogenates to optimize amounts used for metabolic activation. Optimum levels for S9 were established and monitored daily. Negative controls included the Ames/Salmonella typhimurium test strains plus and minus S9, and appropriate solvent controls. Revertant (rev) colonies per petri plate were counted electronically using a Biotran II automated colony counter (New Brunswick Scientific Co., Inc., Edison, NJ).

Crude bp cuts and their fractions were assayed for mutagenicity at concentrations of 2, 4, 8, 10, 20 and 50 µg or 0.2, 0.4, 0.8, 1.0, and 2.0 µg per petri plate. Each concentration was assayed in duplicate. Stock solutions of coal liquid test materials were prepared at concentrations of 10,000 µg/ml in DMSO and stored at -80°C under nitrogen when not in use.

A positive Ames test yielded a response (rev/plate) at least 2-fold above background. Specific mutagenic activity was expressed as rev colonies S. typhimurium TA98/µg test material (rev/µg), and estimated by linear regression

of dose-response data. In addition to a maximum response of at least 2 times background for at least one concentration in the dose-range, the correlation coefficient between, and concentration of, test material plate counts was at least 0.8 unless otherwise indicated. Experiments were replicated several times in order to obtain the average mutagenic potency in rev/μg and to estimate recovery of genetic activity in the chemical fractions. The average value was multiplied by the weight percent (wt%) of a given fraction to yield a weighted mutagenic activity in relation to the crude bp cut or fraction.

FLUCTUATION TEST

The fluctuation test was also conducted with S. typhimurium TA98. This test is an adaptation of the Luria, Delbruck fluctuation assay (1941) using the "microtitre" technique described by Gatehouse (1978) and Gatehouse and Delow (1979). Cells of S. typhimurium TA98 in liquid exposed to SRC-I fractions for 4 hours and aliquots (ca 10^6-10^7 cells) were diluted in small volumes of histidine deficient liquid medium for selection of mutants. The test was scored by changes in turbidity and pH owing to growth of mutants (histidine prototrophs) induced by test materials. The level of statistical significance (P value) was determined by X^2 statistic incorporating Liddle's continuity correction (1976). Exposure concentrations of coal liquid materials were 0.1 and 10 μg or 10 and 100 μg depending on the material being assayed.

FORWARD MUTATION TEST

Forward mutation induced by SRC-I materials in Salmonella typhimurium TM677, was measured by resistance to the purine analog 8-azaguanine (8-Ag). We used a modification of the method of Skopek et al. (1978) where length of exposure to test material was increased from 2 to 4 hours. Metabolic

activation was provided by Aroclor-induced S9 homogenates. Levels of S9 were optimized against the same reference compounds used in the standard Ames assay. The coal liquid materials were tested for mutagenicity at 5, 10 and 20 µg/ml or 0.5, 1.0 and 2.0 µg/ml exposure medium. An overnight growth period in broth not containing 8-Ag allowed expression of induced mutation in target cells. The overnight cultures of target cells were plated onto Minimal E Medium hard agar plates (Skopek et al. 1978) containing enough 8-Ag at a final concentration of 50 µg/ml. Viability of exposed cells needed to calculate specific mutagenic activity was determined by dilution plate counts onto Minimal E Medium without the inhibitor 8 Ag. Forward mutants represented by 8-Ag resistant clones were scored by automatic colony counter (Biotran II) about 36 hours after plating. The 8-Ag resistant fraction was expressed as:

$$\frac{\text{number of resistant clones on 8-Ag containing media}}{\text{number of clones on media without 8-Ag}} \times \text{Dilution Factor}$$

A positive response relative to historical control data was one greater than 1.4×10^{-4} mutants per viable colony-forming unit (MUTANTS/VIAB CFU x 10^{-4}).

PROPHAGE INDUCTION (INDUCTEST)

Prophage induction was measured with Escherichia coli, K12 (8177) using a modification of the method of Moreau et al. (1978). E. coli is lysogenic for a special form of the bacterial virus lambda which is induced to undergo a lytic cycle after primary damage to its DNA. Phage produced by the lytic cycle were detected by plaque formation with E. coli K12 (6340), a strain sensitive to the phage in E. coli (8177). Chemical or metabolic activation was provided by Aroclor-induced S9 homogenates, as described above for the Ames assay.

The test was performed by exposing about 10^7 growing cells of E. coli 8177 (to test material) for 6 hours with or without S9 metabolic activation. The exposure step for prophage induction was similar to that used in the fluctuation and forward mutation assays. After exposure, 1000 to 10,000 cells were removed from the exposure mixture, killed with chloroform to release induced phage. The number of phage were then titrated by plaque formation. Coal liquid materials were assayed at concentrations of 5, 10 and 20 µg/ml exposure medium. Plaques were counted manually. Response was expressed in terms of plaque form units per plate (PFU/PLATE).

NITROSATION

Nitrosation experiments were conducted using a modification of the procedure described by Yoshida and Matsumoto (1978). Sodium nitrite solution was prepared by dissolving 600 mg of the salt in 30 ml 0.3 HCl (v/v). A 0.3% hydrochloric acid solution was used as a control on the acidic nitrite solution. Both acidic solutions were filter-sterilized prior to use. To begin nitrosation experiments, 100 to 1500 µg test chemical or 100 to 5000 µg of coal-derived material were added to 2 ml of acidic nitrite solution or the acid solution without nitrite. The complex fractions or pure chemicals were also added to 2 ml DMSO, the solvent used to introduce test materials in the Ames Salmonella assay. Both the acidic and DMSO mixtures were mixed vigorously and allowed to stand 90 min at room temperature (about 25°C). From 5 to 50 µl of acid or DMSO mixture were removed and added to the 45°C liquid top-agar already containing the other components of the Ames test minus the activating enzymes. Activating enzymes in the hepatic, Aroclor (1254)-induced crude microsomial (S9) homogenates were added last. Contents of the top-agars were

then poured on the agar plates. The pH of top-agar varied from 6.6 to 7.2, depending on the volume of acidic solution added. The range in pH did not affect sensitivity of the assay.

RESULTS

Fractions of SRC-I bp cuts were prepared by chemical class fractionation (Later et al. 1981) and assayed for genetic activity. Forward and reverse mutation in S. typhimurium and prophage induction in E. coli provided the genetic endpoints for the screening assays. For comparison of test results with the SRC-I samples, the average positive control test results obtained from reference chemical mutagens are presented in Table 3.1.

Unfractionated (crude) bp cuts exhibited mutagenic activity in the standard Ames and fluctuation assays with S. typhimurium TA98 (Figures 3.1a and 3.1b). The highest mutagenic activity in the standard Ames test was localized in 700°F to 750°F and 750°F to 800°F bp cuts, with lower activity in the 650°F to 700°F and 800°F to 850+°F bp cuts. The fluctuation test provided similar data except that the maximum level of mutagenesis was induced by the 800°F to 850+°F bp cut. No mutagenic activity was detected in either assay in bp cuts below 700°F. All genetic activity in crude bp cuts and their fractions (see below) was S9 dependent. (Only data obtained with complete metabolic activation are presented here.)

Results of the 8-Ag forward mutation assay were different from those obtained in the reverse mutation assay (Figure 3.1c). Nearly all forward mutagenesis was induced by the 600°F to 650°F and 650°F to 750°F bp cuts, and the crude bp cuts greater than 700°F did not induce significant levels of

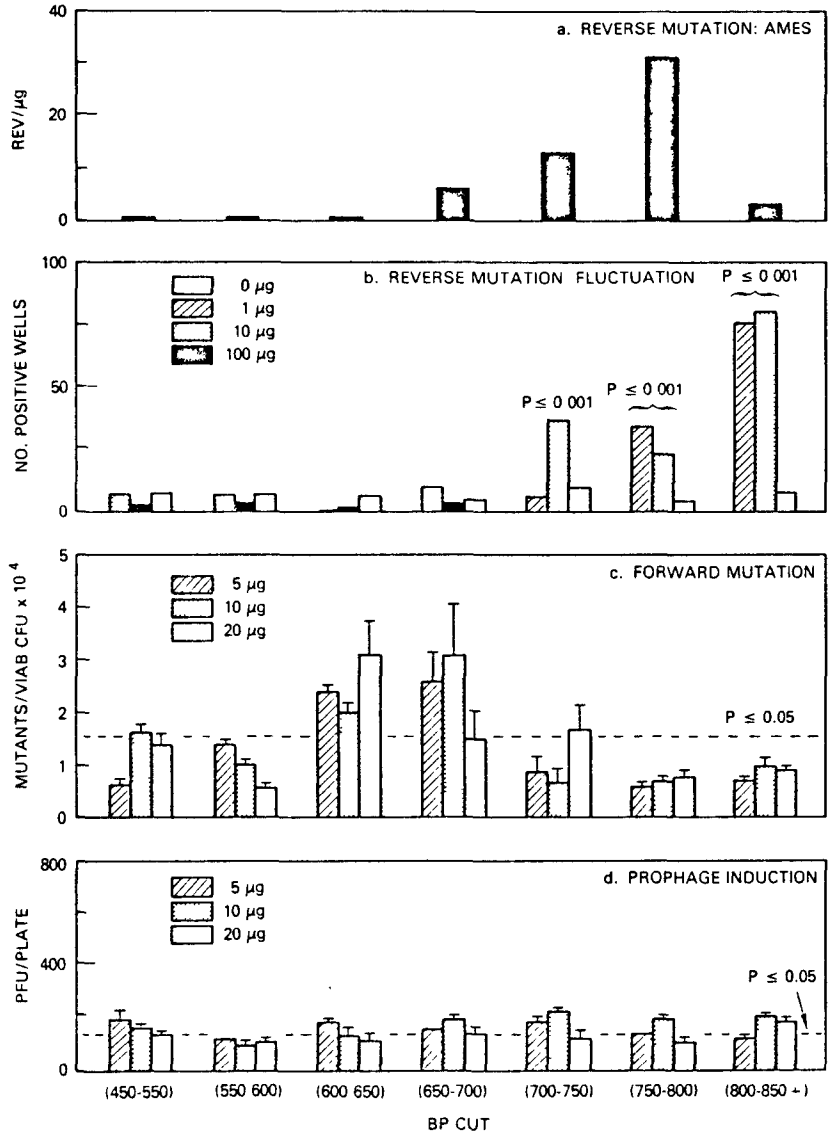


FIGURE 3.1. Genotoxicity of Crude SRC-I bp Cuts. (a) Mutagenic potency in standard standard Ames test with S. typhimurium TA98 estimated by linear regression of dose-response data. Maximum responses all with $P \leq 0.001$. (b) Mutagenicity in the Microtitre version of the fluctuation test with S. typhimurium TA98. P values as indicated above histograms. (c) Forward mutation with S. typhimurium TM677. Average (i.e., historical) negative controls ±SE indicated by solid horizontal and dashed lines, respectively. The response values (histograms) represent the mean of the three determinations. Unless otherwise indicated, the SE of the responses (histograms) was less than 10% of the mean value. P values as indicated on histograms. (d) Prophage induction with E. coli 8177 as the largest and E. coli 6304 as the indicator cell. The response values represent the mean of two determinations ±SE.

response in the forward mutation assay. A positive response is indicated by any value that exceeded the 5 percent level of significance based on historical negative controls.

No trend was evident for genetic response versus bp cut with the prophage induction test (Figure 3.1d). Nearly all distillate cuts induced a low but significant level of prophage induction compared to the averaged negative controls. A positive response is defined as described above for the forward mutation test.

Each crude SRC-I bp cut was separated into chemical class fractions which were then analyzed for genetic activity. The A-1 fractions were highly enriched in aliphatic components but were essentially free of polyaromatic hydrocarbon (PAH) or polar constituents. The A-1 fractions were essentially without mutagenic activity in the standard Ames and fluctuation tests (Figure 3.2a and 3.2b). Similarly, the A-1 fractions failed to induce significant levels of forward mutation in the 8-Ag assay (Figure 3.2c). Prophage induction was barely above background response for several bp cuts, and there was no indication of peaks of activity associated with any bp cuts.

All of the A-2 (or PAH-enriched) fractions were inactive in the standard Ames test (Figure 3.3a). Lack of Ames mutagenic activity might be anticipated in the A-2 fractions from the bp cuts less than 700°F in view of their low content of 5-ring (i.e., potentially mutagenic) PAH. Conversely, the concentrations of 5-ring PAH in the bp cuts greater than 700°F was high. For example, the concentration of BaP in the A-2 fraction from the 800°F to 850^{+}°F bp cut exceeded 2500 ppm BaP, but this fraction was still innactive in the standard Ames test. Lack of genetic activity in the standard Ames assay for

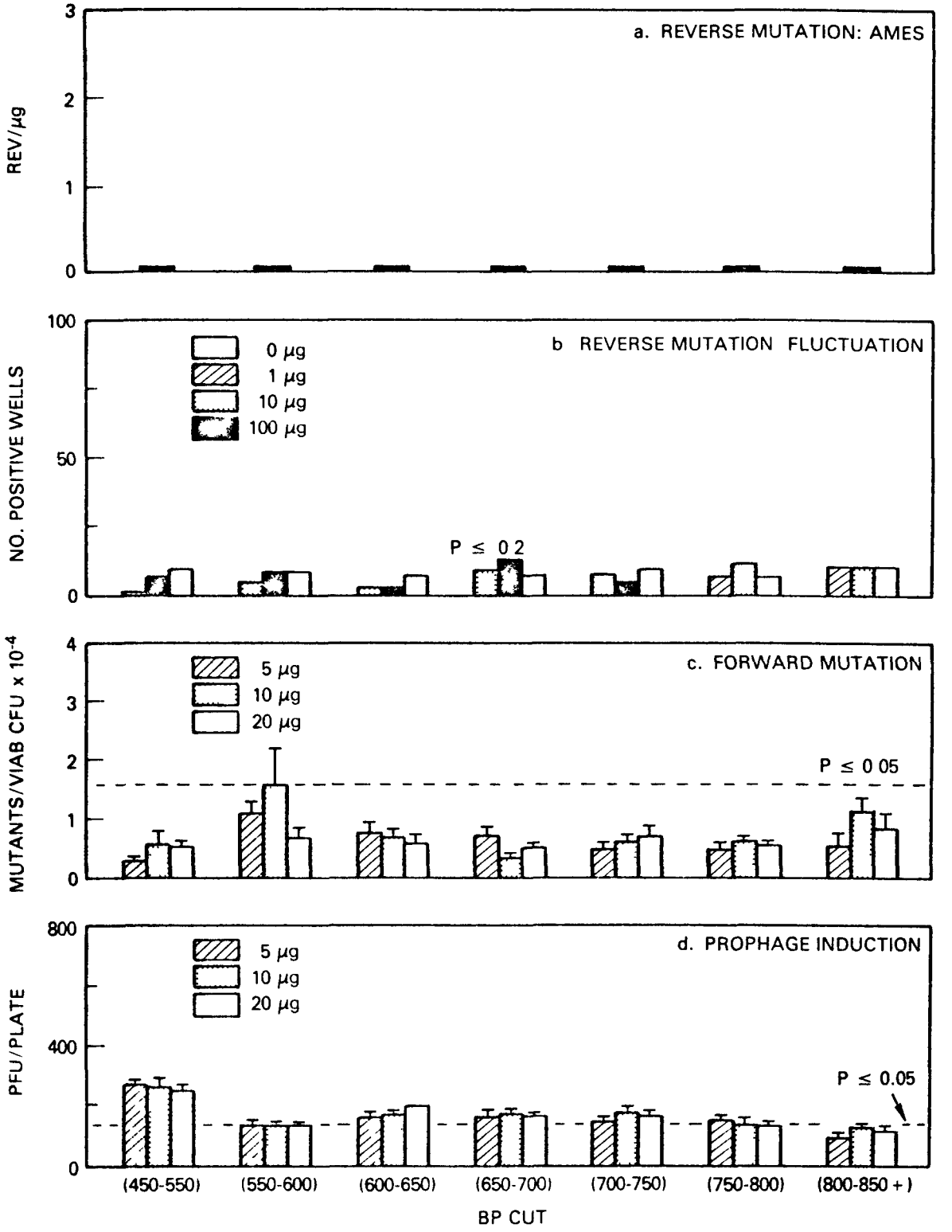


FIGURE 3.2. Genotoxicity of Aliphatic (A1) Alumina Column Fractions from the SRC-I bp Cuts. (a) Standard Ames mutagenic potency from dose-response data, S. typhimurium TA98. (b) Fluctuation assay response in the "Microtitre" version of the fluctuation test with S. typhimurium TA98. (c) Forward mutation response with S. typhimurium TM677. (d) Prophage induction with E. coli 177. For details see Figure 3.1.

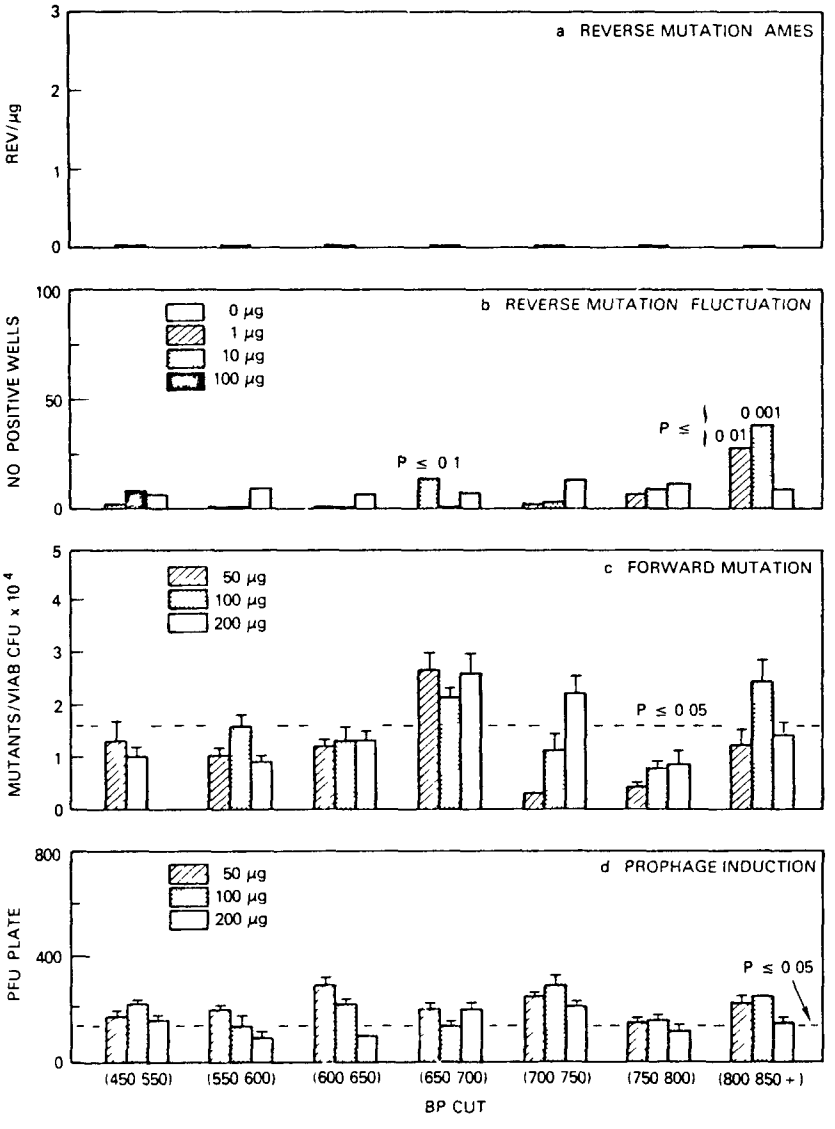


FIGURE 3.3. Genotoxicity of Polyaromatic Hydrocarbons (PAH), A2 Alumina Column Fractions from the SRC-I bp Cuts. (a) Standard Ames mutagenic potency from dose-response data, S. typhimurium TA98. (b) Fluctuation assay response in the "Microtitre" version of the fluctuation test with S. typhimurium TA98. (c) Forward mutation response with S. typhimurium TM677. (d) Prophage induction with E. coli I77. For details see Figure 3.1.

PAH-enriched fractions of the high-boiling SRC-I bp cuts confirms earlier observations with similar materials from both SRC-I Process Solvent and SRC-II Heavy Distillate (Pelroy and Petersen 1981; Pelroy et al. 1981).

In contrast to the lack of activity in the standard Ames test, the A-2 fraction from the 800°F to 850^{+}°F bp cut was positive in the fluctuation test. All other A-2 fractions from bp cuts below 800°F were negative in the fluctuation assay confirming results of the standard Ames test. It should be noted that both the standard Ames and fluctuation tests utilize the same target cell, S. typhimurium TA98. Thus differences in response appear to be attributable to the assay system and not the test strain employed for the mutagenic assays.

Low genetic activity with no apparent dose-response was observed in the prophage induction test (Figure 3.3c). A single peak of genetic activity for A-2 fractions in the forward mutation test was localized in bp 700°F to 750°F (Figure 3.2b). All other A-2 fractions were negative in the forward mutation assay.

The total nitrogen polycyclic (N-PAC) or A-3 fractions from bp cuts above 700°F were highly active against S. typhimurium TA98 in both the standard Ames and fluctuation tests (Figures 3.4a and 3.4b). Response of A-3 fractions in the standard Ames test was greatest in the 700°F to 750 and 750°F to 800°F bp cuts, and dropped sharply in the 800°F to 850^{+}°F bp cut (Figure 3.4a). The fluctuation assay showed strong response to all A-3 fractions from bp cuts greater than 700°F. While the dose range was limited, it appears that the A-3 fractions from bp cuts 750°F to 800°F and 800°F to 850^{+}°F were the the most genetically active of the total nitrogen fractions.

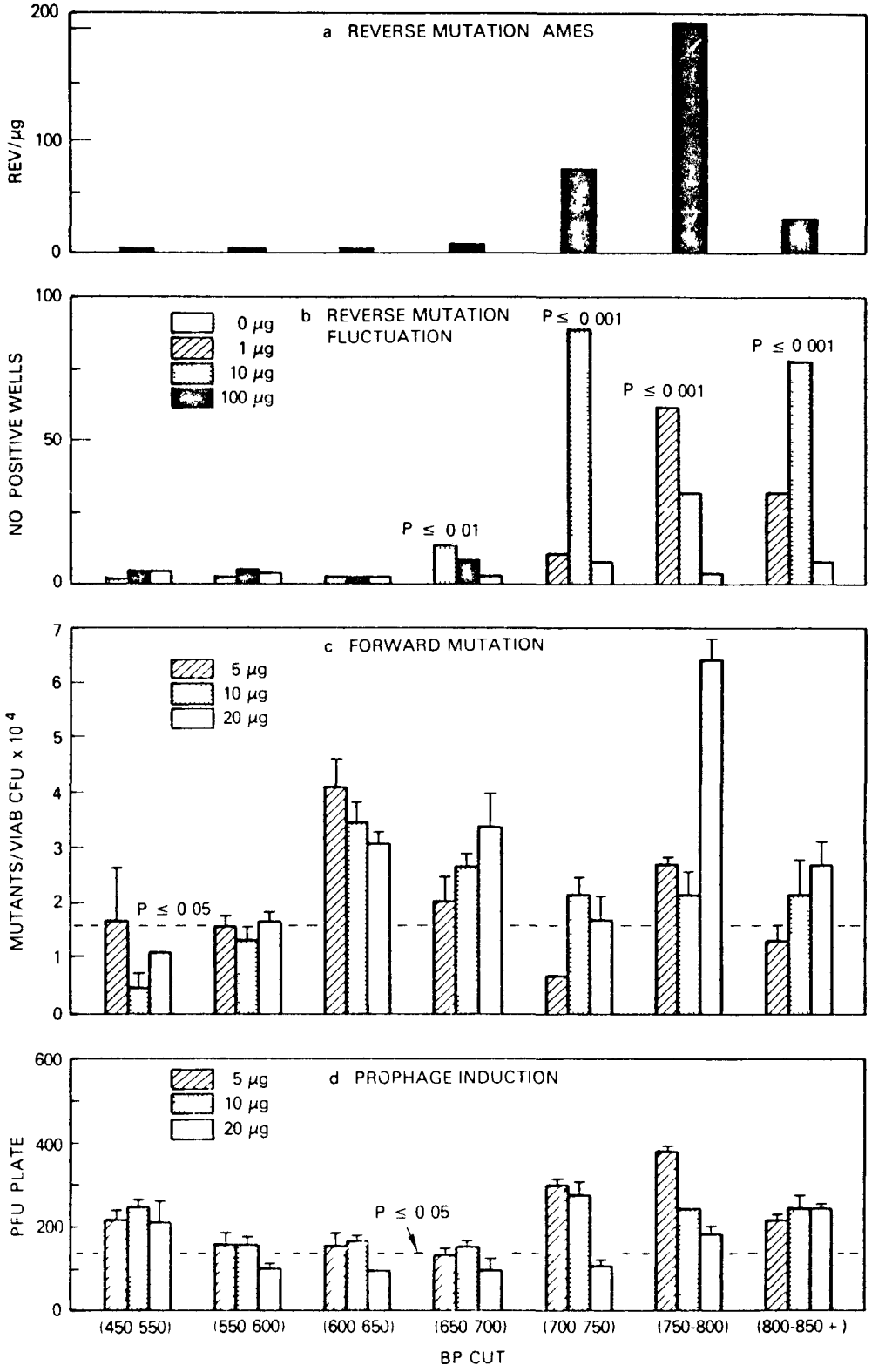


FIGURE 3.4. Genotoxicity of Nitrogen Polycyclic (N-PAC) A3 Alumina Column Fractions from the SRC-I bp Cuts. (a) Standard Ames mutagenic potency from dose-response data, S. typhimurium TA98. (b) Fluctuation assay response in the "Microtitre" version of the fluctuation test with S. typhimurium TA98. (c) Forward mutation response with S. typhimurium TM677. (d) Prophage induction with E. coli I77. For details see Figure 3.1.

In contrast to results obtained with S. typhimurium TA98, response to A-3 fractions in the forward mutation assay with S. typhimurium TM677 was bimodal. It appeared that most of the mutagenic activity was associated with the A-3 fractions from the two bp cuts in the 600°F to 700°F range. A second, minor peak of activity was also found in the A-3 fraction from the 750°F to 800°F bp cut. The A-3 fractions from remaining bp cuts were innactive or marginally active in the forward mutation assay. It is noteworthy that S. typhimurium TA98 (reverse mutation) in the standard Ames and fluctuation tests apparently is more sensitive to mutagens in the A-3 fractions from the higher bp cuts, while S. typhimurium TM677 (forward mutation) mainly responds to mutagens in A-3 fractions from lower temperature bp cuts.

Response in the prophage induction assay of A-3 fractions was also bimodal (Figure 3.4d). One minor peak of activity was observed for low molecular weight materials boiling from 450°F to 550°F and a major part of most activity was associated with the A-3 fraction from bp cuts 750°F to 800 and 800°F to 850^{+}°F.

The A-4 or hydroxy polyaromatic hydrocarbon (HPAH) fractions from SRC-I bp cuts showed little or no mutagenic activity in any of the microbial screening assays (Figure 3.5). The 800°F to 850^{+}°F bp cut did show a weak mutagenic response with S. typhimurium TA98 in the standard Ames test. Fluctuation and forward mutation test results were not significantly above background (Figure 3.5b and 3.5c).

A low but significant level of prophage induction indicating primary DNA damage was observed for all but one of the A-4 fractions (Figure 3.5d). As

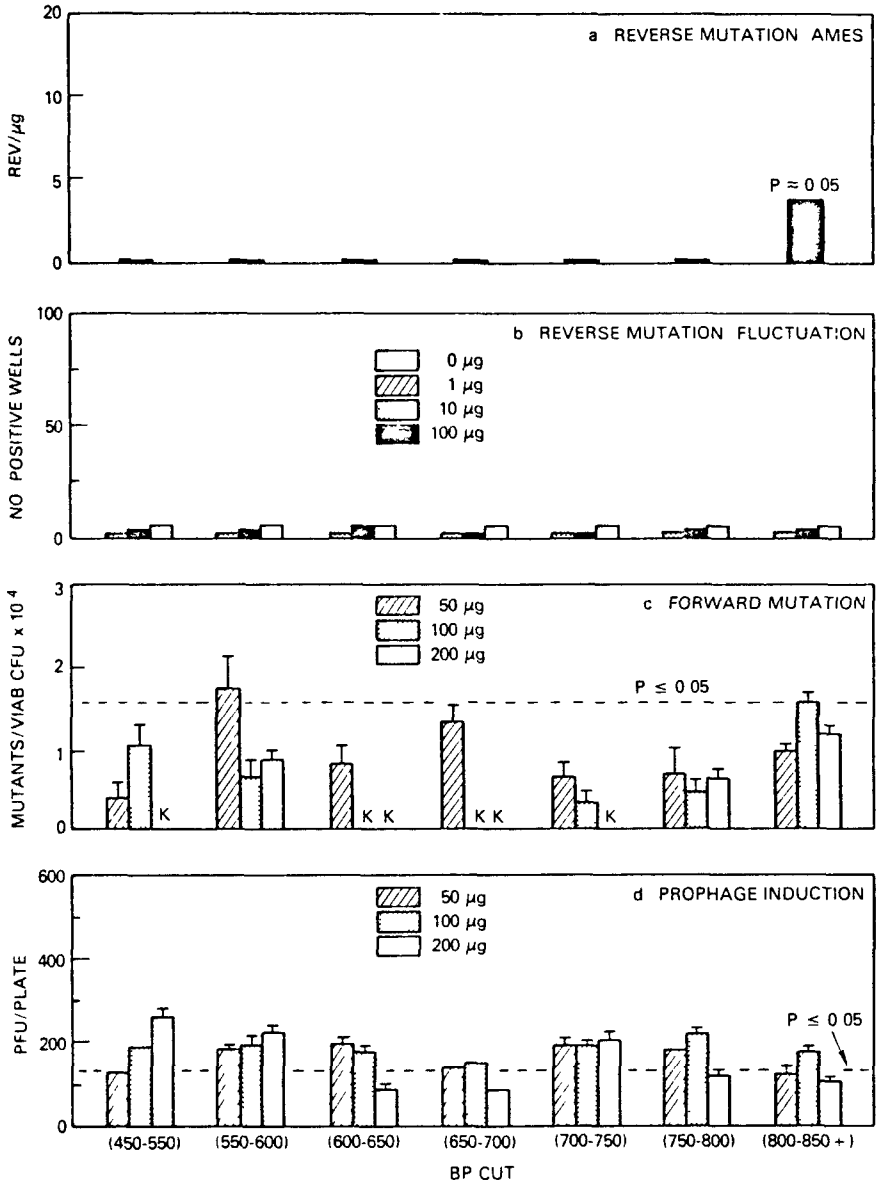


FIGURE 3.5. Genotoxicity of Hydroxypolyaromatic (HPAH), A-4 Alumina Column Fractions from the SRC-I bp Cuts. (a) Standard Ames mutagenic potency from dose-response data, S. typhimurium TA98. (b) Fluctuation assay response in the "Microtitre" version of the fluctuation test with S. typhimurium TA98. (c) Forward mutation response with S. typhimurium TM677. (d) Prophage induction with E. coli 177. For details see Figure 3.1. Capital letter "K" indicates killing of target cells to the extent that response was not observed.

observed for A-3 fractions, there was an indication of some bimodality for prophage induction with one peak for bp cut 450°F to 550°F and another peak for bp cuts above 700°F.

The N-PAC (or A-3 fractions) were subfractionated into chemical classes enriched in carbazoles (S-1), APAH (S-2), and azaarenes (S-3). The "S" fractions were then assayed for genetic activity (Figures 3.6 to 3.11).

Based on considerable evidence previously obtained with high-boiling SRC liquids, we expected the APAH-enriched (S-2) fractions from bp cuts above 700°F to show the highest genetic activity in microbial tests.

The S-1 (i.e., carbazole-rich) subfractions from the A-3 fractions of the 700°F to 750, 750°F to 800 and 800°F to 850+°F bp cuts were mutagenically active against S. typhimurium TA98 in both the standard Ames and fluctuation tests (Figures 3.6a and 3.6b). Distribution of activity versus bp was similar to that in the A-3 fractions. For example, the standard Ames test was positive only for the 750°F to 800 and 800°F to 850+°F bp cuts. The fluctuation test was positive for the S-1 fractions from all three bp cuts above 700+°F. None of the S-1 fractions from bp cuts below 700°F was mutagenically active against S. typhimurium TA98. Based on standard Ames test data, potency of the active S-1 fractions was about 10 to 20% that of the corresponding parent A-3 fractions (Figure 3.4a versus Figure 3.6a).

Results of the forward mutation test with S-1 bp cut fractions and S. typhimurium TM677 showed significant activity in all bp cuts from 600°F to 850+°F (Figure 3.6b), although the strongest response was for those fractions obtained from cuts above 700°F. No indication of bimodality was observed.

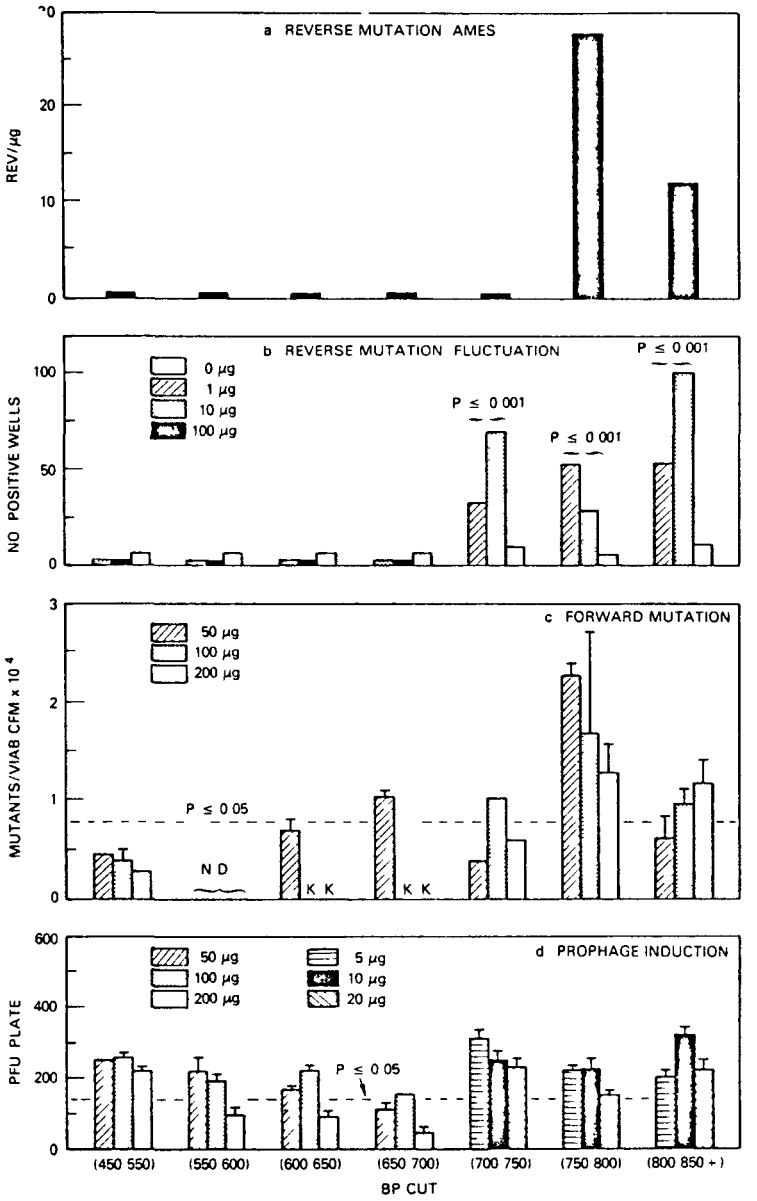


FIGURE 3.6. Genotoxicity of Secondary Polyaromatic Nitrogen Hydrocarbon (2°-PAHN), S1 Silicic Acid Column Subfractions of the N-PAC Chemical Fraction of the SRC-I bp Cuts. (a) Standard Ames mutagenic potency from dose-response data, S. typhimurium TA98. (b) Fluctuation assay response in the "Microtitre" version of the fluctuation test with S. typhimurium TA98. (c) Forward mutation response with S. typhimurium TM677. (d) Prophage induction with E. coli 177. For details see Figure 3.1. μD means not determined and "K" as defined in Figure 3.5.

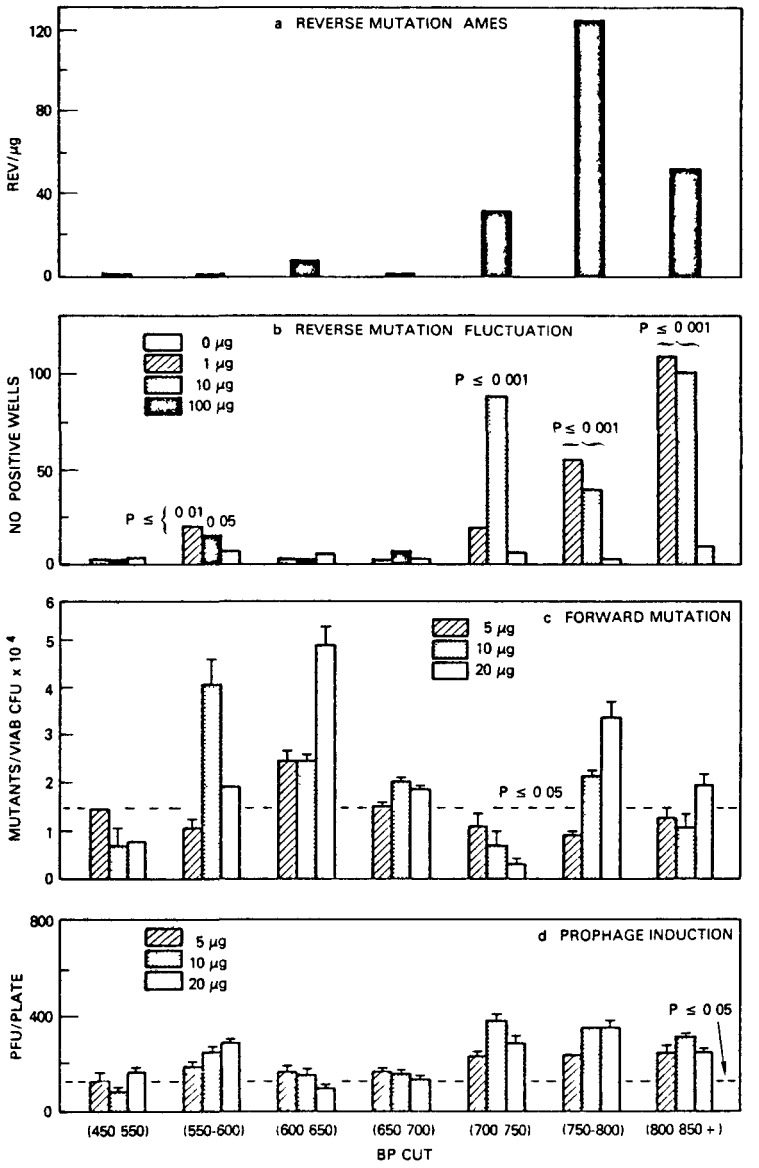


FIGURE 3.7. Genotoxicity of Amino Polyaromatic Hydrocarbon (APAH), S2 Silicic Acid Column Subfractions of the N-PAC Chemical Fraction of the SRC-I bp Cuts. (a) Standard Ames mutagenic potency from dose-response data, S. typhimurium TA98. (b) Fluctuation assay response in the "Microtitre" version of the fluctuation test with S. typhimurium TA98. (c) Forward mutation response with S. typhimurium TM677. (d) Prophage induction with E. coli 177. For details see Figure 3.1.

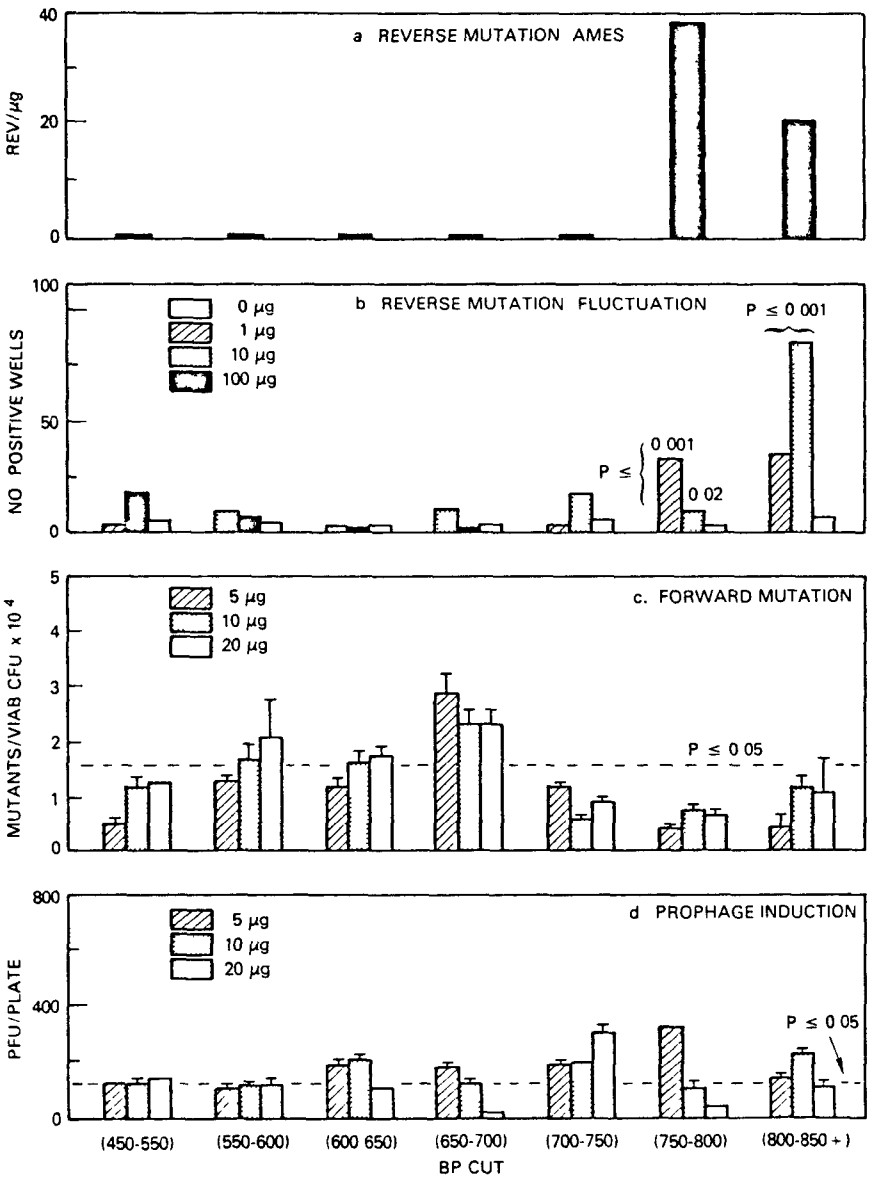


FIGURE 3.8. Genotoxicity of Tertiary Polyaromatic Nitrogen Hydrocarbon (3°-PAHN), S3 Silicic Acid Column Subfractions of the N-PAC Chemical Fraction of the SRC-I bp Cuts. (a) Standard Ames mutagenic potency from dose-response data, S. typhimurium TA98. (b) Fluctuation assay response in the "Microtitre" version of the fluctuation test with S. typhimurium TA98. (c) Forward mutation response with S. typhimurium TM677. (d) Prophage induction with E. coli 177. For details see Figure 3.1.

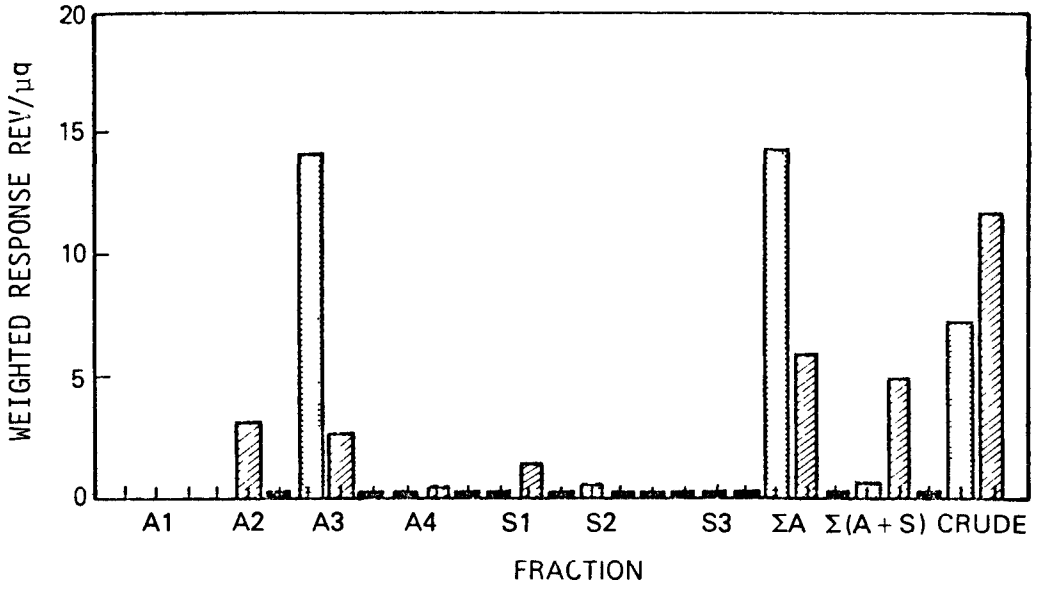


FIGURE 3.9. Standard Ames Test Mutagenic Activity of Chemical Class Fractions and Subfractions of bp Cut 700-750°F Before and After Treatment with Nitrous Acid. (▦) indicates untreated fractions; (▨) indicates nitrous acid treated fractions. ΣA indicates recombined A1-A4 alumina column fractions. Σ(A+S) indicates recombined A1, A2 and A4 plus the recombined S1-S3 (silicic acid) fractions.

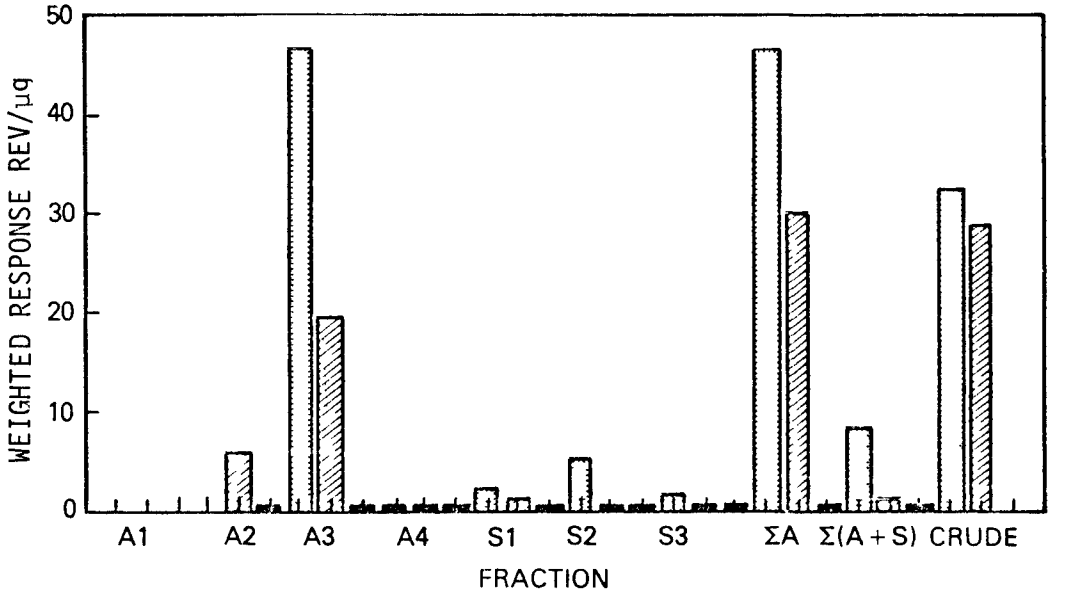


FIGURE 3.10. Standard Ames Test of bp Cut 750-800°F Chemical Fractions Before and After Nitrous Acid Treatment. For details see Figure 3.9.

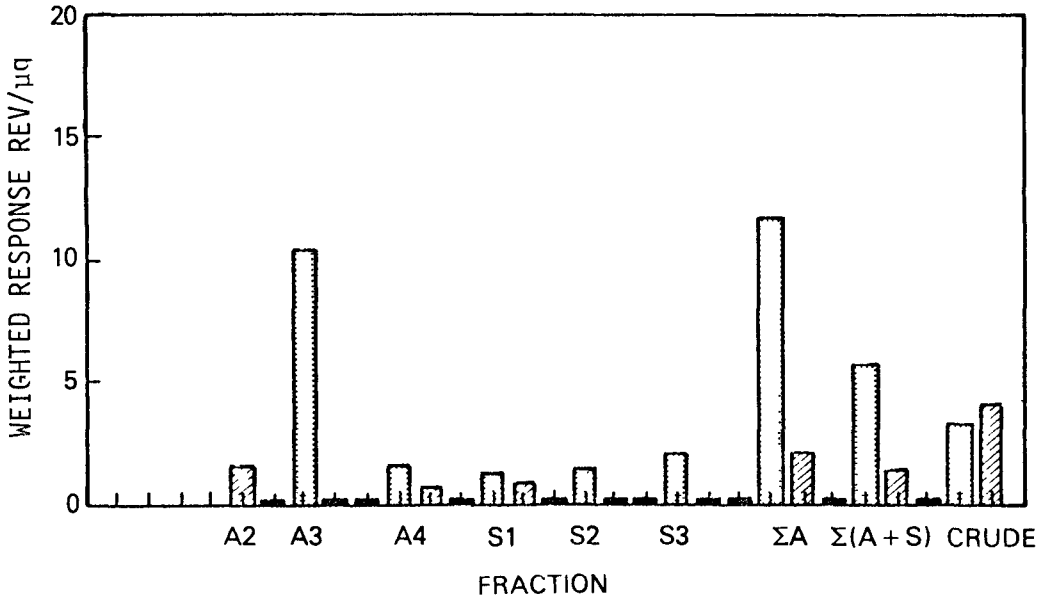


FIGURE 3.11. Standard Ames Test of bp Cut 800-850+°F Chemical Fractions Before and After Nitrous Acid Treatment. For details see Figure 3.9.

Nearly all S-1 subfractions were active in the prophage induction test (Figure 3.6d). However, the level of activity was low, with a suggestion of bimodality.

As expected, the APAH-rich S-2 fractions from bp cuts above 700°F were more active against S. typhimurium TA98 than were corresponding S-1 or S-3 fractions (Figures 3.6-3.8). The S-2 fractions from bp cuts below 700°F were negative in the standard Ames test.

Results of fluctuation tests on S-2 fractions with S. typhimurium TA98 agree with those obtained in the standard Ames test. Data for these fractions suggest increasing mutagenic activity with bp for bp cuts above 700°F. The 800°F to 850^{+}°F bp cut showed the highest potency. Fluctuation data also show that S-2 fractions from bp cuts below 700°F were either inactive or of reduced mutagenic potency compared to bp cuts above 700°F. Genetic activity

detected by the fluctuation test in the S-2 fraction of the 550°F to 600°F bp cut was unexpected. No activity was detected in either the 450°F to 550°F or 600°F to 650°F bp cut which bracketed the 550°F to 600°F bp cut. This suggests that compounds from the S-2 fraction of the 550°F to 600°F bp cut which were responsible for mutagenic activity against TA98 are uniquely associated with compounds that boil in this temperature range.

Results of the forward mutation and prophage induction assays with S-2 fractions were again somewhat different from those obtained in assays with S. typhimurium TA98. As in the A-2 and A-3 fractions, response in the forward mutation assay was greatest from 550°F to 650°F. A minor peak was associated with components from the 750°F to 800°F bp cut.

Prophage induction by S-2 fractions was also bimodal with a minor peak associated with bp cut 550°F to 600°F and the major peak in bp cuts above 700°F (Figure 3.7c).

Mutagenic activity in azaarene-enriched, S-3 fractions against S. typhimurium TA98 was concentrated in the 750°F to 800 and 800°F to 850^{+}°F bp cuts (Figures 3.8a and 3.8b). Similarly the 800°F to 850^{+}°F S-3 fraction was highly active, and the 750°F to 800°F S-3 fraction less active in the fluctuation test. A trace of mutagenic activity in the fluctuation test was also associated with the S-3 fraction from bp 700°F to 750°F. The corresponding Ames tests on bp 700°F to 750°F subfraction were negative.

Nearly all forward mutation activity of the S-3 fraction was in bp cut 650°F to 700°F. No significant forward mutation activity was observed in bp cuts above 700°F with S. typhimurium TM677.

A weak response was observed in the prophage induction assay with S-3 fractions from bp cuts above 700°F and for two bp cuts below 700°F (600°F to 650°F, and 650°F to 700°F bp cuts).

NITROUS ACID AND ADDITIVITY EXPERIMENTS

Fractions from the three most active SRC-I bp cuts (700°F to 850^{+}°F) were analyzed for mutagenicity in the standard Ames test, with and without nitrous acid treatment. Loss of mutagenicity in the Ames test after nitrosation of test materials is diagnostic for APAH as determinant mutagens (Pelroy and Stewart 1981). Briefly, the APAH are rapidly converted to phenols with concomitant loss of mutagenic activity.

Figure 3.9 shows that substantial losses in total mutagenic activity occurred after nitrosation of the A and S fractions of the three SRC-I bp cuts between 700°F and 850^{+}°F. Mutagenicity of A-3 fractions decreased over 90% in the 800°F to 850^{+}°F bp cut; about 60% in the 750°F to 800°F bp cut; and 80% in the 700°F to 750°F bp cut. Most mutagencity associated with the S-1 and S-3 subfractions was also destroyed by nitrosation (Figure 3.9).

Some mutagenically active components were formed in both the A-2 and A-3 fractions after nitrosation treatment. In A-2 fractions, these were probably oxidation products from PAHs which are direct-acting mutagens (Haugen et al. 1981). The origin of direct-acting mutagens formed in the A-3 (total nitrogen) fractions after nitrosation is uncertain. However, they may arise from nitroso compounds formed from carbazoles, which are abundant in A-3 fractions. Thus, the indirect mutagenicity of SRC-I bp cuts in the Ames test is nitrous-acid sensitive and apparently caused by APAH. This conclusion is consistent with the distributions of the large-ring (mutagenically active) APAH in various SRC-I bp cuts (see section 2).

Data in Figure 3.9 also demonstrate that A-3 fractions account for most mutagenic activity in crude bp cuts. However, subfractionation of the A-3 fractions (to "S" subfractions) resulted in substantial losses in recovered genetic activity in the standard Ames test. Loss of activity was not due to loss of mass, as recoveries in the BYU fractionation procedure were nearly 100%. Although selective inactivation of APAH is a possibility, activity of APAH in A-3 fractions may have been enhanced because of composition.

DISCUSSION

Genotoxicity versus Boiling Point

Genetic response in relation to boiling point (i.e., to average molecular weight of SRC-I constituents) varied according to the screening test. With few exceptions, reverse mutation with *S. typhimurium* TA98 (standard Ames and fluctuation test) responded mainly to mutagens contained in bp cuts above 700°F (bp cuts 750°F to 800°F and 800°F to 850^{+}°F). Only trace activities were detected by TA98 for bp cuts below 700°F.

Conversely, the forward mutation assay with *S. typhimurium* TM677 was more sensitive to compounds that distilled between 600°F and 700°F than constituents in bp cuts above 700°F. The distribution of mutagenic activity in the forward mutation assay with *S. typhimurium* TM677 was also bimodal for N-PAC compounds with major peaks in the 600°F to 700°F and 750°F to 800°F ranges. The PAH constituents showed only one peak of mutagenic activity in the 650°F to 700°F bp cut.

Activity in the prophage induction test was low over the entire temperature range of bp cuts (i.e., 450°F to 850^{+}°F). However for certain fractions, notably A-3, this test also indicated bimodality with activity in bp

cuts above and below 700°F. Thus reverse mutation with S. typhimurium TA98 was mainly responsive to higher molecular weight mutagens in the greater than 700°F bp cuts and forward mutation with S. typhimurium TM677 to lower molecular weight mutagens in the less than 700°F bp cuts. In general, prophage induction, although weak, followed forward mutation rather than reverse mutation in its response to DNA active agents in the bp cuts.

Genotoxicity Versus Chemical Class

There were fewer inconsistencies among the responses of the microbial screening assays to chemical class fractions than there were to bp cuts. Consistent with previous work on SRC-I PS and SRC-II HD, the N-PAC components were mutagenically more active than other chemical classes, and APAH accounted for most activity associated with SRC-I bp cuts.

The prophage induction test provided a generally weak response for N-PAC and all other chemical class fractions, regardless of bp cut. Thus, the inductest, as presently used, is not appropriate for screening SRC-I bp cuts for genotoxins.

Aliphatic fractions of SRC-I bp cuts were all genetically inactive except for a slight response in the prophage induction assay. Slightly more surprising was the near lack of genetic activity in the PAH fractions which contained relatively high concentrations of 5-ring polyaromatic compounds such as benzo(a)pyrene in bp cuts above 750°F, and especially in the 800°F to 850^{+}°F bp cut. However S. typhimurium TA98, when used in the fluctuation test, was able to detect significant genetic activity in the PAH fraction from bp cut 800°F to 950^{+}°F. The same tester strain in the standard Ames assay did not

detect genetic activity in PAH fractions from any bp cut. Thus, the fluctuation test, and in particular the fluctuation test with one or more of the Ames tester strains, may be the most appropriate screening assay for the complex PAH fractions.

4.0 MOUSE SKIN TUMOR INITIATION ACTIVITY OF SRC-I COAL LIQUID RELATIVE TO BOILING POINT

Early investigations by Condra and Weil (1960) suggested that carcinogenicity of coal-derived materials increased with increasing temperature of formation. Moreover, epidemiological studies at gas manufacturing and coking facilities suggested that workers exposed to high temperature pollutants were at greater risk from respiratory cancer than workers exposed to low temperature materials (Kawai et al.). Recent studies (PNL-3189) demonstrated that SRC-II heavy distillate (bp, 550°F to 850°F) was carcinogenic to mouse skin when applied 3X weekly, while SRC-II light distillate (bp, ambient to 380°F) was not. Calkins et al. (1982) and Mahlum (submitted to J. Appl. Toxicol. 1982) found that skin tumor initiating activity was also higher for higher boiling crude coal liquids than for lower boiling ones.

Results of the above studies indicated it would be useful to have a systematic study of the tumorigenic activity of coal-derived materials relative to distillation temperature. Concentration of activity in defined distillation cuts might afford an opportunity to identify the chemical agents responsible and to aid in designing methods of preventing worker exposure to those agents. Therefore, several distillation cuts from SRC-I PS were tested for initiation/promotion activity.

METHODS

Female CD-1 mice (Charles River, Portage, Maine) 6 weeks old were housed five per cage on standard bedding material (Sani-Cel, Paxton Processing Co., Paxton, IL) with food (Wayne 4 Lab-Blox, Allied Mills, Libertyville, IL) and tap water available libitum. Prior to administration of test material, animals

were ear tagged for identification; weighed; shaved; and assigned to a test group, 28 to 30 mice per group. Fifty μl of each test material were diluted 1:1 with acetone, and 50 μl of the diluted material were applied to the shaved backs of the mice. Two groups of animals were initiated with 50 μg of dimethylbenz(a)anthracene DMBA (Eastman Chemical Company) or benzo(a)pyrene (Sigma Chemical Company) to serve as positive controls. Additionally, 50 μg of several polyaromatic amine compounds (9-amino phenanthrene, 3-aminopyrene, 6-aminochrysene, 2-amino anthracene) were applied as reference initiators. Two weeks post-initiation, 50 μl of phorbol myristate acetate (Sigma Chemical Company, 0.1 mg/ml of acetone) were applied to the initiated area twice weekly for six months. Mice were shaved as necessary throughout the study, usually once per week. Animals were observed regularly for tumor growth. Sketches of tumor location and size were recorded each week to document papilloma development.

RESULTS

The cumulative tumor incidences and total tumor yield in mice initiated with acetone, BaP or DMBA (negative and positive controls) are shown in Figures 4.1 and 4.2. Only 1 out of 30 mice (3.3%) initiated with acetone developed a tumor. In contrast, the BaP and DMBA groups had 90 and 100% tumor incidence, respectively. Figure 4.2 shows that total tumor yield was higher for DMBA than for BaP.

There was some initiating activity for SRC-I bp cuts below 700°F as indicated by tumor incidence (Figure 4.3) or total tumor yield per group (Figure 4.4). However, tumor incidence and yield were significantly higher for mice initiated with 700°F to 750°F, 750°F to 800°F, 800°F to 850^{+}°F, or crude PS than for those initiated with lower bp material. The 800°F to 850^{+}°F cut was especially active.

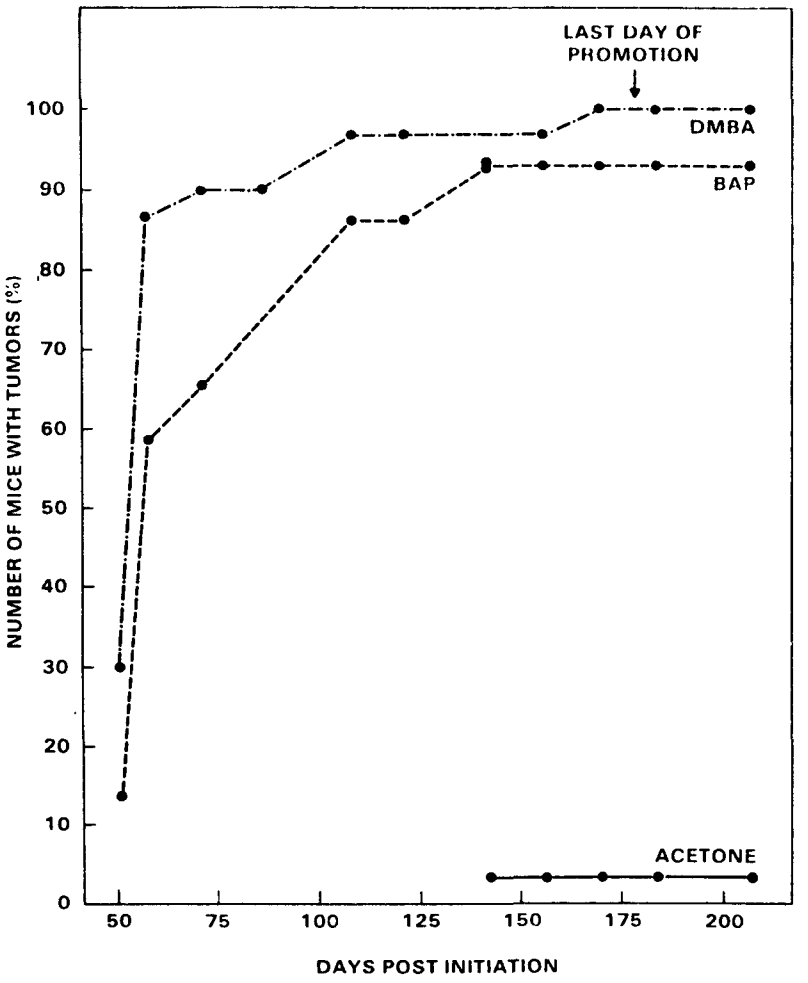


FIGURE 4.1. Mouse Skin Tumor Response after Initiation with Acetone (control), Benzo[a]pyrene (BaP), or Dimethylbenzanthracene (DMBA) Followed by Promotion with Phorbol Myristate Acetate.
1A. Percent tumor incidence versus time.
1B. Cumulative tumor yield per group versus time.

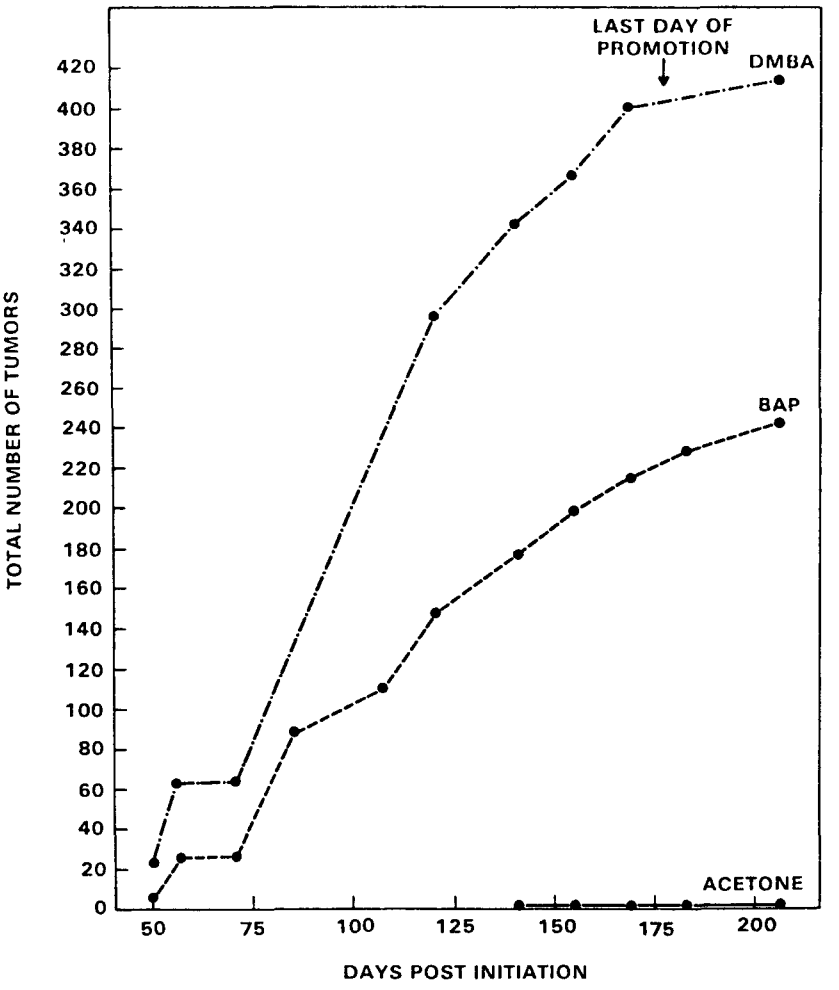


FIGURE 4.2. Cumulative Mouse Skin Tumor Yield After Initiation with Positive Controls Followed by Twice Weekly Promotion with Phorbol Myristate Acetate.

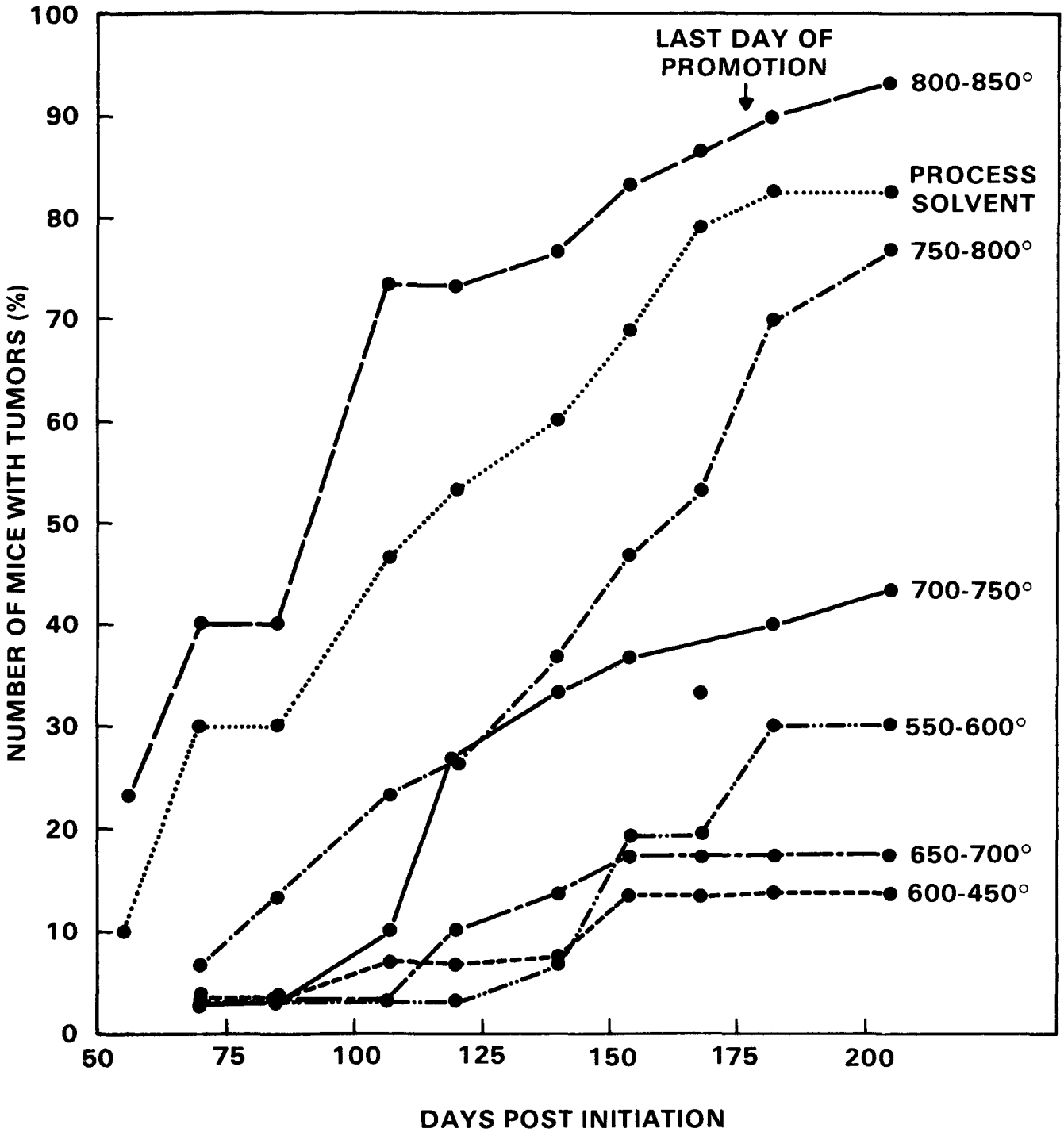


FIGURE 4.3. Mouse Skin Tumor Incidence After Initiation with SRC-I Distillates Followed by Twice Weekly Promotion with Phorbol Myristate Acetate.

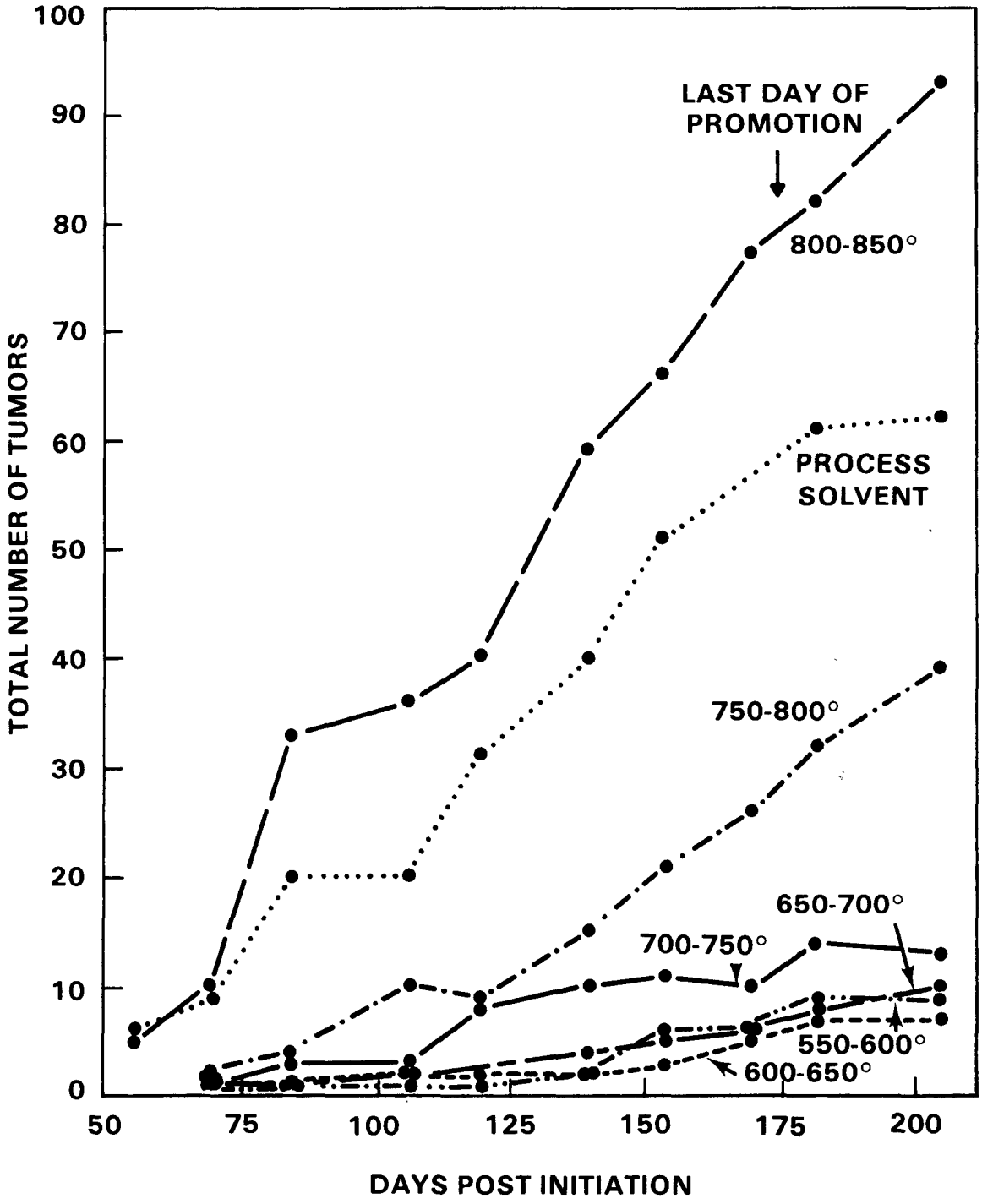


FIGURE 4.4. Cumulative Mouse Skin Tumor Yield After Initiation with SRC-I Distillates Followed by Twice Weekly Promotion with Phorbol Myristate Acetate.

Results from mice initiated with the pure aromatic amines, 2-aminoanthracene, 9-aminophenanthrene, 1-aminopyrene, or 6-aminochrysene are shown in Figures 4.5 and 4.6. Mice initiated with 2-AA and 6-AC showed a similar response to that seen for PS, and the 750°F to 800°F and 800°F to 850^{+}°F bp cuts.

DISCUSSION

Results indicate that skin tumor initiating activity of SRC-I PS generally increases with bp. Although there was low activity below 700°F, activity was significantly higher for bp cuts above 700°F. In other experiments we found that SRC-II liquid boiling below 700°F lacked activity, but over 700°F the material showed progressively higher activity as bp increased. Activity of the SRC-I 800°F to 850°F bp cut was higher than that for the SRC-II 800°F to 850°F bp cut. However, bp cuts from the two processes were not fully comparable since the SRC-I cut also contained residual material (bottoms). Nonetheless, the highest boiling point material from both the SRC-I and -II processes showed tne highest initiating activity.

Calkins et al. prepared distillates from crude SRC-II, H-coal, and Exxon Donor Solvent oils as well as from petroleum crudes and evaluated the distillates for initiating activity using an assay similar to that used here. Although the distillation ranges studied by Calkins et al. were different from those used here, the data obtained generally support the concept that tumor-initiating activity increases with bp. Their data suggest a somewhat lower activity in the residual material that remains after distillation. However, their upper distillation temperature was about 100°F higher than that used in

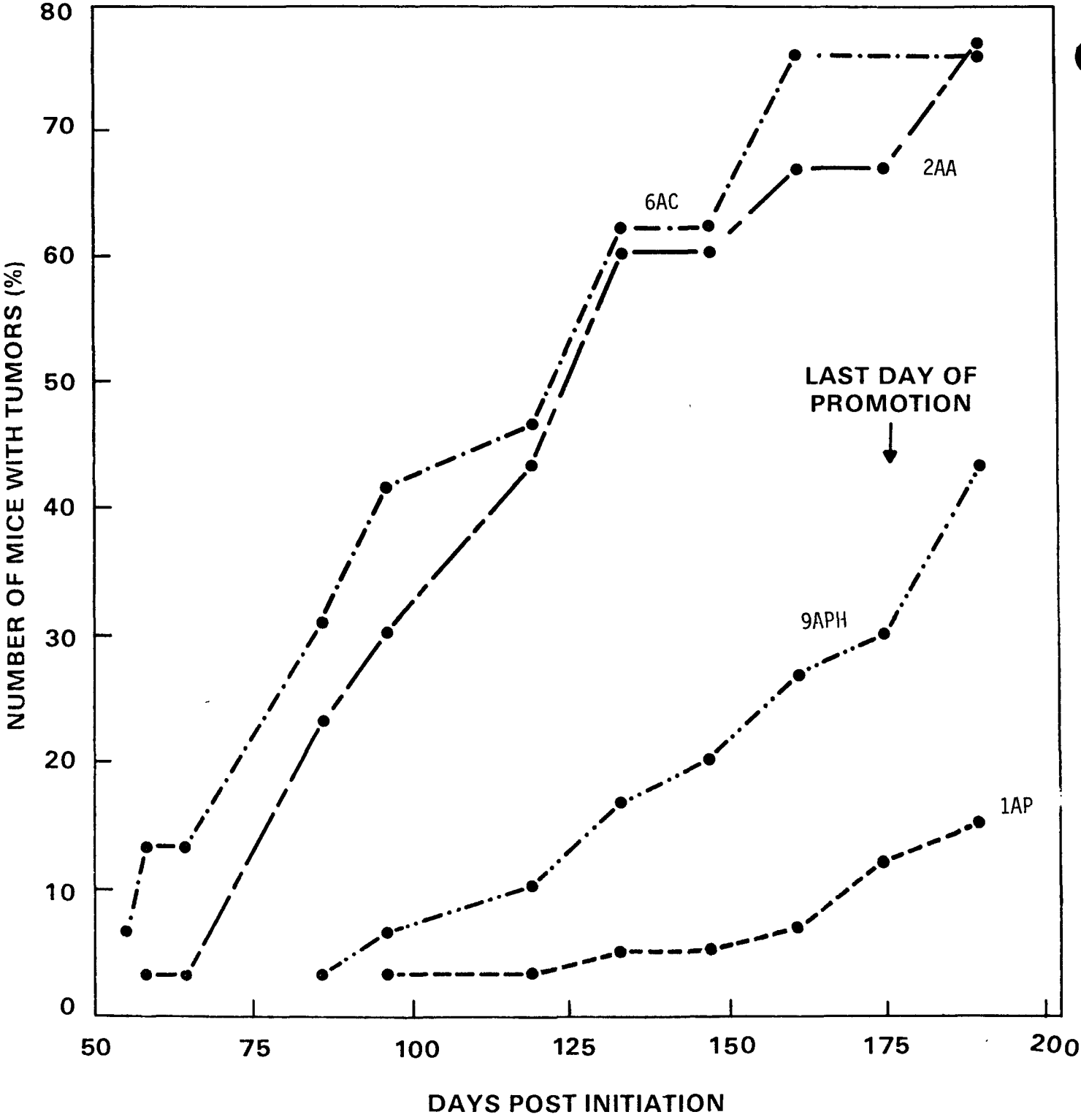


FIGURE 4.5. Mouse Skin Tumor Incidence After Initiation with 9-Aminophenanthrene (9-APH), 12-Aminoanthracene (2-AA), 1-Aminopyrene (1-AP), or 6-Aminochrysene (6-AC) Followed by Twice Weekly Promotion with Phorbol Myristate Acetate.

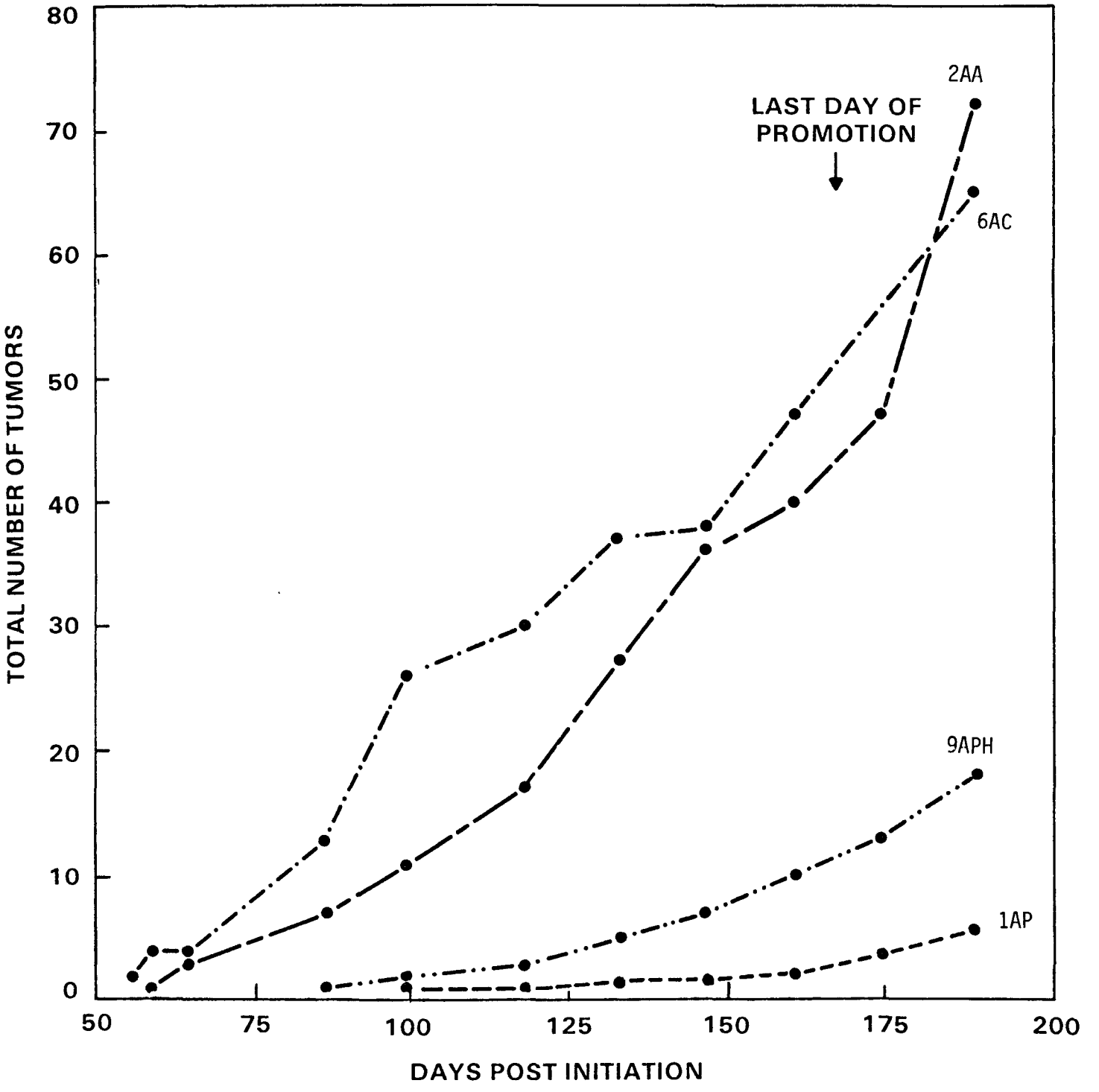


FIGURE 4.6. Cumulative Mouse Skin Tumor Yield After Initiation with Several Aromatic Amines Followed by Twice Weekly Promotion with Phorbol Myristate Acetate. (Designations for APAH are the same as in Figure 4.5.)

this study. Therefore, we plan to specifically evaluate the material remaining after distillation at 850°F to determine if initiating activity is higher than it is in the 800°F to 850°F cuts.

Although there is some agreement between the results of the IP and Ames assays, there are also both qualitative and quantitative differences. Highest initiating activity was found with the 800°F to 850^{+}°F bp cut, while highest Ames activity was in the 750°F to 800°F cut. Moreover, low initiating activity was found for cuts boiling between 550°F and 700°F, with the 700°F to 750°F cut showing moderate activity. In contrast, mutagenic activity was not observed for materials boiling below 750°F unless the material was fractionated before testing. Mutagenic activity occurred in fractionated materials boiling above 650°F.

5.0 DISCUSSIONS AND CONCLUSIONS

Although overall results from mutagenicity and IP assays give the same general picture of response related to boiling point, there were some differences for specific materials. The greatest initiating activity was found in the 800°F to 850^{+}°F cut while activity against TA98 in the standard Ames test was highest in the 750°F to 800°F cut. Using the microlitre version of the fluctuation test with TA98 however, the similarity between IP and microbial mutagenicity is striking (see Figure 5.1).

A low level of initiating activity was found for cuts boiling between 550°F and 700°F with the 700°F to 750°F cut showing moderate activity. In contrast, mutagenic activity was not observed for materials boiling below 750°F unless the material was fractionated before testing; with prior chemical fractionation, mutagenic activity could be observed in cuts boiling above 650°F.

Although several of the coal-derived liquids possessed significant initiating activity, they were much less active than DMBA or BaP as judged by incidence or total yield of tumors. It should be noted that much larger initiating doses of the coal liquids (about 25 mg) are being used relative to the PAH or APAH controls (50 μg). In each case, only one dose level is used. Therefore, until a measure of dose response is available for the coal-derived materials, comparison of relative activities must be viewed with caution.

It should also be stated that the initiation/promotion tests were carried out with crude (unfractionated) SRC-I bp cuts. Thus we are unable from these data to relate carcinogenesis to any particular chemical class. Nevertheless, it might be anticipated that the PAH fractions would be major contributors

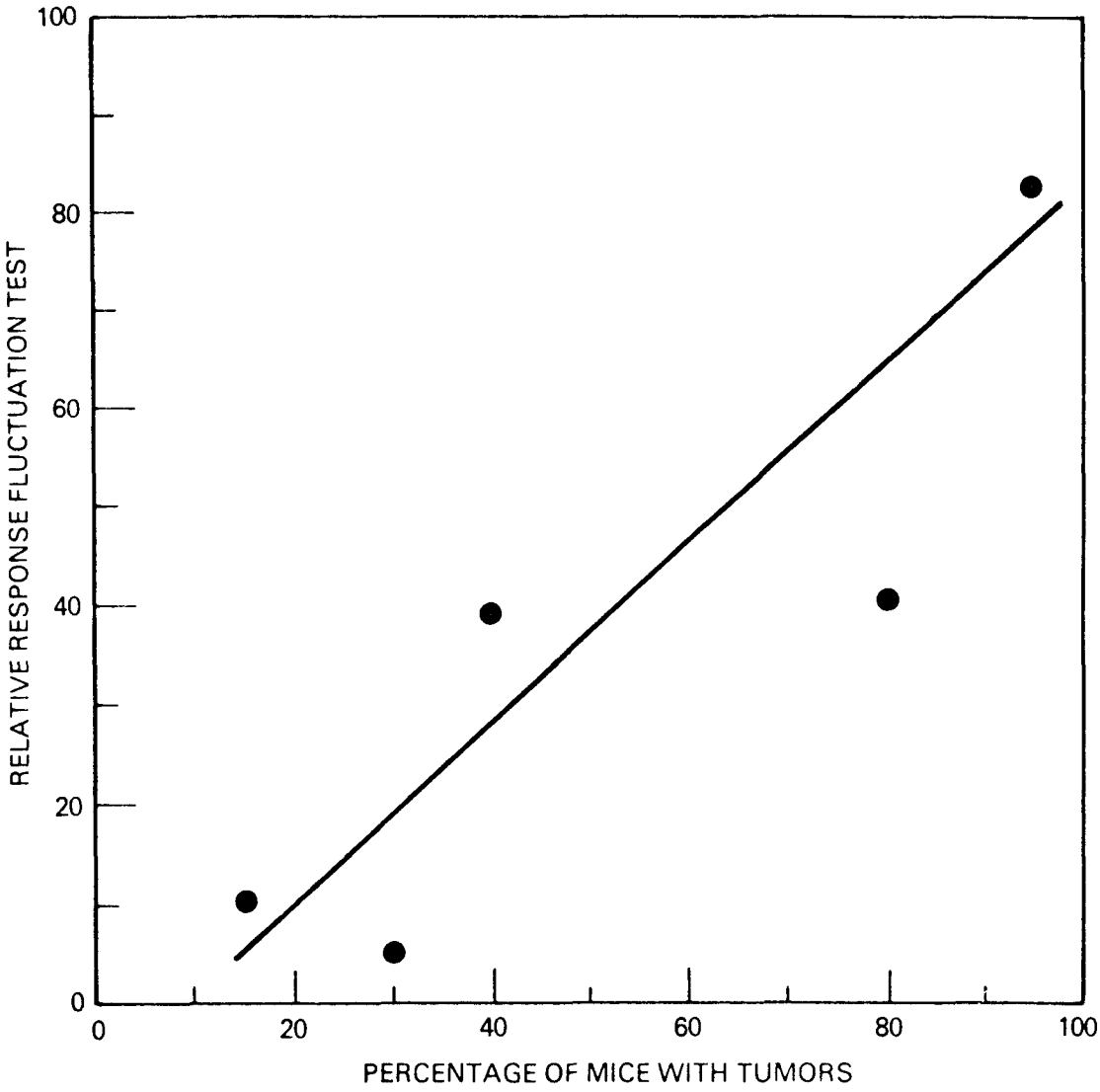


Figure 5.1 Relationship of the Response in the Fluctuation Test to that Obtained From Initiation/Promotion Skin Painting for the Crude Boiling Point Cuts.

since the mouse skin is exquisitly sensitive to carcinogenesis induced by individual PAHs. In this regard the sensitivity of the microbial screening assays to the complex PAH fractions is of interest in that only the fluctuation test was able to detect mutagenic activity in any of the PAH fractions. Conversely, these microbial tests, most notably those involving TA98, were highly sensitive to N-PAC and in particular, to the APAH constituents of the N-PAC.

This sensitivity to APAH has been recognized for some time, although certain workers have tended to discount the significance of Ames test results since on chemically differentiated fractions they often do not correlate well with skin painting results. The value of the Ames test for detecting APAH in extremely low concentrations and in complex mixtures, however, should not be underestimated, particularly in view of the fact that the most widely recognized and documented instances of industrial-related cancers in humans, traceable to a given agent, are traceable to APAH. Furthermore, APAHs are known to give rise to systemic cancers which are difficult to manage clinically. Effects in humans of industrial exposure to PAH, on the other hand, are not yet clear. Although TA98 is not particularly sensitive to those specific compounds known to be human carcinogens, such as 2-aminonaphthalene and 4-aminobiphenyl, the test is effective in screening for APAH in general, and specific chemical analysis to determine quantitatively the levels of specific APAH are now relatively straightforward.

Several features regarding the chemical composition of the bp cuts as it relates to the initiation/promotion results are of some interest. Although overall PAH content remained fairly constant above 700°F, the actual concentrations of individual PAH changed considerably, with the highest concentrations

of several known skin carcinogens being reached in the 800°F to 850^{+}°F cut. Concentrations of N-PAC having 4 or more aromatic rings also increased in the 800°F to 850^{+}°F cut although compared to neutral PAH such as DMBA and BaP, N-PAC carcinogens which have been studied such as dimethyl benzacridine are relatively weak carcinogens. Since chemical class fractions were not tested in IP however, no firm conclusions as to the compound classes responsible for the initiation activity can be reached at this point.

Experiments with nitrous acid, which selectively destroys the APAH, indicate that most of the microbial mutagenic activity is due to APAH. At this time, comparable experiments have not been performed to identify specific components responsible for tumor initiating activity. However, the data obtained in this study suggest it may be possible to narrow the choice of possibilities by further fractionating the 800°F to 850^{+}°F cut and assaying the resulting subfractions for intiating activity.

6.0 REFERENCES

Ames, N., J. McCann and E. Yamasaki. 1975. "Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test." Mutation Res. 347-364.

Calkins, W. H., J. F. Deye, R. W. Hartgrove, C. F. King and D. F. Krahn. 1982. "Synthetic crude oils from coal mutagenicity and tumor-initiation screening tests." In Coal conversion and the Environment, ed. by D. D. Mahlum, R. H. Gray and W. D. Felix, pp. 462-470. CONF-801039, NTIS, Springfield, VA.

Gatehouse, D. 1978. "Detection of Mutagenic Derivatives of Cyclophosphamide and a Variety of Other Mutagens in a "Microtitre" Fluctuation Test, without Microsomal Activation." Mutation Res. 229-296.

Gatehouse, D., and G. Delow. 1979. "The Development of a "Microtitre" Fluctuation Test for the Detection of Indirect Mutagens, and Its Use in the Evaluation of Mixed Enzyme Induction of the Liver." Mutation Res. 60, 239-252.

Guerin, M. R., C-h Ho, T. K. Rao, R. B. Clark, and J. L. Epler. 1980. Env. Res. 23, 42.

Guerin, M. R., et al. 1981. Fuel 60, 282-288.

Ho, C-h, B. R. Clark, M. R. Guerin, B. D. Barkenbus, T. K. Rau, J. L. Epler. 1981. Mutation Res. 85, 335-345.

Kawai, M., H. Amamoto, and K. Harda. 1967. "Epidemiologic study of occupational lung cancer." Arch. Environ. Health 14, 859-864.

Later, D. W., M. L. Lee, K. D. Bartle, R. C. Kong, and D. L. Vassilaros. 1981. Anal. Chem. 53, 1612-1620.

Later, D. W., M. L. Lee and B. W. Wilson. 1982. "Selective Detection of Amino Polycyclic Aromatic Hydrocarbons in Solvent Refined Coal." Anal. Chem. 54, 117.

Liddle, D. 1976. "Pratical Tests of 2 x 2 Contingency Tables." The Statistician 25, 295-304.

Luria, S., and M. Pelbruck. 1943. "Mutations of Bacteria from Virus Sensitivity to Virus Resistance." Genetics 28, 491-511.

Moreau, P., A. Bulone and R. Devoret. 1976. "Prophage Lambda Induction in Escherichlira Coli K12 Evr A Uvr B: A Higher Sensitive Test for Potential Carcinogens." PNAS 73, 3700-3704.

Pelroy, R. A. and B. W. Wilson. 1980. Fractional Distillation as a Strategy for Reducing the Genotoxic Potential of SRC-II Coal Liquids: A Status Report. PNL-3787, Pacific Northwest Laboratory, Richland, Washington, (NTIS) Springfield, Virginia.

Pelroy, et al. 1981. "Comparison of the Mutagenicities of Fossil Fuels." Mutation Res. 90, 233-245.

Pelroy, R., and M. Petersen. 1981. "Mutagenic Characterization of Synthetic Fuel Materials by the Ames/Salmonella System." Mutation Res. 90, 309-320.

Pelroy, R. A., and B. W. Wilson. 1982. "Relative Concentrations of Polyaromatic Primary Amines and Azaarenes in Mutagenically Active Nitrogen Fractions from a Coal Liquid." Mutation Res. 90, 309.

Pelroy, R. A., and D. L. Stewart. 1982. "The Effects of Nitrous Acid on the Mutagenicity of Two Coal Liquids and Their Genetically Active Chemical Fractions." Mutation Res. 90, 297-308.

PNL-3189. 1979. Biomedical Studies on Solvent Refined Coal Liquefaction Materials: A Status Report. Pacific Northwest Laboratory, Richland, Washington, (NTIS) Springfield, Virginia.

PNL-3464. 1980. Initial Chemical and Biological Characterization of Hydrotreated Solvent Refined Coal (SRC-II) Liquids: A Status Report. Pacific Northwest Laboratory, Richland, Washington, (NTIS) Springfield, Virginia.

Renne, R. A., L. G. Smith, and D. D. Mahlum. 1982. "Epidermal carcinogenicity of some crude fossil fuels in mice: A preliminary report." In Coal Conversion and the Environment, ed. by D. D. Mahlum, R. H. Gray and W. D. Felix, pp. 471-481. CONF-801039, NTIS, Springfield, VA.

Skopek, T. R., H. L. Liber, D. A. Kaden, R. A. Hites, and W. C. Thilly. 1979. "Mutation of Human Cells by Kerosene Soot." JNCI 63, 309-314.

Weil, C. S., and N. I. Condra. 1960. "The hazards to health in the hydrogenation of coal." Arch. Environ. Health 1, 187-193.

Wilson, B. W., and R. A. Pelroy. 1979. Proceedings 27th ASMS, Seattle, Washington.

Wilson, B. W., R. A. Pelroy, and J. T. Cresto. 1980. Mutation Res. 79, 193-202.

Wilson, B. W., M. R. Petersen, R. A. Pelroy, and J. T. Cresto. 1981. Fuel 60, 289-294.

Yoshida, D., and T. Matsumoto. 1978. "Changes in Mutagenicity of Protein Pyrolysates by Reaction with Nitrite." Mutation Res. 58, 35-40