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September 1983

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
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PNL-4836

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Printed in the United States of America
Available from
National Technical Information Service
United States Department of Commerce
5285 Port Royal Road
Springfield, Virginia 22161

NTIS Price Codes
Microfiche A01

Printed Copy

Pages	Price Codes
001-025	A02
026-050	A03
051-075	A04
076-100	A05
101-125	A06
126-150	A07
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276-300	A013

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COMPARISON OF IN VITRO AND IN VIVO STUDIES WITH COAL LIQUIDS FROM THE SRC-11 PROCESS

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SUMMARY

Coal liquids obtained from the solvent refined coal-II process and fractions prepared from these liquids have been assayed in a number of in vitro and in vitro systems for biological activity. The in vitro systems include: 1) the standard Ames Salmonella typhimurium reverse mutation assay, 2) the S. typhimurium fluctuation test; 3) forward mutation assay in S. typhimurium (8-Ag) test; 4) prophage induction (INDUCTEST); 5) Syrian hamster ovary (SHE) cell transformation assay; and 6) Chinese hamster ovary (CHO) cell mutation assay. In addition, both initiation/promotion (I/P) and chronic skin-painting assays were used as measures of tumorigenesis.

In general, materials shown to be carcinogenic in the chronic skin-painting assay were also positive in the other assays. The failure of the Ames assay to respond to the neutral polycyclic aromatic hydrocarbon (PAH) fraction of SRC-II heavy distillate (HD) was a notable exception. Quantitatively, the Ames assay was more sensitive to nitrogen-containing compounds (particularly primary aromatic amines) and less sensitive to mixtures of PAH. The mammalian systems, both in vitro and in vivo, showed greater responses to the neutral PAH than to the nitrogen-containing compounds.

Activity in all biological systems increased with increasing boiling point of the material tested. The 850°F distillation cut produced the highest activity in all assays. Materials boiling below 700°F resulted in little or no activity in any of the assays used.

The I/P assay ranked the materials studied in the same order as did the chronic skin-painting assay; however, the results of the two assays diverged quantitatively, particularly for certain distillate cuts.

Despite the lack of quantitative agreement between the in vitro microbial and in vivo skin-painting assays, the in vitro assays remain valuable screening tools for complex mixtures. Sufficient information now exists to qualify the use of the in vitro assays for complex mixtures and to increase their reliability.

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1.0 INTRODUCTION

The Salmonella typhimurium mutagenesis (Ames) assay has been used extensively over the past several years to screen chemicals for potential carcinogenicity. A large data base comparing mutagenesis data with the carcinogenicity of pure compounds indicates that the Ames assay is a useful predictor of carcinogenic activity. Although the Ames test has also been applied to complex mixtures of organic compounds, the data base has been insufficient to determine whether it is a good predictor of carcinogenicity.

More recently, several mammalian-cell-culture systems which measure transformation or mutagenesis have been employed to study pure chemicals and complex organic mixtures. Results from these studies have also been used as indicators of potential carcinogenicity. However, the in vitro data base for complex mixtures is also limited, and it is not clear how well the results of these assays corresponded with those from in vivo animal carcinogenesis studies.

In the course of evaluating the toxicologic properties of coal liquefaction materials, we had the opportunity to assay several coal liquids, their boiling point (bp) cuts, and their chemical fractions in several systems, including: 1) Ames, 2) Syrian hamster embryo (SHE) transformation, 3) Chinese hamster ovary (CHO) cell mutation, 4) mouse skin initiation/promotion (I/P), and 5) chronic mouse skin-painting. Although not every material was assayed in every system, a sufficient data base was developed to permit an estimate of the degree of correlation among assays.

Previous studies have indicated that the mutagenic and carcinogenic activity of coal liquids is confined to higher-boiling constituents (Pelroy and Wilson 1981; Mahlum, 1983). This observation provided a basis for one approach to studing the biological activity of coal liquids using several assays and for identifying the components responsible for that activity. We have, therefore, fractionally distilled coal liquids with wide boiling ranges into 50°F bp cuts as described below. These cuts from SRC-II liquids, as well as chemical fractions prepared from the cuts, have been used in these studies. Where appropriate, data for other materials, including SRC-I liquids, are used for additional comparisons.

2.0 SAMPLES AND SEPARATION METHODS

Sample sources for SRC-II liquids and other materials used for comparative purposes have been described in an earlier PNL Status Report (PNL-3189 1979). The solvent fractionation used with heavy distillate (HD) has been described previously (PNL-3474 1981). A set of six process distillation samples were used for the assays of solvent refined coal (SRC)-II bp cuts. First, a full-boiling-range (>300-850°F) blend of SRC-II material was obtained during the processing of Powhatan Mine No. 5 coal on Process Development Unit P-99, operated by Gulf Science and Technology Co. at Harmarville, Pennsylvania. The full-boiling-range material was fractionally distilled to produce the following bp cuts: 300-700°F, 700-750°F, 750-800°F, 800-850°F, and >850°F bottoms. The precision of the distillation was such that 95% of the material within a given cut had its bp within that temperature range.

The five distillate cuts were fractionated by adsorption-column chromatography to obtain discrete aliphatic, polycyclic aromatic hydrocarbons (PAH), nitrogen-containing polycyclic aromatic compounds (NPAC) and hydroxylated PAH class fractions. Details of this separation procedure, using neutral alumina as an adsorbent and hexane, benzene, chloroform, and tetrahydrofuran/ethanol as eluents, have been described previously (Later et al. 1980). All four fractions were dried and weighed to obtain chemical class distribution data. The PAH and NPAC fractions from the benzene and chloroform elutions were analyzed for their respective chemical composition and assayed for biological activity. Table 2.1 shows the weight distribution of material among the four chemical classes.

TABLE 2.1. Chemical Composition of SRC-II bp Cuts

	Percentage by Weight[a]					
Compound Class	300-700°F	700-750°F	750-800°F	800-850°F	>850°F	300->850°F[b]
Aliphatic hydrocarbons	32	15	15	4	2	13
PAH	27	45	43	47	44	41
NPAC	12	21	29	31	35	13
Hydroxylated PAH	15	11	12	16	19	16
Total Recovery	86[c]	92	99	98	100	83[c]

[a]Average of three separate determinations

[b]Full-boiling-range blend

[c]The low recovery observed for this sample probably reflects the volatile nature of this relatively low-boiling material.

To facilitate the qualitative/quantitative study of its mutagenic nature, the NPAC fraction was subfractionated by a second adsorption column chromatographic step. Briefly, the NPAC fraction from the alumina adsorption step was separated on a silicic acid adsorbent using benzene and ethyl ether eluents to provide three fractions enriched in carbazoles (S1), amino polyaromatic hydrocarbons (APAH, S2) and azaarenes (S3), respectively. The APAH fraction, which contained both carbazoles and azaarenes, was not further purified for the work presented here.

3.0 MICROBIAL MUTAGENESIS STUDIES

The SRC-II distillate cuts described above were evaulated in a battery of in vitro assays for their capacity to induce mutations and/or DNA damage in microbial systems.

3.1 AMES ASSAY

Agar plate mutagenicity assays utilized the Ames histidine reversion test (Ames et al. 1975) and S. typhimurium strain TA-98. Dimethylsulfoxide (DMSO) was used as a solvent for all test materials. Fixed concentrations of 2-aminoanthracene (2-AA), benzo[a]pyrene (BaP) and a complex (isooctane-soluble) basic fraction from SRC-II HD were tested against Aroclor 1254-induced rat liver activating enzymes (S9) to optimize amounts used for metabolic activation.

Optimum levels for S9 were established and monitored daily. Negative controls included Ames/S. typhimurium test strains with and without S9, and appropriate solvent controls. Revertant (rev) colonies were counted electronically, using a Biotran II automated colony counter (New Brunswick Scientific Co., Inc., Edison, New Jersey).

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/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 twice that of background. Specific mutagenic activity was expressed as rev colonies of S. typhimurium TA-98/µg test material (rev/µg) and was estimated by linear regression of dose-response data. To be considered positive, a mutagen had to express a maximum response of at least two times background for at least one concentration in the dose range, as well as a linear correlation coefficient of at least 0.8. Experiments were replicated several times to obtain the average mutagenic potency in rev/µg and to estimate recovery of genetic activity in the chemical fractions. The average value was multipled by the weight percentage of a given fraction to yield a weighted mutagenic activity in relation to the crude bp cut or fraction.

3.2 FLUCTUATION TEST

The fluctuation test was also conducted with S. typhimurium TA-98. This test was adapted from Luria and Delbruck (1941) using the "microtitre" technique described by Gatehouse (1978) and Gatehouse and Delow (1979). Cells of S. typhimurium TA-98 in liquid exposed to the test materials for 4 hours and aliquots ($\sim 10^6$-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 the chi-square statistic, incorporating Liddle's (1976) continuity correction. Exposure concentrations of coal-liquid materials were 0.1 and 10 µg or 10 and 100 µg, depending on the material being assayed.

3.3 FORWARD MUTATION ASSAY

Forward mutation induced by SRC materials in S. typhimurium TM-677 was measured by resistance to the purine analog 8-azaguanine (8-Ag). We used a modification of the method of Skopek et al. (1978), increasing the length of exposure to the test material 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 liquids 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 that did not contain 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 to achieve a final concentration of 50 µg/ml. The viability of exposed cells needed to calculate specific mutagenic activity, was determined by dilution plate counts onto Minimal E Medium without the 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:

$$\frac{\text{Number of Resistant Clones on 8-Ag-Containing Medium}}{\text{Number of Clones on Medium without 8-Ag}} \times \text{Dilution Factor}$$

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

3.4 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 for the Ames assay.

The test was performed by exposing approximately 10^7 growing cells of E. coli 8177 to test material for 6 hours, with or without S9 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 and 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 plaque-forming units/plate (PFU/PLATE).

3.5 RESULTS OF MICROBIAL ASSAYS

Figure 3.1 illustrates the response obtained in the four microbial assays with the SRC-II distillate cuts described previously. In general, higher activity was associated with materials boiling over 700°F. The major exception to this pattern occurred with the 8-Ag test, in which the activity peaked in the 750-800°F cut with relatively little response found in the >850°F distillate.

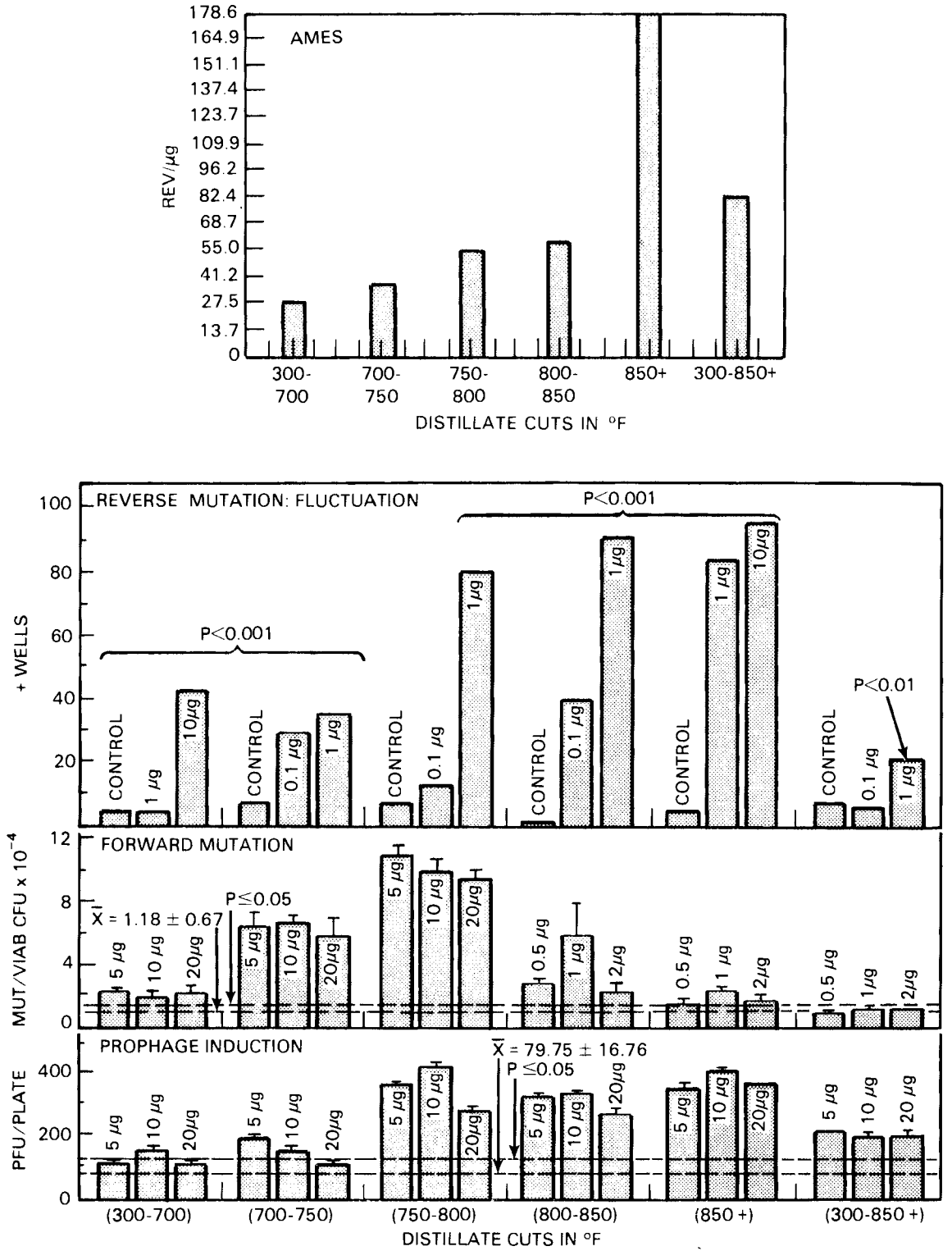

FIGURE 3.1. Response of the Ames, Fluctuation, 8-Ag and Prophage Induction (Inductest) Assays to Discrete bp Cuts Derived from an SRC-II Coal Liquid Boiling from 300->850°F. In the Fluctuation Test, the Control was Treated Only With the Vehicle.

Since the 800-850° and >850°F cuts produced the greatest response in the various assays, they were fractionated on alumina by the method of Later et al. (1980) in an attempt to identify the chemical classes responsible for the mutagenic activity. The response of the Ames assay to these chemical classes prepared from the 800-850° and >850°F cuts was compared to the response obtained with similar fractions prepared from the relatively inactive 300-700° distillate (Figure 3.2). Of these fractions, the highest activity (280 rev/μg) was associated with the NPAC from the 800-850° cut. The NPAC fraction from the >850°F cut also possessed substantial activity (150 rev/μg), while the 300-700°F NPAC had only low levels (<20 rev/μg). The activity in the aliphatic and PAH fractions was barely detectable for any of the cuts; the hydroxy polycyclic aromatic hydrocarbon (HPAH) fractions from both the 800-850° and >850°F cuts had low but significant activity.

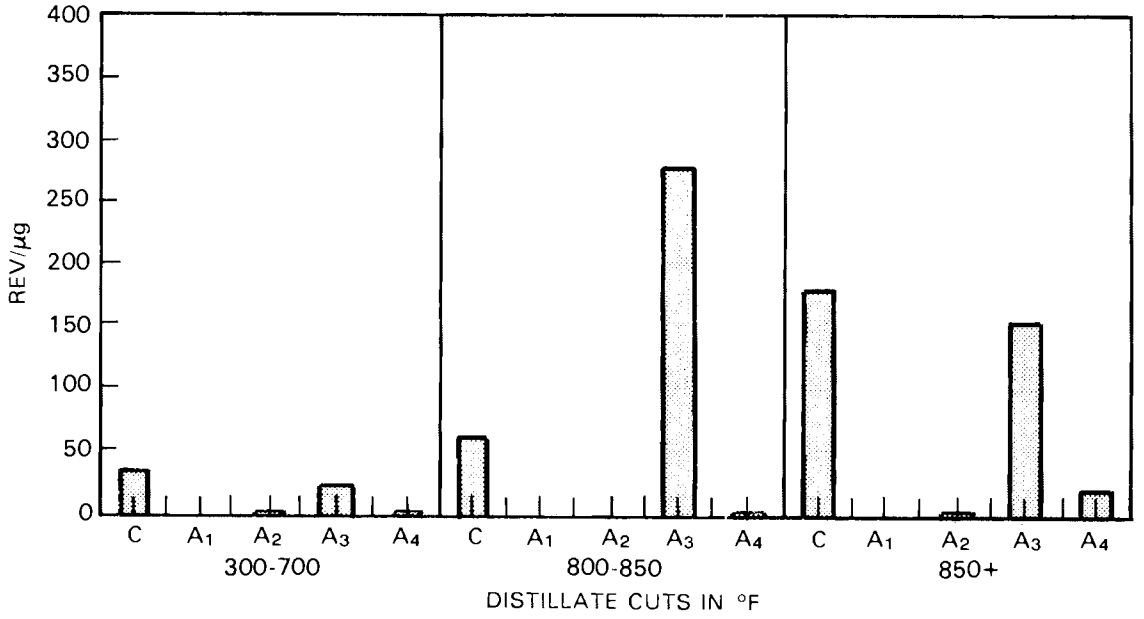

FIGURE 3.2. Response of the Ames Assay to the Aliphatic (A1), Neutral PAH (A2), NPAC (A3) and HPAH (A4) Fractions from the 300-700°, 800-850° and >850°F Distillate Cuts. The Unfractionated Distillates are Designated "C."

Since the PAH fractions from the 800-850° and >850°F cuts contained known mutagens, such as BaP, they were assayed in the other microbial systems to determine whether those assay systems were better able to detect neutral PAH mutagens contained in a complex matrix. These experiments suggested that all systems showed some response to the PAH from the 800-850°F and >850°F cuts (Figure 3.3). The fluctuation test appeared to be the most sensitive, although the dose-response relationships are not very clearcut.

Because we wished to determine whether the microbial systems other than the Ames assay were also sensitive to the NPAC fractions, we tested them and found that those from the 800-850° and the >850°F cuts were active in all sys-

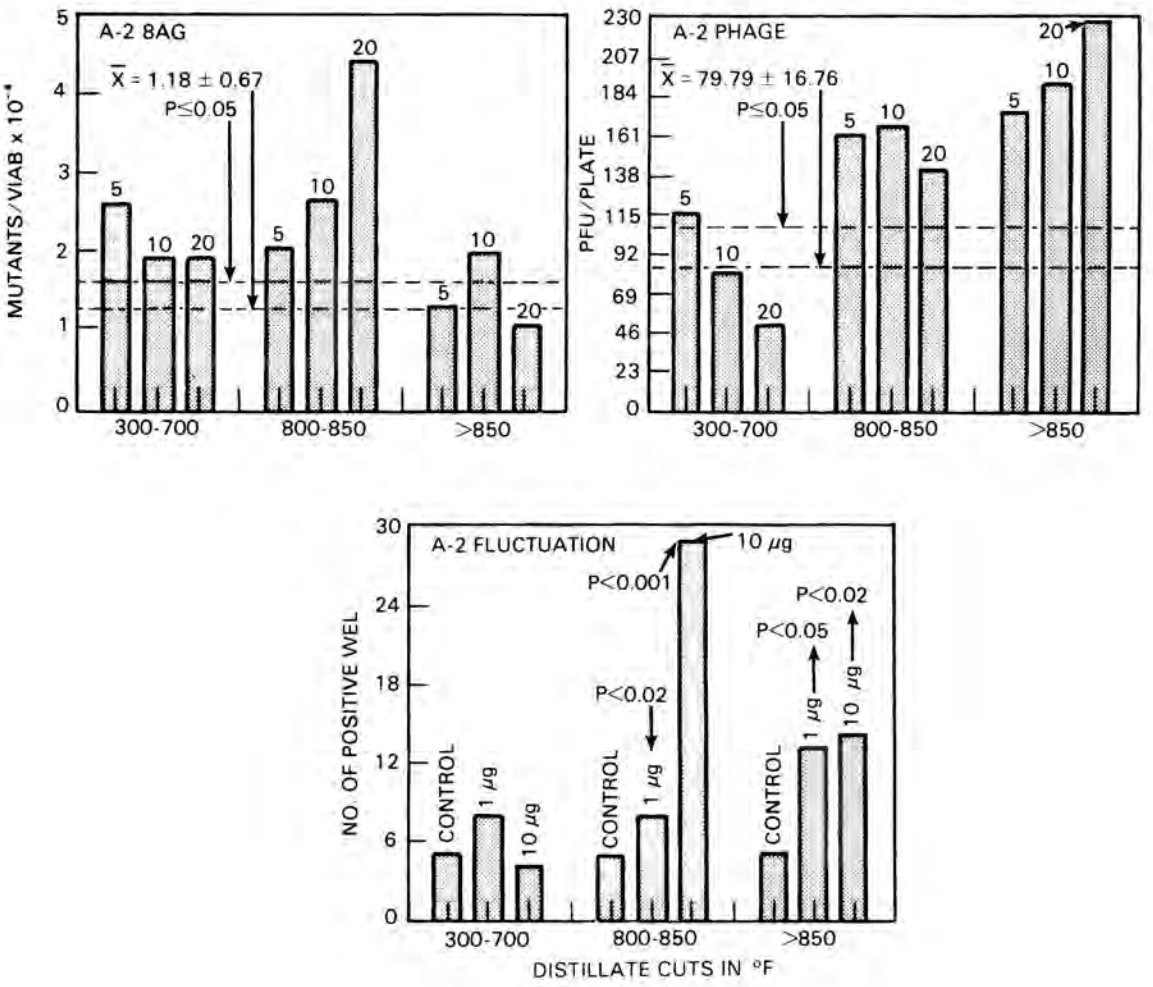

FIGURE 3.3. Response of the 8-Ag, Fluctuation and Phage Induction Assays to the Neutral PAH Fraction from the 300-700, 800-850° and >850°F Distillate Cuts. In the Fluctuation Test, the Control was Treated Only with Vehicle.

tems (Figure 3.4), although the 8-Ag assay did not show a very high response to the >850°F NPAC fraction. In all the microbial systems, response to the 300-700°F NPAC was only marginal.

We further fractionated the NPAC fractions which contained carbazoles, aromatic amines and azaarenes on silicic acid to separate these components. Figure 3.5 shows their responses in the standard Ames assay. The carbazoles and azaarenes from the 800-850°F cuts produced a significant mutagenic response; the aromatic amine fraction was eight to ten times more active. The silicic acid fractions from the 300-700°F cut produced no significant response.

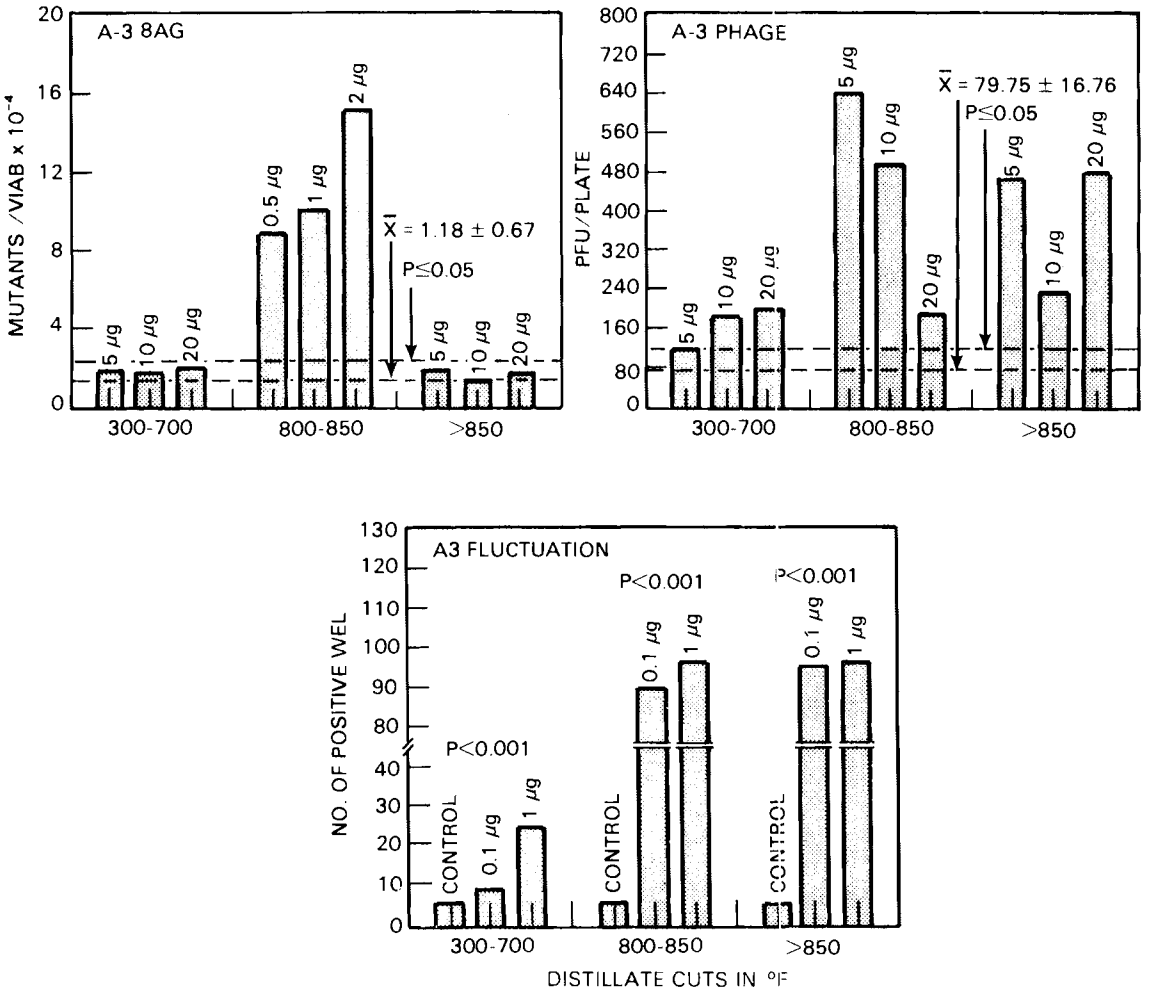

FIGURE 3.4. Response of the 8-Ag, Fluctuation and Phage Induction Assays to the NPAC Fraction from the 300-700°, 800-850° and >850°F Distillate Cuts. In the Fluctuation Test, the Control was Treated Only with Vehicle.

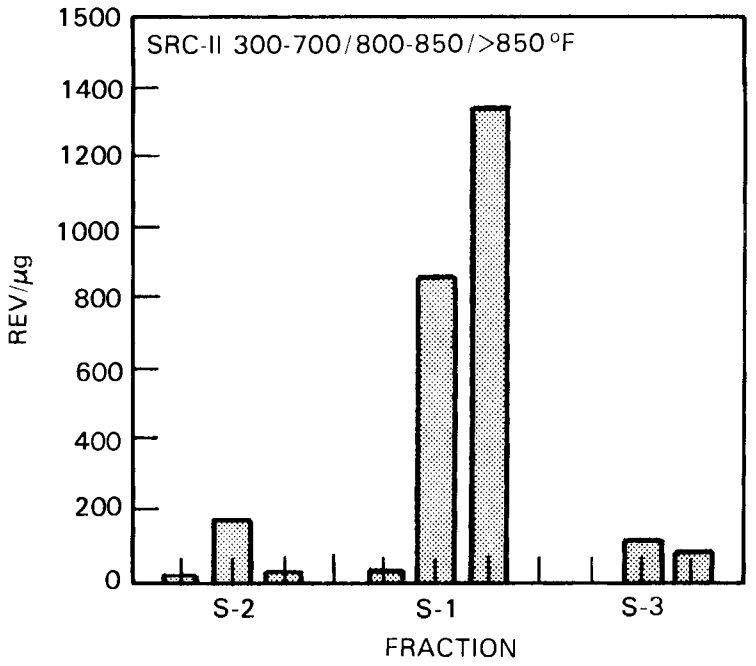

FIGURE 3.5. Response of the Ames Assay to the Carbazole (S1), APAH (S2) and Azaarene (S3) Fractions Prepared from the NPAC Fraction from the 300-700°, 800-850° and >850°F SRC-II Distillate Cuts.

4.0 MAMMALIAN-CELL ASSAYS

Two mammalian-cell (SHE transformation and CHO forward mutation) assays were used to assess the genotoxicity of the complex organic mixtures resulting from the SRC-II coal liquefaction process. In the transformation assays, SHE cells were exposed to test compounds and monitored to determine whether they underwent morphological and biological changes that resulted in unrestricted growth analogous to that of a cancer cell (transformation). Chemicals capable of transforming cells in this manner (in vitro) were considered to be candidate carcinogens. The mutation assay used CHO cells as the indicators for measuring forward mutation rates caused by exposure to these same or similar compounds.

4.1 STANDARD TRANSFORMATION ASSAY

The transformation assay is based on the method of Pienta (1977), as modified for use with complex mixtures of hydrocarbons by Frazier and Andrews (in press). In this procedure, primary cell cultures of SHE, strain ELA/ENG (Engle Labs), at 14 days of gestation, were prepared as described by DiPaolo et al. (1971). Cells were trypsinized after 2 days in culture (before monolayer was reached), and 3 x 10^3 cells were seeded into each 6-mm well of Linbro® quad plates (12 wells/group) containing 3 ml complete medium without antibiotics.

Twenty-four hours after seeding, the test chemical was added to the medium. Dilutions of the test chemical were made in DMSO, and 15 µl of the appropriate dilution were added to each well. The chemical was added to 12-16 plates/dilution. The cells were exposed to the test material in the presence or absence of activating enzymes. After 24 hours the reaction mixture was removed, and the cells were fed with fresh growth medium. Cells were then incubated for at least 8 days to allow colony formation, then fixed, stained and counted. Corresponding amounts of the solvent were added to the control plates; control plates were then handled in the same manner as the treated plates (exposure and refeeding).

4.2 CHO/HGPRT MUTATION ASSAY

A well characterized assay system for measuring mutation frequency in mammalian cells has been described by Hsie et al. (1978). Modification of that assay necessary to conduct our experiments included the use of activating enzymes prepared from rat liver homogenates (Kuroki et al. 1979) to detect compounds that required metabolic activation. A second modification involved the use of six-well Linbro® plates and, as a result, only 50,000 cells were seeded into each 24-mutant-selection petri dish. This reduction in cells was necessary to obtain optimal mutation frequencies. Otherwise, with 100,000 or more cells/60-mm petri dish, the expression of mutant cells was inhibited by nonmutant cells because of cooperativity between cells.

Each monolayered culture contained approximately 1 x 10^6 exponentially growing cells. Cells were rinsed with saline, and graded doses of test materials were added both with and without activating enzymes. Cells were incubated at 37°C for 5 hours. A typical experiment consisted of controls (positive, negative and solvent), as well as three doses of test compounds. Triplicate samples were tested for each dose.

Following exposure, cultures were rinsed, and cells were removed with trypsin. They were then washed to remove trypsin, counted, and 150 cells/well (six-well Linbro® chamber) were plated to determine cell survival. Remaining cells ($\sim 1 \times 10^6$) were plated in growth medium and incubated at 37°C. These cultures were transferred and diluted back to 1×10^6 cells at 2-day intervals. After an 8-day expression period, cells were again trypsinized, washed and counted. The cloning efficiency and mutation frequencies were then determined.

To obtain the cloning efficiency, each flask of cells was diluted, and one six-well plate was seeded with 150 cells; another plate was seeded with 300 cells/well. Cells were in 4 ml of hypoxanthine-free growth medium.

For mutant selection, each well (of the six-well plate) was seeded with 50,000 cells in 4 ml of selective medium (containing 6 µg/ml of 6-thioguanine). A total of 1.2×10^6 cells (24 wells) were screened for each data point. Plates were incubated at 37°C in a 100% humidified incubator in a 5% CO_2:95% air atmosphere. Dishes were fixed and stained (dilute crystal violet) after 7 days. Colonies were counted, and mutation frequency was calculated by dividing the total number of mutant colories by the number of cells/plate, corrected for the cloning efficiency.

4.3 ACTIVATING ENZYMES FOR MAMMALIAN-CELL ASSAYS

Hepatic microsomal enzymes were induced in Sprague-Dawley rats with Aroclor, according to the procedure of Kuroki et al. (1979). This method for preparing activating enzymes differs slightly from those used for microbial (Ames) assays. Rat livers were minced in the presence of HEPES-sucrose (HS) buffer (0.2 M sucrose and 2.0 mM HEPES, pH 7.4) and the cells were gently disrupted (by hand), using a dounce-type tissue homogenizer. The homogenate was checked, using a phase-contrast microscope, to ensure that cell cytoplasmic membranes were disrupted while most nuclear membranes remained intact. The resultant material was centrifuged at 9000 x g for 10 minutes at 4°C, and the supernatant was recentrifuged for 1 hour at 105,000 x g. The 105,000 x g pellet was resuspended in HS buffer, assayed for protein, diluted to 20 mg/ml, and divided into aliquots that were frozen at -80°C until used. This preparation was designated S15. Since activating-enzyme preparations can vary greatly in their properties, tests were conducted under a range of S15 concentrations and with a number of known mutagens (belonging to different chemical classes) to optimize mutation frequencies.

Reaction mixtures consisted of 1.5 ml of medium, 0.5 ml of activating enzymes and cofactors, and 15 µl of DMSO containing the appropriate concentrations of test material. To expose the cells, fresh culture medium was added to each culture, followed by a 0.5-ml mixture of liver enzymes and cofactors. Cofactors included: glucose-6-phosphate, NADP, Mg^{+++}, Ca^{+++}, and PO_4. The concentration of protein (rat liver activating enzymes) was 0.6 mg/ml. After gentle mixing, the test compound (in solvent) was added to each culture, and the culture fluids were mixed a second time.

4.4 RESULTS

A series of SHE transformation experiments were conducted to test and characterize the assay system. Figure 4.1 illustrates the main features and

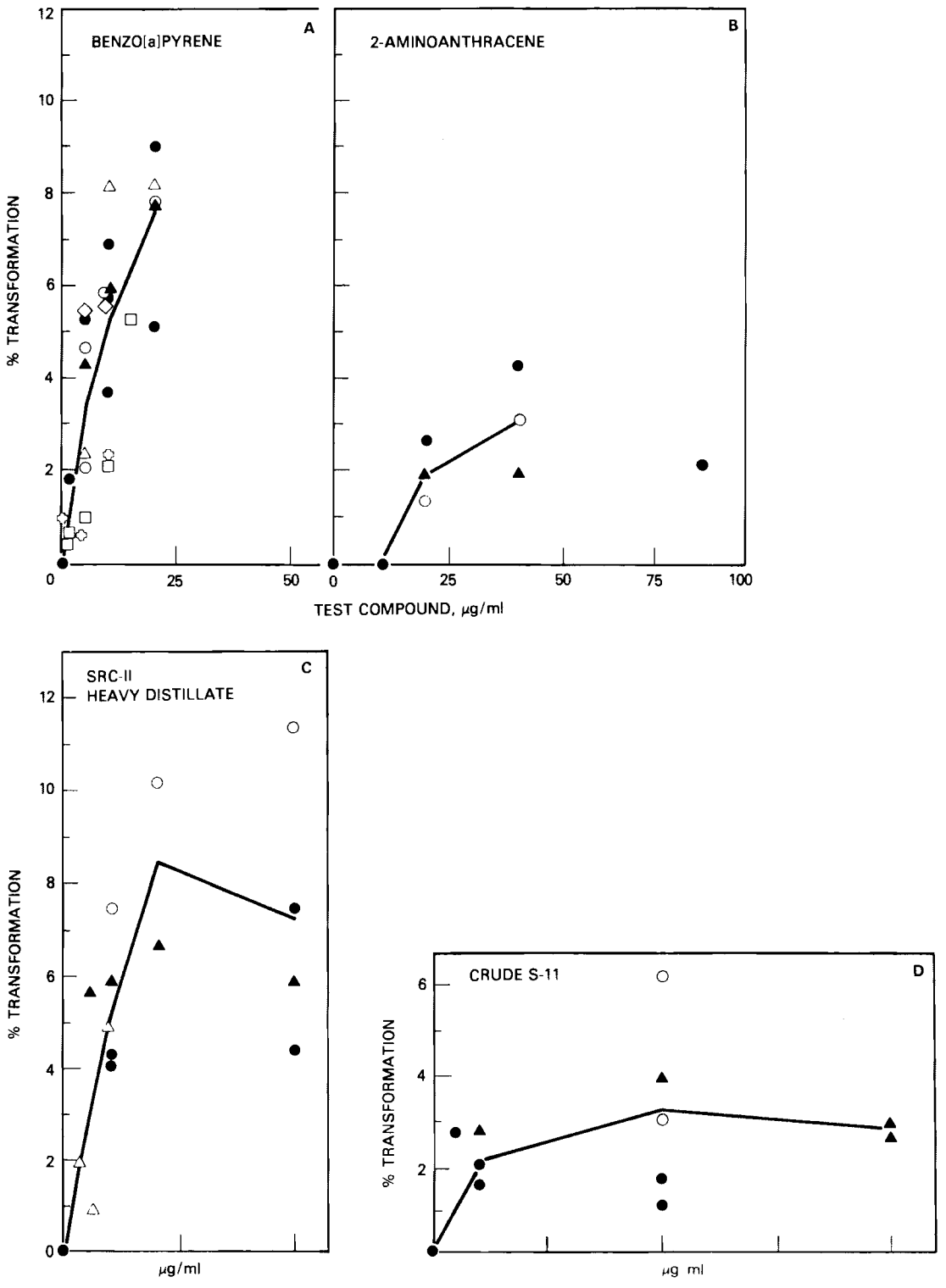

FIGURE 4.1. Response of the SHE Cell Transformation Assay to: A. BaP; B. 2-AA; C. SRC-II HD; and D. Livermore Shale Oil (S-11). The Different Symbols Represent Individual Data Points from Separate Experiments.

problems inherent in evaluating data from this assay. Figure 4.1A shows that a relatively good dose-response curve is obtained when a pure compound (BaP) is used and when the results of several experiments are pooled. Likewise, it is apparent that a linear dose response was obtained only in a relatively narrow dose range (1-10 μg/ml). Conversely, with 2-AA, the dose-response range (Figure 4.1B) was great, with an apparent threshold (minimum effective dose). Responses obtained with complex mixtures of organic compounds also varied widely. For example, SRC-II HD (Figure 4.1C) showed a reasonable dose response but only over a narrow dose range. In contrast, the shale oil caused transformation but without any apparent dose-related relationship (Figure 4.1D).

For complex organic mixtures, we generally found that doses of 10 μg/ml were either in the linear portion of the dose-response curve or near a maximum response (e.g., shale oil). We have therefore chosen the 10-μg/ml dose level to use in this report for comparing the various test materials, in order to facilitate summarizing a large amount of data.

Table 4.1 compares several crude products. High-boiling-range materials from both SRC-I process solvent (PS) and SRC-II HD were active in transformation assays. The lower- and middle-boiling-range SRC materials did not cause transformation even at doses much higher than 10 μg/ml (data not shown). However, because of the high volatility and insolubility of the lower-boiling-range fractions, our results may not be good indicators of genetic activity.

TABLE 4.1. Syrian Hamster Embryo Assay Results for Several Crude Petroleum, Coal, and Shale Liquids

Sample		% Transformation (at 10 μg/ml)
SRC-I	Wash solvent	0
	Light oil	0
	Process solvent	1.9
SRC-II	Light distillate	0
	Middle distillate	0
	Heavy distillate	5.9
Prudhoe Bay petroleum crude		0.2[a]
Wilmington petroleum crude		0.3[a]
Livermore shale oil		1.9

[a]Transformation at 100 μg/ml

Consistent but low transforming activity was detected for the two crude petroleums (Wilmington and Prudhoe Bay) tested. Since a characteristic of the

SHE cell assay is the lack of spontaneous transformation, these results suggest that these two crude oils contain candidate carcinogens.

Although the SHE transformation assay is not considered quantitative, the percent transformation provides an approximation of the activity to be expected relative to positive controls. Thus, it appears that SRC-II HD is a highly active transforming agent.

Following detection of transforming activity in crude materials, experiments were initiated with chemically fractionated materials to minimize interference from cytotoxicity and to separate and identify biologically active components in these complex mixtures. Initially, an acid-base solvent fractionation scheme (Pelroy 1981) was used. Standard Ames assays indicated that only the basic tar, neutral tar and basic fractions of PS, HD and shale oil were genetically active. These fractions were also active in the SHE transformation assay, as were the PAH and neutral fractions from SRC-II HD (Table 4.2). Likewise, PAH fractions from SRC-I PS and shale oil had considerable activity. These results indicated that there were genotoxic materials not being detected by the Standard Ames assay.

TABLE 4.2. Transforming Activity of Solvent-Extracted Fractions of SRC PS and HD and Shale Oil in SHE Cells

Sample	% Transformation (at 10 µg/ml)
Shale oil- crude	1.9
Basic fraction	2.5
PAH fraction	2.6
SRC-I PS - crude	2.6
Basic fraction	4.5
PAH fraction	1.7
SRC-II HD - crude	5.9
Basic fraction	4.4
PAH fraction	5.4
Neutral fraction	1.7
Basic tar	4.5
Neutral tar	3.1
BaP	5.1
2-AA (20 µg/ml)	2.0

Since earlier studies indicated no activity was associated with light and middle distillates, we attempted to segregate genetic activity by selective distillation. Experiments were conducted using SRC-II materials with various boiling ranges. Generally, genetic activity increased with increasing temperature (Table 4.3), with the highest-boiling SRC-II cuts having activity comparable to pure BaP. Similarly, the activity of SRC-I cuts increased with in-

TABLE 4.3. Syrian Hamster Embryo Cell Transforming Activity of SRC Distillate Cuts

Sample		% Transformation (at 10 µg/ml)
SRC-II	>850°	5.8
	800-850°	3.3
	300-700°	0.4
SRC-I	800-850°	1.5
	700-750°	0.6
	BaP	5.7
SRC-II	387-700°	0.2
	700-750°	0.3
	750-800°	3.7
	800-850°	6.8
	>850°	8.9
	BaP	10.3
SRC-II	639-689°	0.2
	705-825°	0.5
	710-730°	0.6
	739-761°	0.7
	645-782°	1.4
	782-878°	1.5
	867-967°	5.5
	914-1055°	8.5
	BaP	6.5
	2-AA	2.2

creased bp; however, the relative activities of the SRC-I samples were lower than those for SRC-II materials.

Distillate cuts were separated into chemical class fractions to determine the classes of compounds responsible for the observed genetic activity. Results from microbial assays indicated that a fractionation scheme which utilized alumina and silicic acid to separate compounds primarily on charge produced most of the genetic activity in the NPAC fraction. However, when these same SRC-II distillate-cut (bp > 850°F) fractions were assayed using SHE cells, transforming activity was present in all four alumina fractions as well as in the APAH and azaarene subfractions.

To identify the active components, we further fractionated SRC-II material using high-performance liquid chromatography (HPLC). In this scheme, aliphatic hydrocarbons and PAH are eluted in Fractions 1 and 2. Fractions 3, 4 and 5 contain mostly carbazoles and azaarenes, with the highest concentrations of APAH found in Fraction 5. Fraction 6 does not contain much material although some monohydroxy PAH elute in this region. Hydroxy and multihydroxy PAH make up most of Fractions 7 and 8. Higher-molecular-weight multiheteroatomic (containing N, S and O) materials elute in Fraction 8. Table 4.4 shows

TABLE 4.4. Syrian Hamster Embryo Cell Transformation in Chemical Class Fractions Prepared by HPLC from SRC-II bp Cuts

Sample	% Transformation (at 10 µg/ml)
SRC-II >850 crude	5.8
#1	9.3
#2	8.8
#3	4.7
#4	2.4
#5	0.5
#6	0
#7	0
#8	0.8 (at 4 µg/ml)
SRC-II 800-850 crude	2.0
#1	3.3
#2	1.9
#3	1.4
#4	0.8

that this separation scheme resulted in better segregation of transforming activity. For example, there appears to be considerable activity in Fractions 1 and 2, which are rich in PAH compounds, with sequentially decreasing activity in Fractions 3, 4 and 5. Fractions 6 and 7 do not have detectable activity. A similar pattern (although lower activity) was observed with the 800-850° bp SRC fractions.

We also evaluated whether hydrotreatment used in the production process to upgrade the SRC-II materials would affect the transforming activity. The results of the SHE transformation assay, which were not quantitative, indicated a decrease in genetic activity following hydrotreatment (Table 4.5). Interestingly, the data suggest that moderate hydrotreatment had a greater effect than severe hydrotreatment.

TABLE 4.5. Effect of Hydrotreatment on SHE Cell Transforming Activity of an SRC-II Fuel Oil Blend

Sample	% Transformation (at 10 µg/ml) Experiment 1	Experiment 2
Feedstock (SRC-II)	2.5	4.7
Moderate hydrotreatment	1.1	1.3
Severe hydrotreatment	2.1	1.9
2-AA (20 µg/ml)	3.4	2.4
BaP (5 µg/ml)	8.6	---

Most of the SRC-II samples were examined using the SHE assay. Recently, the CHO mutation assay was adopted in our laboratory (after many of the SHE experiments were completed). It appears that the CHO assay, as modified and adapted for use in our laboratory, has several advantages over the SHE assay. First, the endpoint (mutation) is an objective determination, while cell transformation is a subjective endpoint. Secondly, the CHO assays provide quantitative dose responses with virtually every compound or mixture we have examined. Thus, we can analyze data by linear regression and express the results as mutants/μg of test material. The results from numerous CHO experiments with positive control compounds are shown in Table 4.6. These compounds were chosen because they represented classes of chemical compounds which are of interest. The relevant pure compounds were included as positive controls and to show that the assay system was capable of detecting those mutagens.

TABLE 4.6. Mutagenicity of Some Selected Chemicals in the CHO Cell Assay (Average from Multiple Experiments)

Sample	Mutants/μg	Range of Activity, μg/ml
2-AA	1.6	20-80
6-AC[a]	2.2	10-50
BaP	11.2	5-15
Dimethylacridine	10.6	10-50
Thiophene	0	
Acetylaminofluorene	0.4	

[a] 6-Aminochrysene

Results for all of the SRC-II materials tested in the CHO assay in our laboratory are summarized in Table 4.7. It is noteworthy that the results are consistent with earlier findings in the SHE assay. More specifically, one can see that the higher-boiling (>850°F) fraction was significantly more active than the 800-850°F fraction. The HPLC fractionation scheme provided a good first step for separating the active components, with the most activity produced in Fractions 2 and 3. Fraction 4 had considerable activity; Fractions 5 and 6 had little. The HPLC fractionation of 800-850°F boiling-range material produced the same pattern of activity, apparently confirming these results. Most importantly, the CHO assay (like the SHE assay) detected considerable activity in HPLC Fractions 1 and 2, which had no genetic activity in the standard Ames assay. The CHO data also confirmed SHE data, indicating that hydrotreatment decreases but does not eliminate genetic activity.

We also attempted to determine whether biological activity was being lost as a result of fractionation by calculating the total activities on a percent-by-weight basis (Table 4.8). Our calculations indicate that the activity detected in the individual fractions may be two to three times that observed in the materials from which the fraction were derived. We therefore conclude that the expression of genotoxicity was inhibited in the crude complex mixtures.

TABLE 4.7. Mutagenicity of Coal Liquids in the CHO Cell Assay

Sample		Mutants/μg	Range of Activity, μg/ml	Correlation Coefficient
800-850°F Crude		0.6	1-25	0.87
	HPLC #1	3.5	1-10	0.6
	#2	2.9	1-10	0.75
	#4	2.1	1-10	0.72
>850°F Crude		1.4	5-20	0.97
	HPLC #1	3.6	1-10	0.92
	#2	6.5	1-25	0.93
	#3	3.3	5-50	0.99
	#4	2.1	5-25	0.92
	#5	0.3	25	ISD[a]
	#6	0.7	5-25	0.96
	#7	0	5-50	1.0
	#8	+	5-10	ISD[a]
Alumina >850°F				
	A1	0.9	1-10	0.9
	A2	4	1-25	0.82
	A3	1.8	1-15	0.7
	A4	1.7	1-10	0.73
Feedstock SRC-II		0.5	2-8	0.87
Hydrotreated feedstock		0.2	7.5-30	0.83

[a]Insufficient number of data points

TABLE 4.8. Weighted Mutagenic Activity Prepared by Separation of HPLC Fractions from the SRC-II >850°F Distillate Cut

Sample		Average Mutants/ μg Test Material	% of Total	Total Mutants	% Activity
>850°F Crude		1.4	100	140	100
HPLC	#1	3.6	38	136.8	97.7
	#2	6.5	7	45.5	32.5
	#3	3.3	16	52.8	37.7
	#4	2.1	10	21	15
	#5	0.3	10	3	1.5
	#6	0.74	14	10.4	7.4
	#7	0	4	0	0
	#8	(1.1)	1	1.1	0.8
TOTAL	--	--	100	270.5	193.2

5.0 INITIATION/PROMOTION (I/P) STUDIES

Work by Calkins et al. (1982) suggests that the I/P system may be useful for screening coal-derived complex mixtures for skin carcinogenis. The I/P assay depends on the concept that skin tumorigenesis consists of at least two events, initiation and promotion. Initiation occurs rapidly, is essentially nonreversible, and likely involves chemical interaction with DNA. Promotion is reversible and requires repeated application of the promoting agent. The concept of I/P may be demonstrated experimentally by applying a single, small dose of an agent such as dimethylbenzanthracene (DMBA) to mouse skin. If the initial application is followed by repeated applications of a promoter, such as phorbol myristate acetate (PMA), tumors develop. If no promoter is used, or if an initiator does not precede application of the promoter, no tumors develop. Complete carcinogens, such as BaP and DMBA, are capable, if high enough doses are used, of both initiation and promotion. However, initiation occurs at smaller doses than those required to promote. Moreover, the PMA is a more effective promoter than equivalent amounts of BaP or DMBA.

We screened numerous coal-derived liquids for their ability to initiate skin tumorigenesis. Many of these have also been assayed in chronic skin-painting studies as well as in many of the in vitro tests.

5.1 GENERAL METHODS

Charles River CD-1 mice were housed, five/cage, on standard bedding material (Sani-Cel, Paxton Processing Co., Paxton, Illinois) with food (Wayne 4% Lab-Blox, Allied Mills, Libertyville, Illinois) and tap water available ad libitum. Prior to administration of the test compound, the animals were ear-tagged for unique identification, then weighed and shaved. Test material was applied to the shaved backs of the mice in a 50-µl volume. Two weeks after initiation, 50 µl of PMA (0.1 mg/ml of acetone) were applied to the initiated area twice weekly for 6 months. The mice were shaved as necessary throughout the study, usually once/week. Animals were observed regularly for tumor growth. The rate of tumor development, the total incidence of tumor-bearing mice, and the number of tumors/tumor-bearing mouse were used to evaluate the activity of the test material.

Materials representing a range of biological activities, as measured in the Ames, SHE, and chronic skin-painting assays, were chosen for this experiment. The materials chosen were SRC-II HD (550 to 850°F), and the basic (BF), basic tar (BTF), neutral tar (NTF), and polynuclear aromatic (PNA) fractions prepared from HD by solvent-extraction. In addition, SRC-II distillates boiling from 300 to 700°F and from 800 to 850°F were tested. The HD, BF, NTF, BTF, PNA fractions, and the 300 to 700°F bp cuts were applied undiluted. The 800-850°F distillate was diluted in acetone (1.0 g/ml) to facilitate application. This represented approximately 55 mg of HD, 50 mg of BF, NTF, BTF, PAH, and 300 to 700°F fractions and 25 mg of the 800 to 850°F cut. Groups of animals initiated with 50 µg of DMBA or BaP served as positive controls. The negative controls received 50 µl of acetone. Two weeks later, all mice received twice-weekly applications of 5 µg of PMA for 24 weeks.

5.2 RESULTS

Although both BaP- and DMBA-initiated mice developed papillomas after promotion with PMA, DMBA was much more active as judged by tumor incidence (Figure 5.1), and total tumor yield (Figures 5.2A and B). Initiation with HD resulted in tumor incidence and tumor yield similar to that of BaP. The NTF was nearly as active an initiator as HD, while the BTF was slightly less active. Mice initiated with either the BF or PAH fractions developed tumors more slowly than the HD, NTF, or BTF groups. The 800 to 850°F distillate cut showed low initiating activity, while the response to the 300 to 700°F cut was the same as to the acetone-initiated controls.

Although one dose of a material was usually tested for initiating activity, we also tested several doses of HD to establish the dose-response relationship. We plotted tumors/group (normalized to 30 mice) after 185 days versus dose (Figure 5.3) and indicated the best line fit.

Additional distillate cuts prepared from an SRC-II fuel oil were evaluated for their initiating activity. Cuts boiling from 700-750°, 750-800°, 800-850°F and the full-boiling range (300-850°F) material were diluted 1:1 with acetone, and 50 µl of the diluted material were applied to the shaved backs of female CD-1 mice. In addition, 50-µg doses of the aromatic amines, 2-AA, 6-aminochrysene (6-AC), 1-aminopyrene (1-AP), and 9-aminophenanthrene (9-APh) were tested for initiating properties, since the microbial studies had demonstrated that most of the Ames mutagenic activity of coal liquids was due to APAH. Mice receiving BaP or DMBA were again included as positive controls. As before, promotion with twice-weekly applications of 5 µg of PMA was begun 2 weeks after initiation.

Tumor incidences in mice initiated with fractions distilled from SRC-II liquid are shown in Figure 5.4. A significant ($P > 0.05$) response was found in all groups, with the 700-750° and 750-800°F cuts producing similar responses (approximately 35 to 40% incidence) after 24 weeks of promotion with PMA. Responses to the 800 to 850°F cut and to the full-boiling-range material (300 to 850°F) were greater than those for the lower-boiling fractions, as indicated by both the final tumor incidence (60%) and total tumor yield (Figure 5.5). The median tumor latency was approximately 150 days for the 800 to 850°F and the 300 to >850°F groups. Responses for all SRC groups, whether measured by tumor incidence or total tumor yield, were less than for the two positive controls, DMBA and BaP.

Results from the groups initiated with the APAH (2-AA, 9-APh, 1-AP, or 6-AC) are shown in Figures 5.6 and 5.7. Responses shown by the groups initiated with 2-AA and 6-AC were similar to those for the 800 to 850° and 300 to >850°F SRC-II materials, as well as to those for PS and its 750 to 800°F cut. The activity of 9-APh was substantially lower and that for 1-AP was not significantly above background.

Since the 800 to 850° and >850°F distillate cuts were the most active as initiators, they were separated into chemical classes by the alumina method as outlined in Section 1.0. These fractions were tested for initiating activity by applying doses of each according to their weight percentage in the original material. For example, 17-mg doses of the distillate cuts were used as refer-

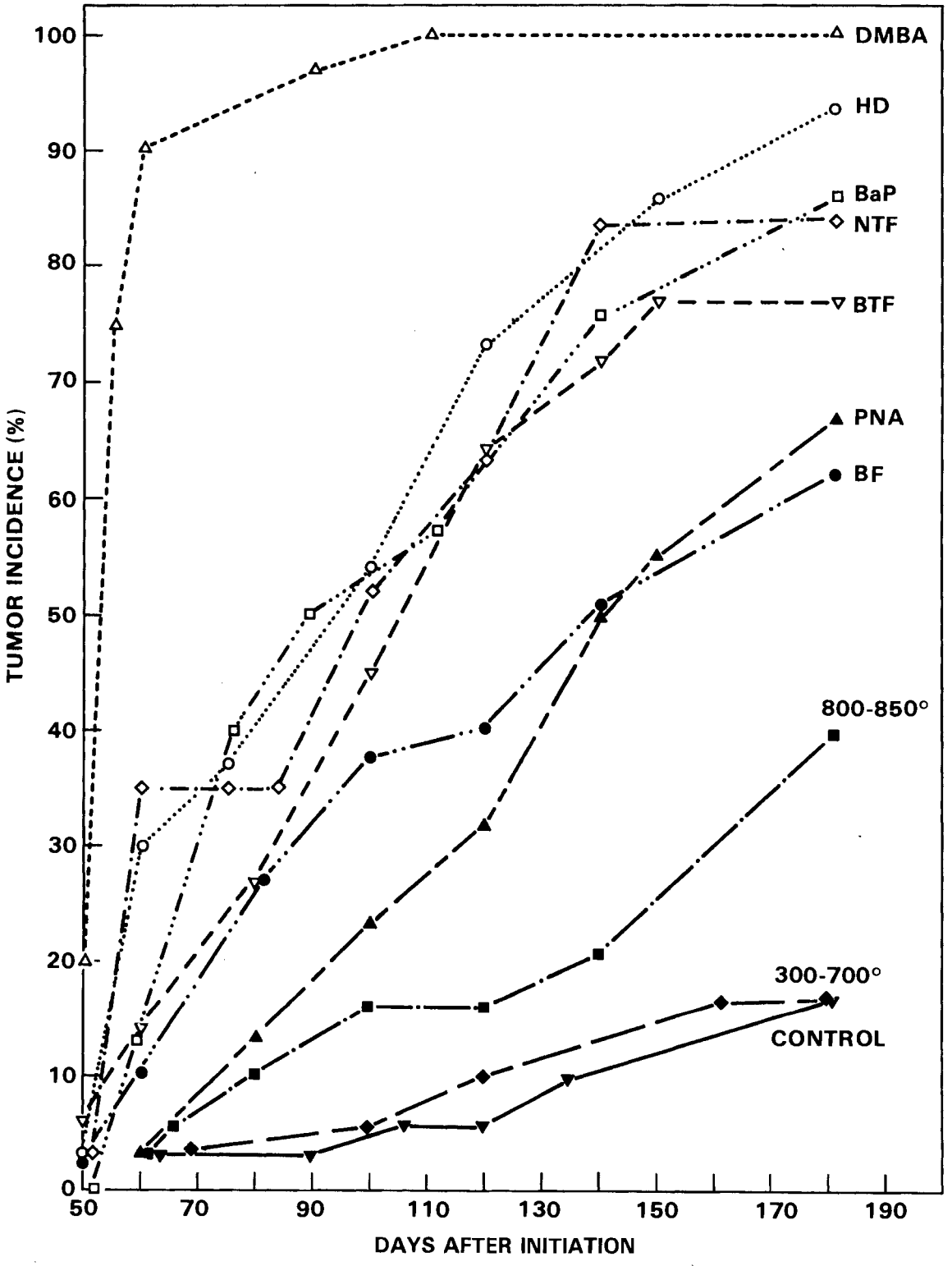

FIGURE 5.1. Tumor Incidence after Initiation with DMBA, BaP or Coal-Derived Liquids. The Designations are: HD, BF, BTF, NTF and PNA (PAH), the Basic, Basic Tar, Neutral Tar and Polynuclear Aromatic Fractions of HD. The bp Cuts are Designated by their Boiling Ranges, 300-700°F and 800-850°F.

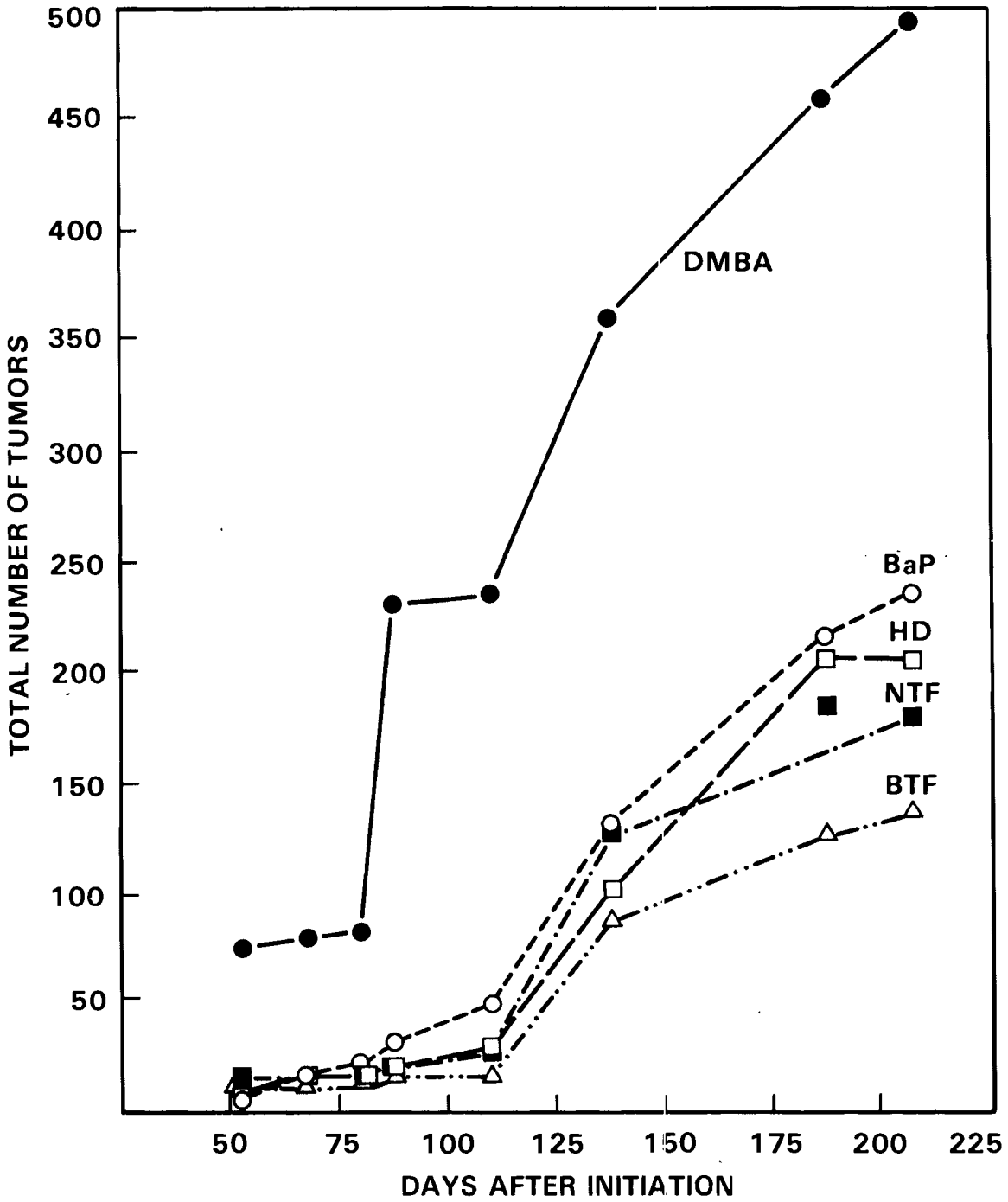

FIGURE 5.2A. Cumulative Skin Tumor Yield (Normalized to 30 Mice/Group) after Initiation with: A. DMBA, BaP, HD, or BTF and NTF Prepared from HD.

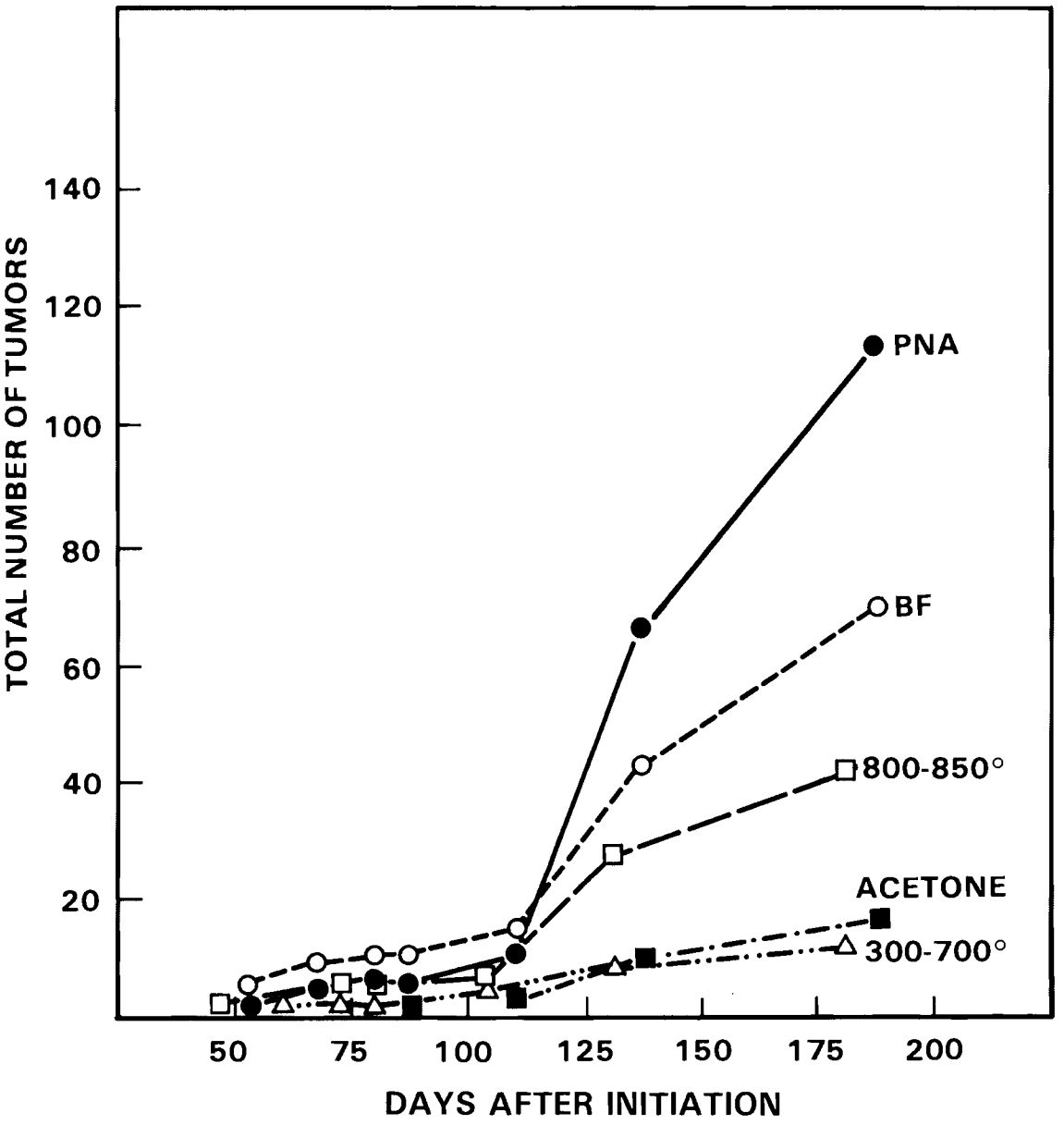

FIGURE 5.2B. Cumulative Skin Tumor Yield (Normalized to 30 Mice/Group) after Initiation with: B. PNA or BF from HD, or the 300-700°F and 800-850°F SRC-II Distillates.

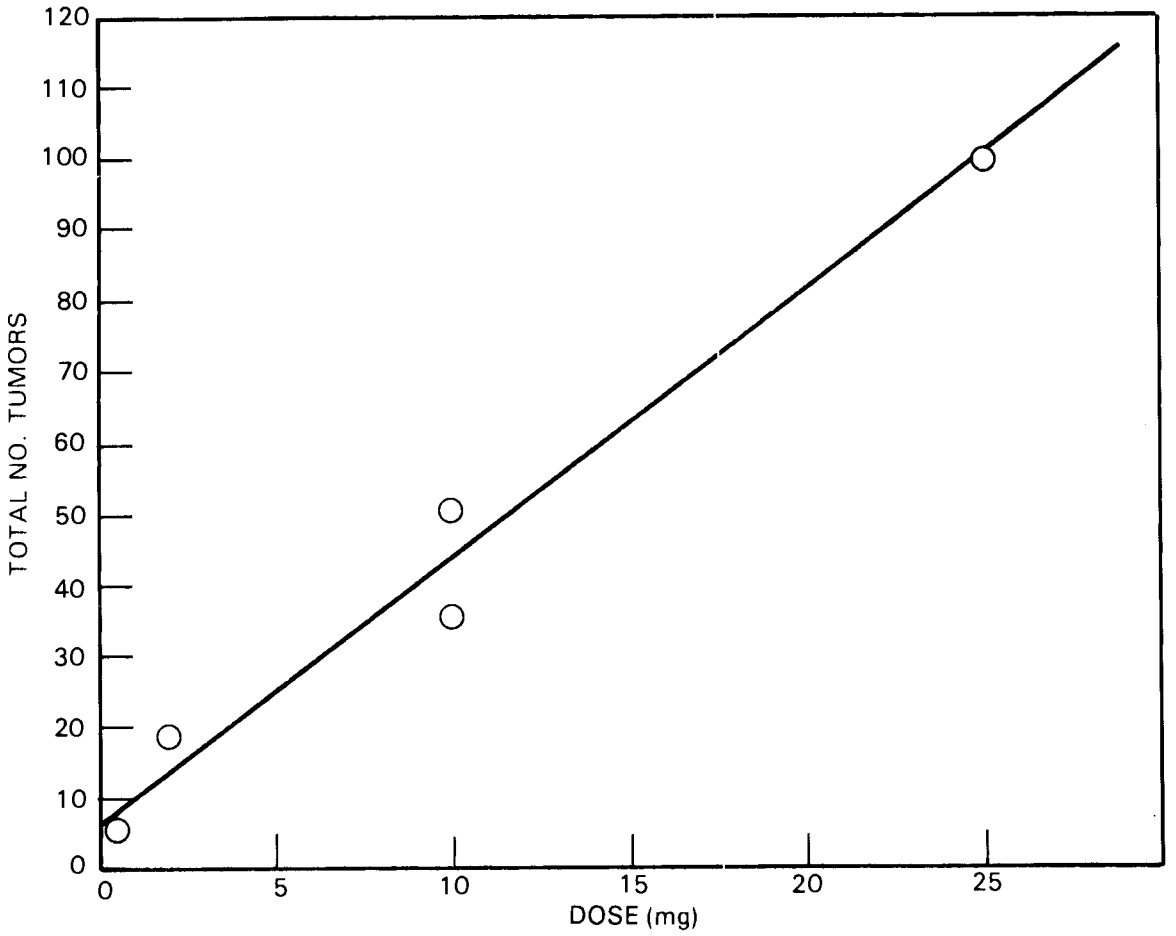

FIGURE 5.3. Dose Response for Initiating Activity of SRC-II HD.

ence materials. Since the neutral PAH fraction comprised about 50% of the cut, approximately 8.5-mg doses of the neutral PAH fractions were used.

The data presented in Figures 5.8 and 5.9 show the tumor yield from this experiment. In each case, the neutral PAH fraction was more active than the parent material. Significant ($P < 0.05$) but lower activity was found for the NPAC fraction. The aliphatic and hydroxy PAH fractions from the >850°F cut showed slight activity while those from the 800 to 850° cut were inactive.

The initiating activity of the NPAC fraction could be due to azaarenes, carbazoles or aromatic amines. It has been shown (Pelroy and Stewart, 1981) that treatment of the NPAC fraction with nitrous acid destroys the mutagenic activity by converting APAH to their corresponding diazonium salts or phenols. We therefore examined the initiating activity of the NPAC fraction from the > 850°F cut after nitrous acid treatment to estimate the contribution of APAH to the overall initiating activity of these materials. Results, shown in Figure 5.10, indicate a substantial loss of activity after nitrous acid treatment, suggesting that the APAH are responsible for most of the activity of the NPAC fraction.

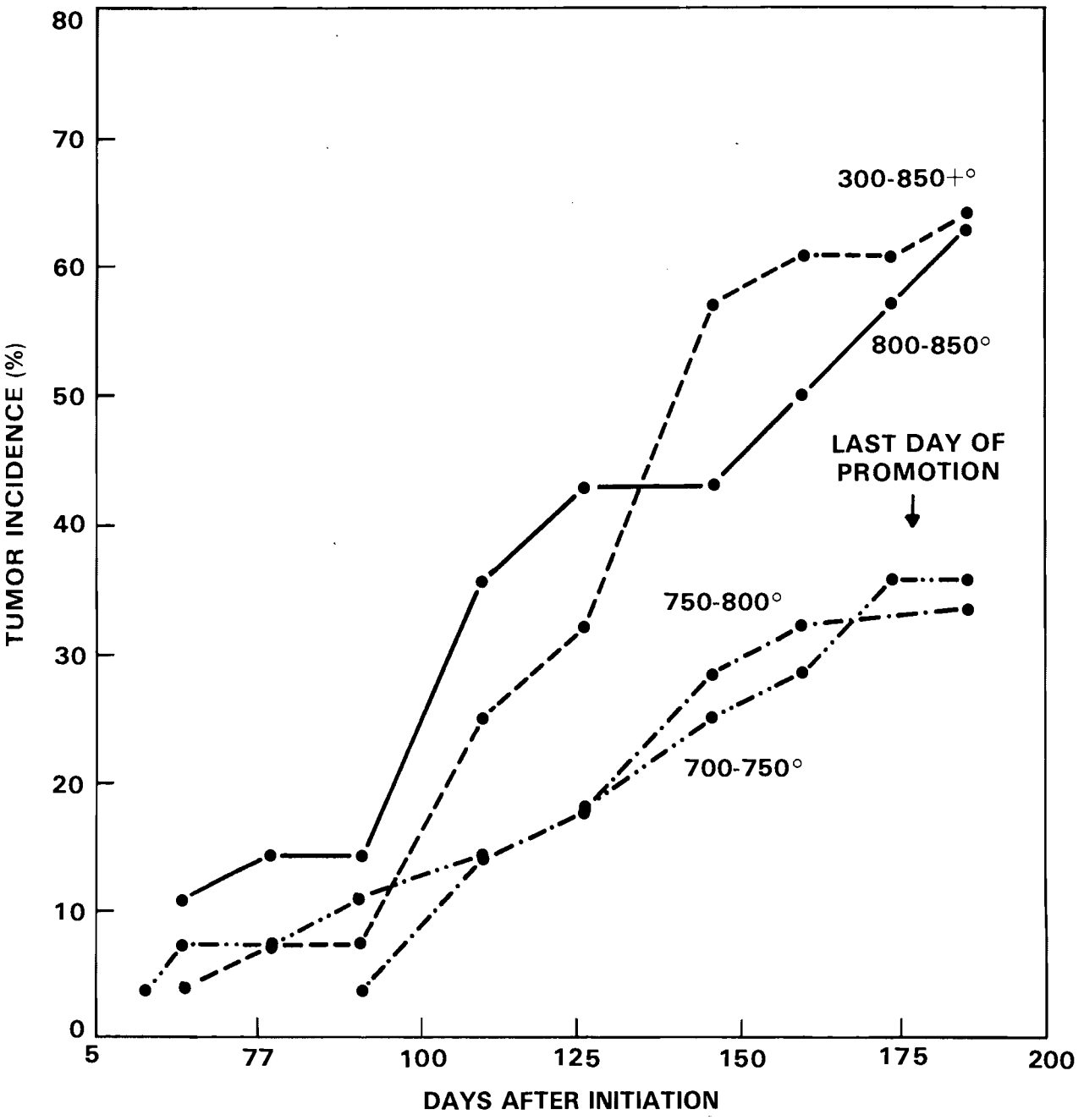

FIGURE 5.4. Tumor Incidence after Initiation with SRC-II bp Cuts.

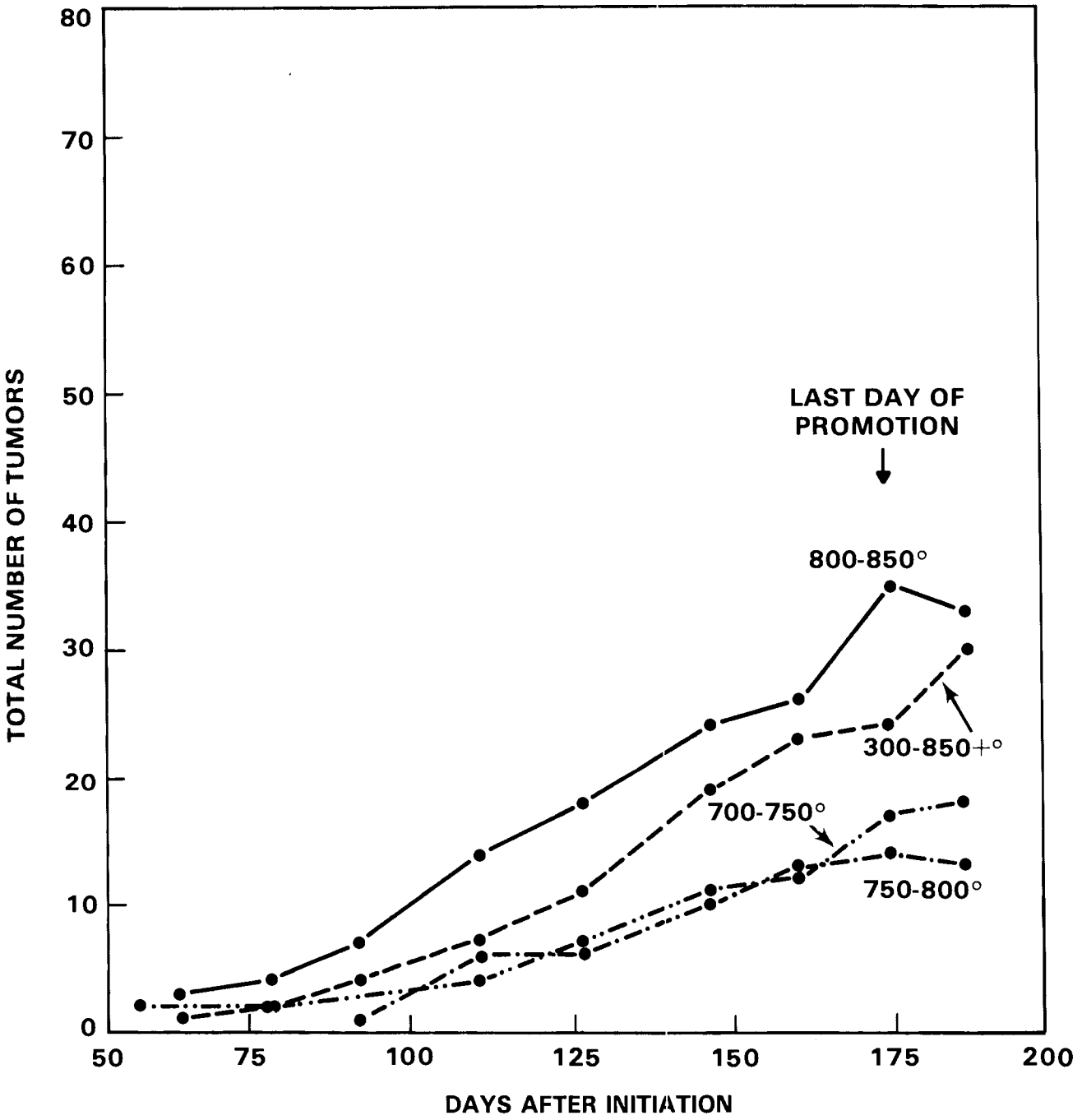

FIGURE 5.5. Total Tumor Yield (Normalized to 30 Mice/Group) After Initiation with SRC-II bp Cuts.

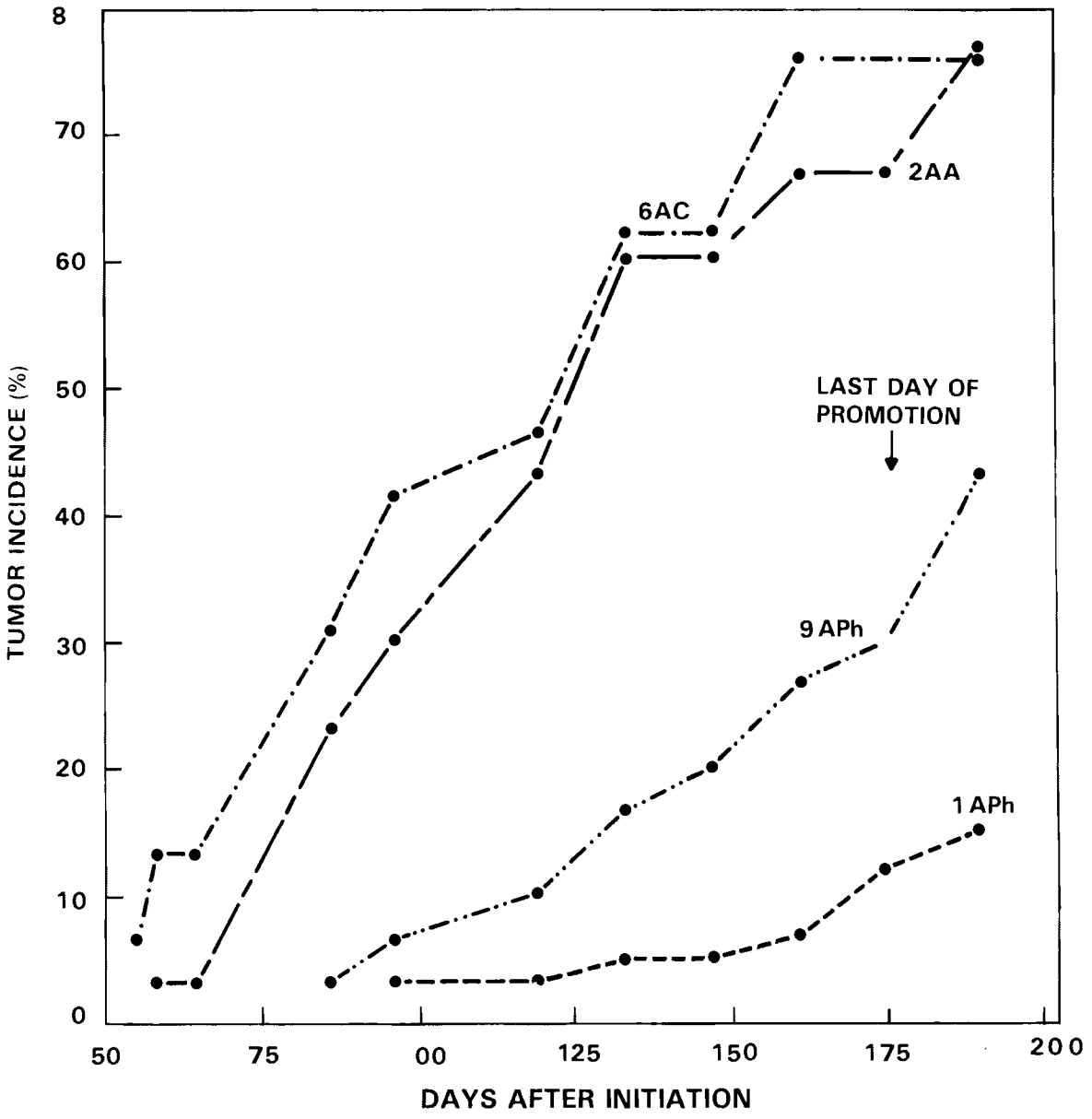

FIGURE 5.6. Tumor Incidence after Initiation with 2-AA, 6-AC, 9-APh or 1-AP.

Moderate hydrotreatment of an SRC-II fuel oil blend (FOB) reduced its microbial mutagenicity by over 90%. Mammalian mutagenicity was also reduced but to a lesser degree. We compared the tumor response of mice initiated with 25 mg of FOB or severely hydrotreated FOB to determine whether the initiating activity was affected by hydrotreatment. Untreated FOB had substantial initiating activity while the hydrotreated FOB contained only slight activity (Figure 5.11).

It had been reported elsewhere that chronic application of a hydrotreated coal liquid resulted in skin dermatitis and kidney damage. These indications that hydrotreated coal liquids were irritants suggested that they might be

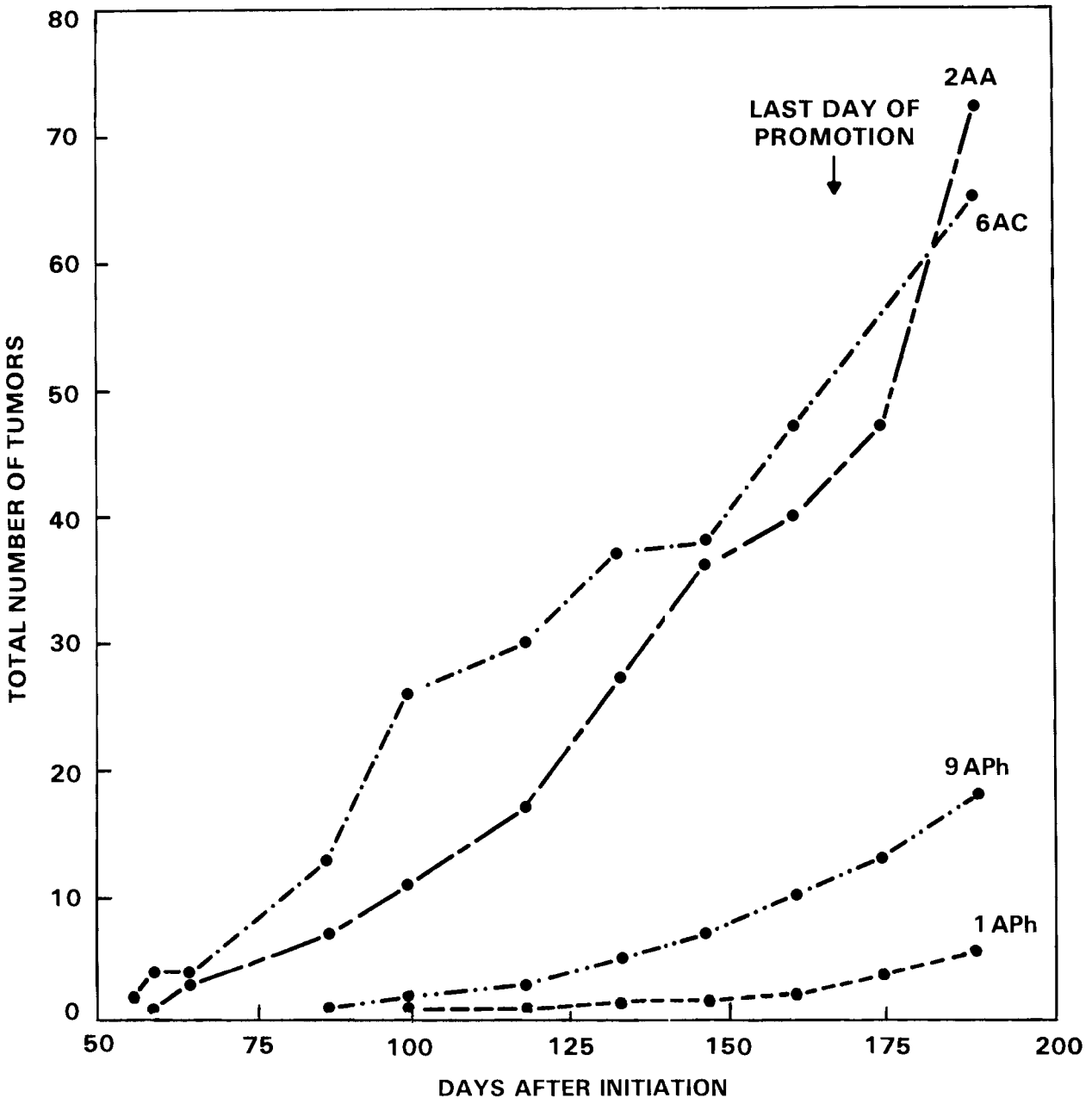

FIGURE 5.7. Total Tumor Yield (Normalized to 30 Mice/Group) after Initiation with 2-AA, 6-AC, 9-APh or 1-AP.

promoters. We therefore evaluated hydrotreated FOB for its ability to promote tumorigenesis after initiation of the skin with DMBA. We initiated with 50 µg of DMBA and, after 2 weeks, promoted twice weekly with hydrotreated FOB diluted with three parts of acetone. Results, shown in Figure 5.12, indicate the presence of promoting activity in hydrotreated FOB, although at the concentration used, the activity was substantially lower than for PMA. Untreated FOB was not tested for promoting activity since it was known to be a complete carcinogen (i.e., contained both initiating and promoting activity). However,

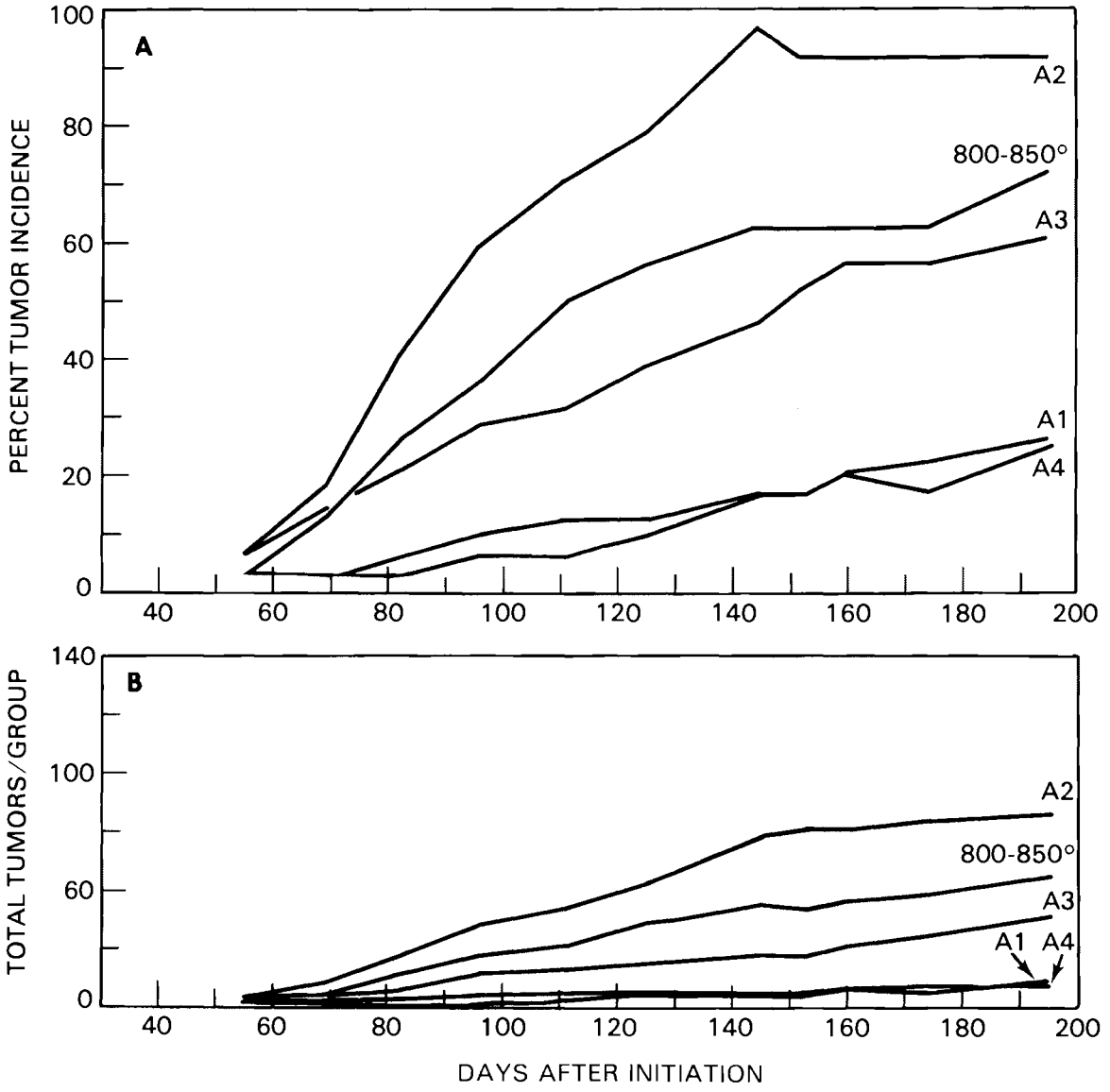

FIGURE 5.8. Mouse-Skin Tumor Response after Initiation with Chemical Class Fractions Prepared from the SRC-II 800 to 850°F bp Cut. A1 = Aliphatics and Olefins; A2 = Neutral PAH; A3 = NPAC; A4 = HPAH. A Dose of 17 mg of the bp Cut was Used; the Fractions were Applied in the same Proportion as They were Found in the Cut (see Table 2.1). Negative Controls Received Methylene Chloride:Acetone as an Initiator. All Mice were Promoted Twice Weekly with 5 µg of PMA in 50 µl Acetone. A. Percent Tumor Incidence Versus Time. B. Cumulative Skin Tumor Yield (Normalized to 30 Mice/Group) Versus Time.

that does not affect the conclusion that hydrotreated FOB still contains promoting activity.

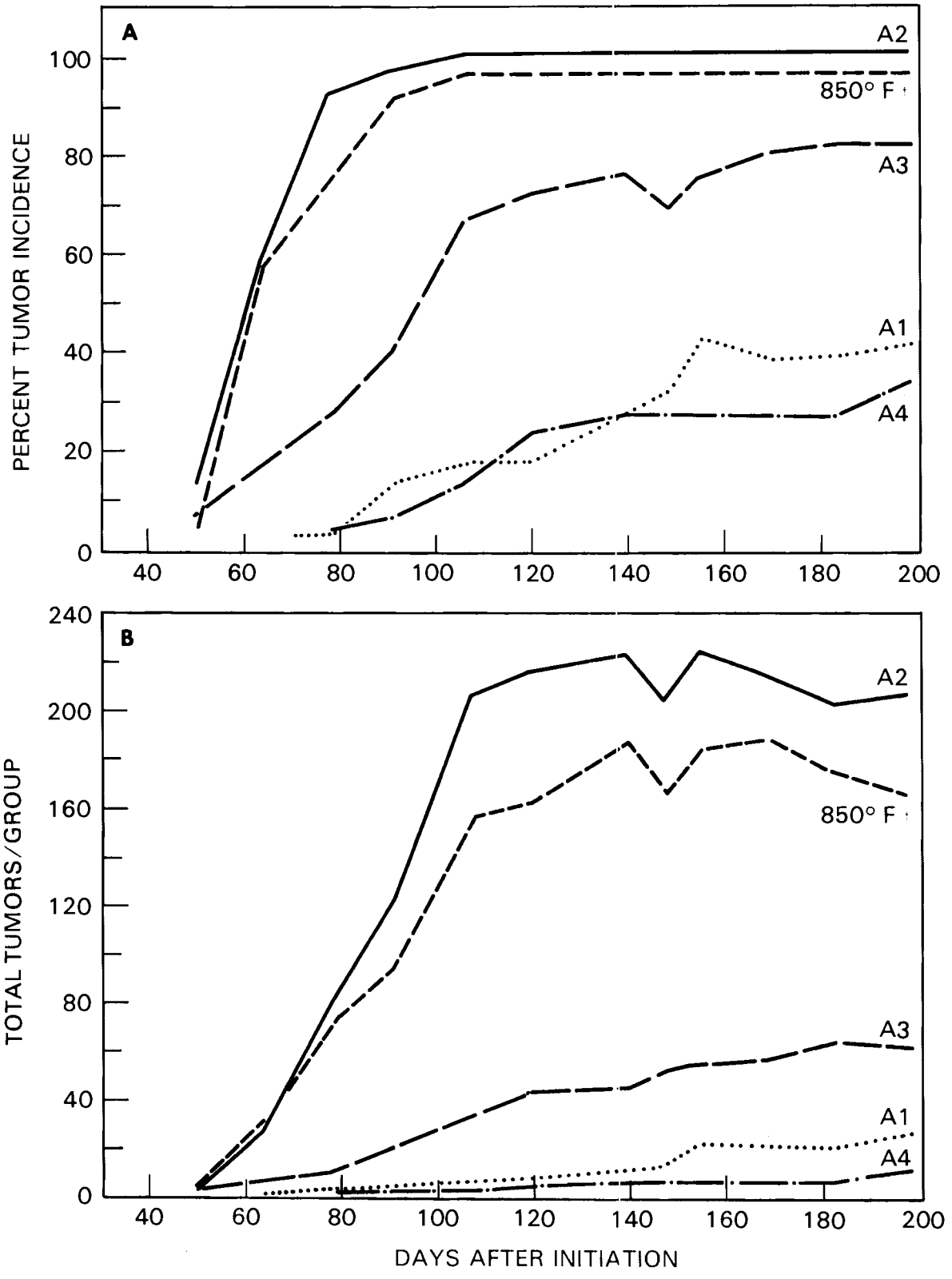

FIGURE 5.9. Mouse-Skin Tumor Response after Initiation with Chemical Class Fractions Prepared from the SRC-II >850°F Distillate Cut (see Figure 5.8 for Fraction Nomenclature). A. Percent Tumor Incidence Versus Time. B. Cumulative Skin Tumor Yield (Normalized to 30 Mice/Group) Versus Time.

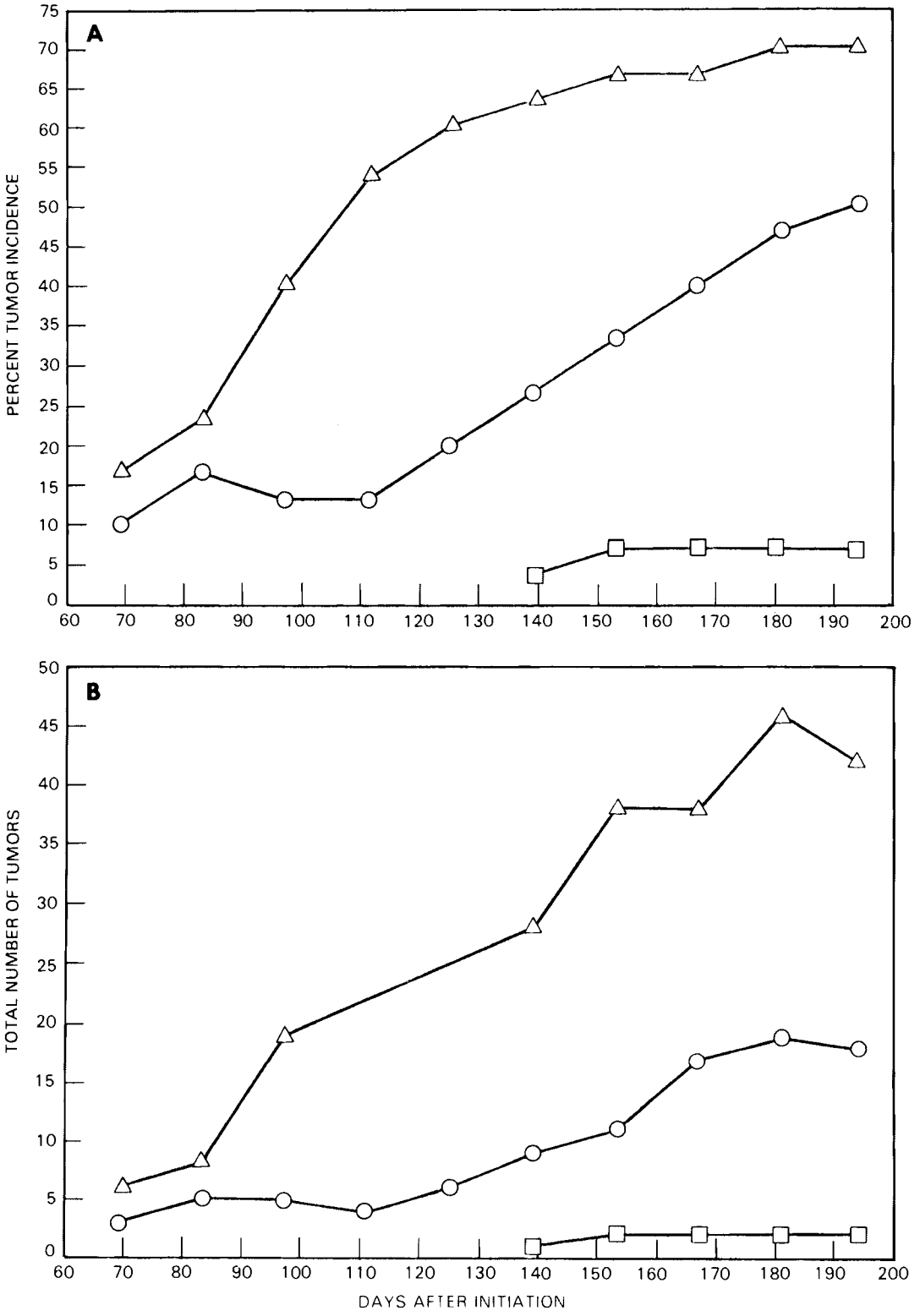

FIGURE 5.10. Effect of Nitrosation on the Skin-Tumor-Initiating Activity of the NPAC Fraction Prepared from SRC-II >850°F bp Cut. A. Percent Tumor Incidence and B. Total Tumor Yield (Normalized to 30 Mice/Group). □ = Acetone Control; Δ = Untreated NPAC; O = Nitrosated NPAC.

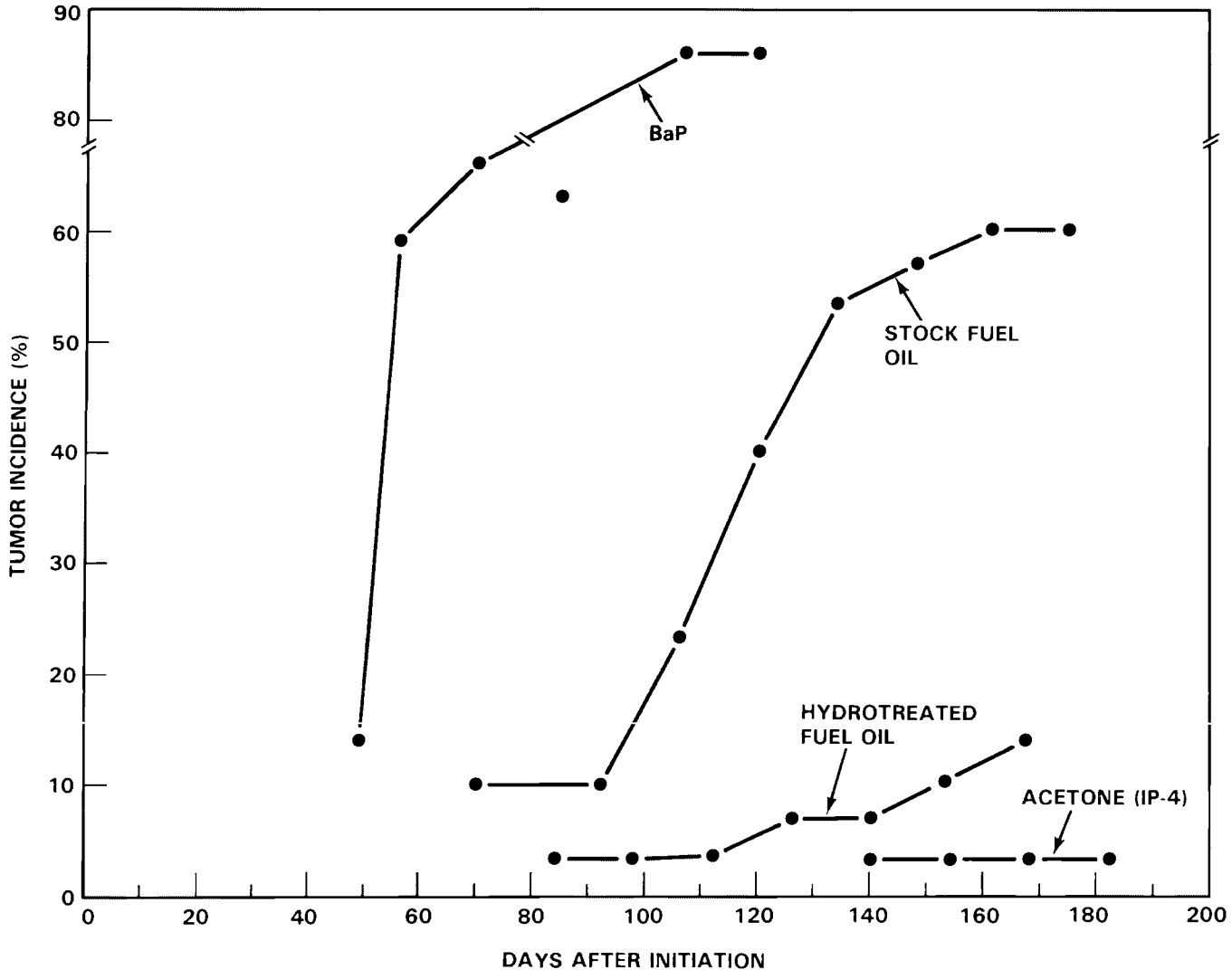

FIGURE 5.11A. Effect of Hydrotreatment on the Skin-Tumor-Initiating Activity of an SRC-II FOB. A. Percent Tumor Incidence.

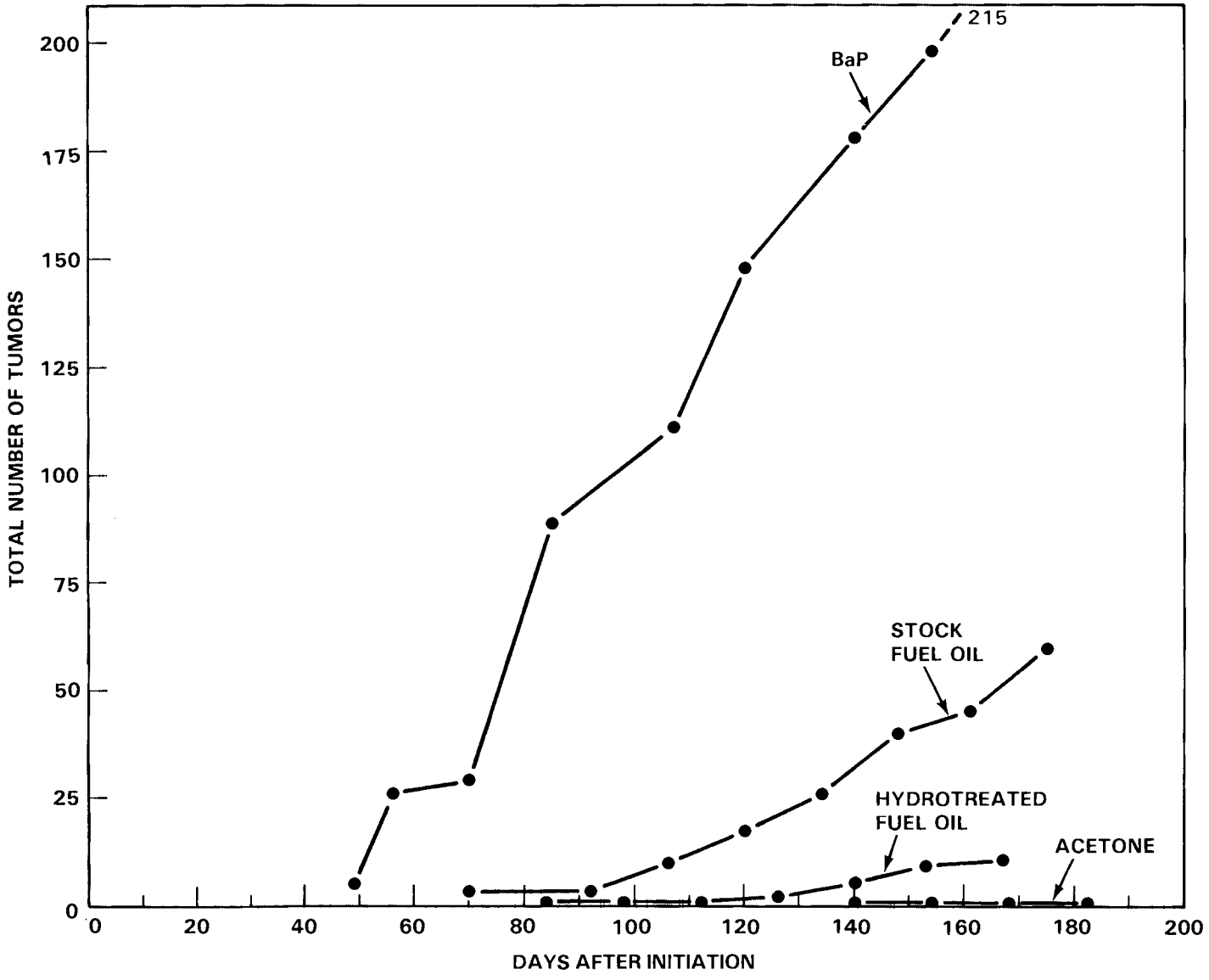

FIGURE 5.11B. Effect of Hydrotreatment on the Skin-Tumor-Initiating Activity of an SRC-II FOB. B. Total Tumor Yield (Normalized to 30 Mice/Group).

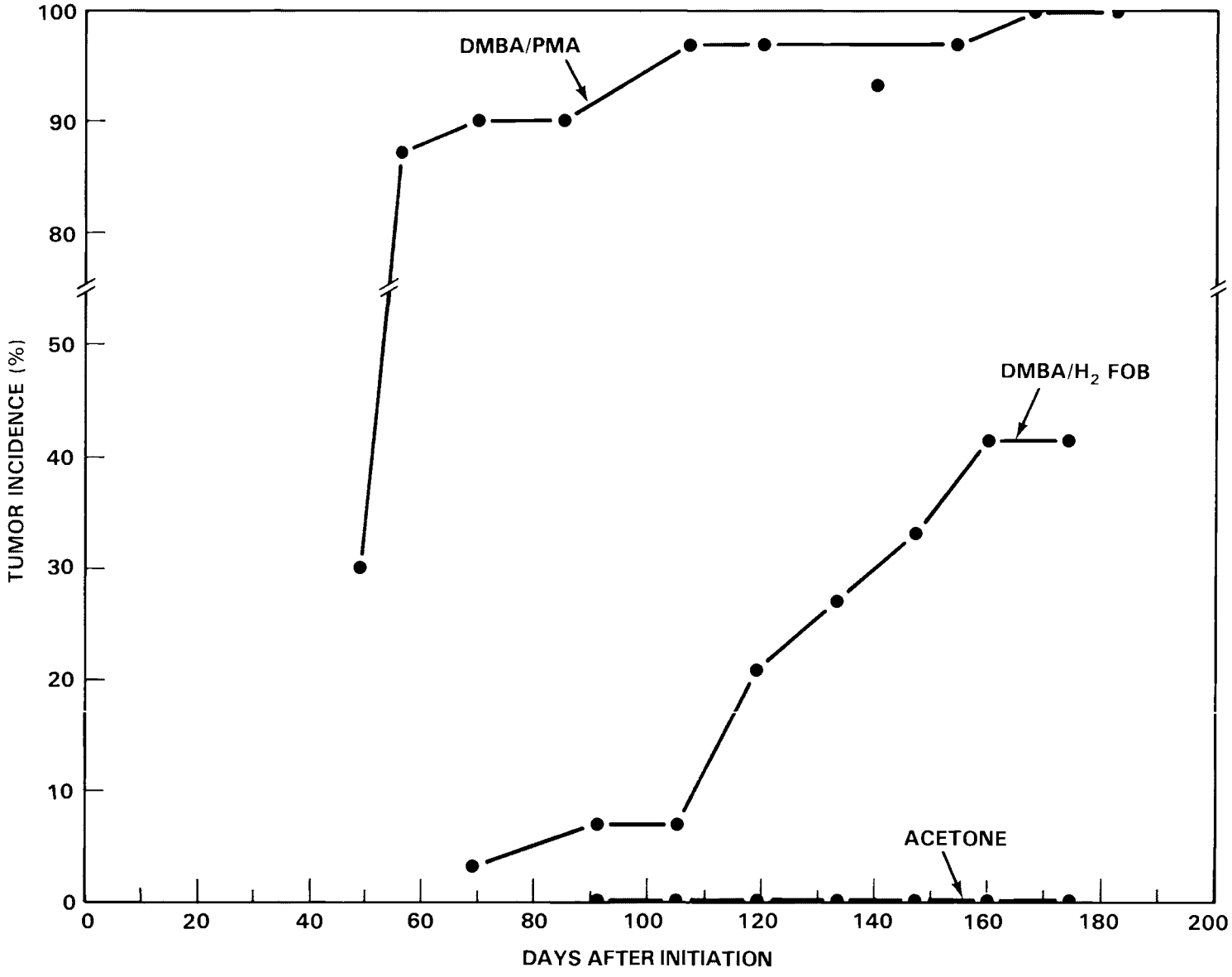

FIGURE 5.12A. Promoting Activity of Hydrotreated SRC-II FOB. Mice were Initiated with 50 µg of DMBA. Two Weeks Later They were Promoted Twice Weekly with Either 5 µg PMA or 50 µl hydrotreated FOB (H_2FOB) Which was Diluted with Three Parts Acetone. A. Percent Tumor Incidence.

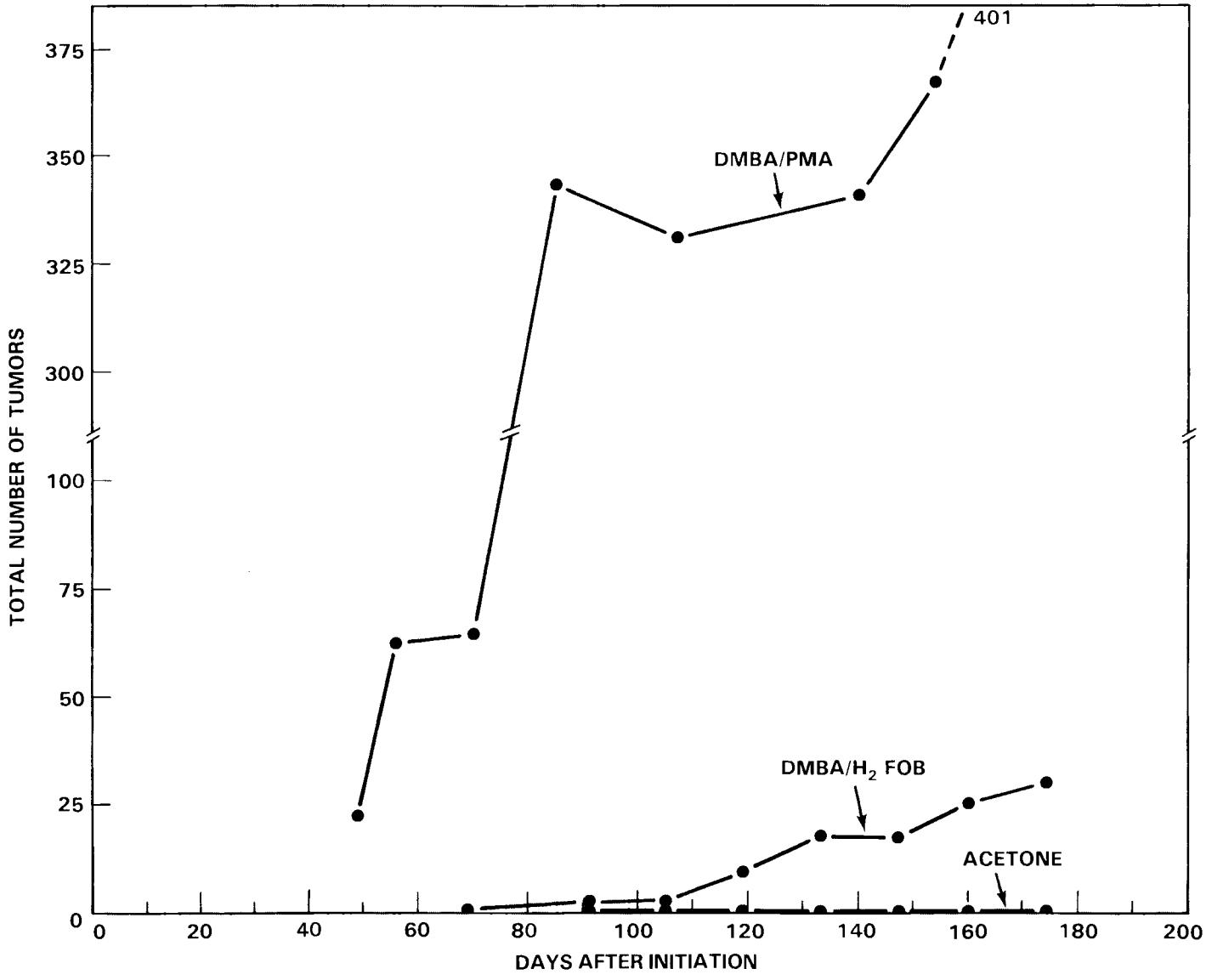

FIGURE 5.12B. Promoting Activity of Hydrotreated SRC-II FOB. Mice were Initiated with 50 µg of DMBA. Two Weeks Later They were Promoted Twice Weekly with Either 5 µg PMA or 50 µl hydrotreated FOB (H_2FOB) Which was Diluted with Three Parts Acetone. B. Total Tumor Yield (Normalized to 30 Mice/Group).

6.0 CHRONIC EPIDERMAL CARCINOGENESIS STUDIES IN MICE

6.1 PHASE I: CRUDE FOSSIL-FUEL MATERIALS AND KNOWN CARCINOGENS

Our Status Report of October 1979 (PNL-3189 1979) described the materials, methods and interim results of the first phase of our epidermal carcinogenesis assays in mice, in which we compared the carcinogenic potential of SRC-II HD and light distillate (LD) with that of crude shale oil, crude petroleum, and two known carcinogens (BaP and 2-AA). The final results of Phase I, with epidermal tumor incidence data confirmed by microscopic examination, are presented in detail in Table 6.1. Figure 6.1 shows epidermal tumor latency data for each group, using the nonparametric Kaplan-Meier procedure. The data confirm our preliminary conclusions: based on tumor incidence and latency, SRC-II HD was the most potent carcinogen of the crude materials assayed, followed by crude shale oil, and crude petroleum. SRC-II LD showed no activity under the conditions of this assay.

In these studies, crude petroleum was applied at a maximum concentration of 40% (weight/weight), whereas the other crude fossil-fuel materials were at a maximum concentration of 50% (see Table 1 of Appendix C, Status Report PNL-3189 October 1979). This difference in maximum concentration was necessitated by the tendency of crude petroleum to precipitate in acetone at a concentration of 50%; also, the viscosity of the 50% solution of crude petroleum in acetone was too high to allow accurate pipetting of the material. After the initial chronic studies were underway, we found that the crude petroleum sample was readily pipettable and did not precipitate at a concentration of 50% in a solution prepared by diluting 30 volumes of cyclohexane to 100 volumes with acetone. In October 1980, we began another chronic assay of the crude petroleum sample at two dose levels, using the acetone/cyclohexane vehicle. The higher dose was 21.2 mg Wilmington crude petroleum in 50 µl of acetone/cyclohexane vehicle, which represented a 50% (weight/weight) concentration of crude petroleum. The lower dose was 2.12 mg of crude petroleum in the same vehicle, representing a 10-fold dilution of the high dose. This study is still in progress; tumor data to date are shown in Figure 6.2 and compared with similar data for mice exposed to Wilmington crude petroleum in acetone.

There was a similar but slightly longer tumor latency at the higher doses for crude petroleum when diluted with acetone/cyclohexane than when diluted in acetone. At the lower doses (10-fold dilution of higher doses) the reverse was true: tumor latency was shorter using the acetone/cyclohexane vehicle. These data may indicate that at the higher doses (21.2 mg or 16.8 mg/application) the point was reached beyond which tumor latency no longer decreases with increasing dose; this effect has been demonstrated with carcinogenic PAH (Bryan and Shimkin 1942). The decreased tumor latency observed with the 2.1-mg dose of crude petroleum in acetone/cyclohexane compared to the dose in acetone may simply reflect the dose difference rather than difference in vehicle. If correct, this observation suggests that our animal test system is more sensitive to small changes in dose at the low dose ranges. However, the difference in latency may also reflect differences in epidermal penetration, metabolism, or other facets of chemical/vehicle/tissue interaction related to chemical composition of the vehicle.

TABLE 6.1. Incidence and Latency Data on Microscopically Confirmed Epidermal Tumors Observed in Mice Exposed to Crude Fossil-Fuel Materials or Known Carcinogens

Material	Concentration (mg/50 µl)	Mice/Group at Start of Study	Mice With Epidermal Tumors	Final Epidermal Tumor Incidence (%)[a]	Latency (No. Days)		
					Minimum	Median	Maximum
Untreated	Control	50	0	0	--	--	--
Acetone	Vehicle	50	0	0	--	--	--
Shale oil	0.21	50	0	0	--	--	--
	2.1	50	41	82	148	358	550
	21.1	50	36	72	95	200	409
Crude petroleum	0.17	50	0	0	--	--	--
	1.7	50	10	20	339	501	708
	16.8	50	39	78	260	442	680
SRC-II HD	0.23	50	22	44	282	542	736
	2.3	50	45	90	72	296	372
	22.8	50	46	92	56	95	147
SRC-II LD	0.2	50	0	0	--	--	--
	2.0	50	0	0	--	--	--
	20.0	50	0	0	--	--	--
2-AA	0.005	50	30	60	465	583	736
	0.05	50	22	44	115	386	485
BaP	0.0005	50	0	0	--	--	--
	0.005	50	46	92	232	358	519
	0.05	50	50	100	106	143	193

[a]This does not take into account interim mortality, i.e., mice without epidermal tumors that died during the study.

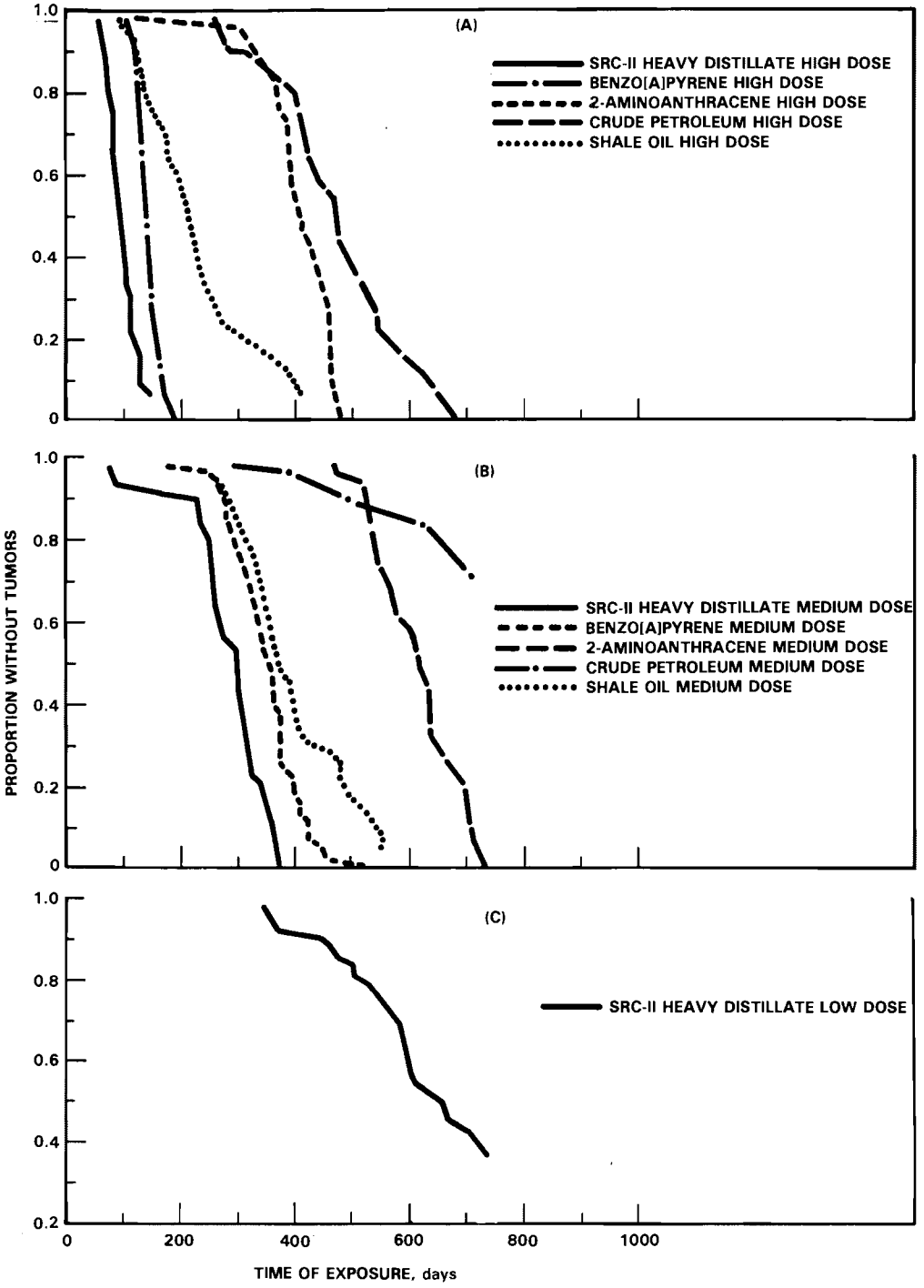

FIGURE 6.1. Tumor Incidence Among Mice Exposed Twice Weekly to SRC-II HD, BaP, 2-AA, Wilmington Crude Petroleum or Parahoe Shale Oil. A. High Dose, B. Medium Dose, and C. Low Dose. For Actual Doses, see Table 6.1.

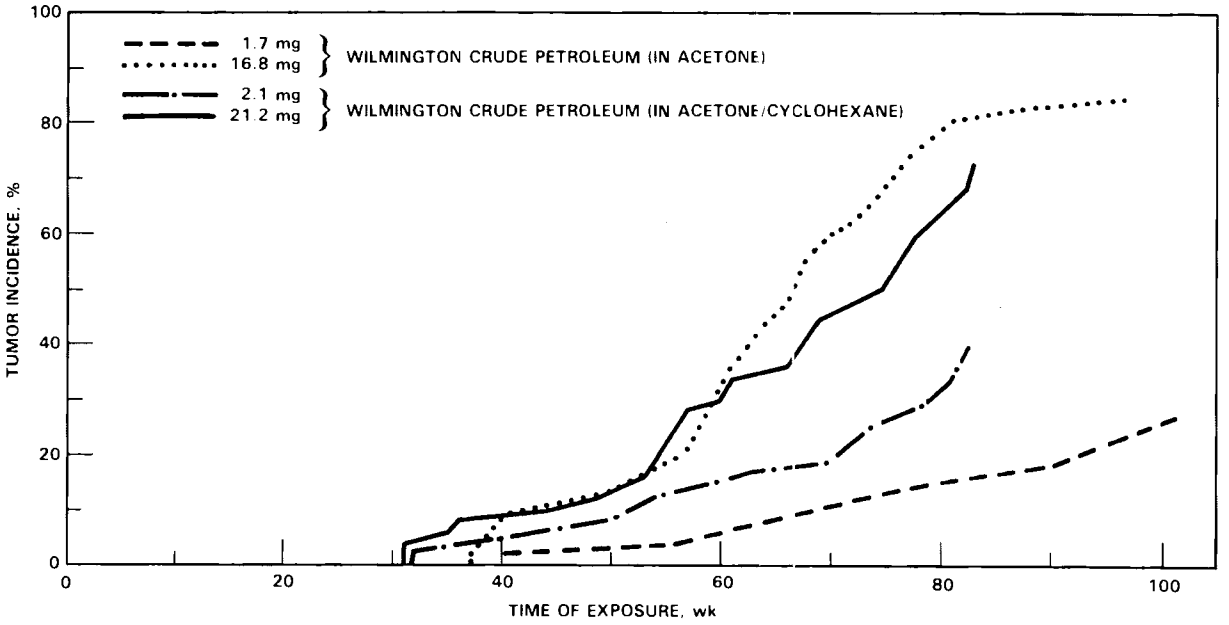

FIGURE 6.2. Comparison of Tumor Incidence in Mice Exposed to Wilmington Crude Petroleum Dissolved in Acetone or in Cyclohexane.

6.2 PHASE II: SOLVENT FRACTIONS OF SRC-II HD

Based on preliminary tumor incidence/latency data for crude materials, and on data from the Ames/Salmonella assay of solvent fractions of HD (Pelroy and Petersen, 1981), we began chronic skin-painting assays on chemically derived solvent fractions of HD, including BF, PAH, BTF, and NTF. Chemical characterization data (Table 6.2) indicated that both of the tar fractions contained high concentrations of NPAC, as well as high concentrations of PAH. The BF contained high concentrations of NPAC and low concentrations of PAH, whereas the converse was true for the PAH fraction.

TABLE 6.2. Concentration (Weight %) of Selected Compound Classes (Aliphatics, PAH, NPAC and HPAC)in SRC-II HD and Its Solvent-Derived Fractions

Material	SRC-II HD	Aliphatics	PAH	NPAC	HPAC
SRC-II HD	100	10	42	20	28
BTF	24	<1	38	35	27
NTF	26	<1	38	24	35
PAH	41	2	81	11	5
BF	1	<1	19	68	10

In choosing materials and doses for this work, we attempted to apply a mutagenic stress to the animals that was equivalent to that in the Phase I experiments; i.e., a dose of the mutagenic fractions (based on Ames data) that had approximately the same amount of mutagenicity as the highest dose of HD applied in our earlier experiments (Table 6.3). For the PAH fraction, which

TABLE 6.3. Exposure Data for Solvent Fractions of SRC-II HD used in Epidermal Carcinogen Studies in Mice

Material	Mutagenic Potency (Ames) Relative to HD	Proportion of HD (Wt %)	Amount of Test Material/Application (mg/50 µl) High Dose	Medium Dose	Low Dose
BF	20X	1.86	1.14[a]	0.23	0.05
PAH[b]		10	2.50[c]	0.50	0.10
NTF	4.3X	21.5	5.25[a]	1.05	0.21
BTF	3X	18.8	6.75[a]	1.35	0.27

[a]Based on "equivalent mutagenic stress":

$$\frac{\text{Mutagenicity of HD}}{\text{Mutagenicity of Fraction}} \times \text{high dose (22.8 mg) of HD} = \text{high dose of fraction}$$

[b]Polycyclic aromatic hydrocarbon

[c]Based on available information on weight % of HD (see text)

was not significantly mutagenic (in Ames assays), we used the percentage of PAH fraction in HD. Data available at the time the study began indicated that that fraction was approximately 10% by weight. More recent data indicate that PAHs make up approximately 41% of this sample of HD. The middle and low doses of each fraction were 5- and 25-fold dilutions, respectively, of the high doses. The amounts of test material applied to each dose group are included in Table 6.3. The two fractions were diluted with 30% methanol in acetone (v/v) because they were not soluble in 100% acetone. Acetone was used as the diluent for the basic and PAH fractions.

Carcinogenesis data for these fractions are presented in Table 6.4 and Figure 6.3. The BTF and the NTF were the most active fractions of HD, according to tumor latency and incidence. The PAH fraction induced a slower response than either tar fraction; the BF had relatively little tumorigenic activity. At the lowest dose of each tar fraction (Figure 6.4), where the doses are similar to the lowest dose of HD in the Phase I study, the tumor latencies were considerably shorter than that for HD.

Results of our chronic mouse-skin assays of solvent fractions of HD partially correlated with the Ames assay results. The BTF and NTF showed high mutagenic activity in the Ames assay (Pelroy and Petersen, 1981) and high carcinogenic activity in the mouse-skin carcinogenesis assay. However, the BF of HD, which was very active in the Ames assay, showed little activity in the mouse skin-painting assay.

Available chemical characterization, mutagenesis, and carcinogenesis data suggested that the carcinogenicity of HD may be due to some synergistic effect

TABLE 6.4. Incidence of Grossly Observed Skin Tumors in Mice after 2 Years' Exposure to Solvent Fractions of SRC-II HD

Material	Concentration (mg/50 µl)	Mice at Risk[a]	Mice with Skin Tumors	Tumor Response (%)	Latency (No. Days) Minimum	Median	Maximum
30% methanol in acetone	Vehicle	8	0	0	--	--	--
Replicate SRC-II HD	2.28	49	49	100	163	291	354
	22.8	50	50	100	44	112	232
Basic fraction	0.05	11	0	0	--	--	--
	0.23	16	0	0	--	--	--
	1.14	34	34	100	323	582	722
PAH fraction[b]	0.10	22	5	23	393	--	--
	0.50	46	46	100	239	428	568
	2.50	49	49	100	171	267	351
Neutral tar	0.21	36	36	100	240	561	711
	1.05	49	49	100	177	281	372
	5.25	50	50	100	99	183	235
Basic tar	0.27	44	44	100	239	435	731
	1.35	49	49	100	162	246	309
	6.75	50	50	100	74	148	204

[a]Number of mice at risk excludes only those mice that died in less than 2 years without developing skin tumors. It includes all live animals in the group plus dead animals with skin tumors.

[b]Polycyclic aromatic hydrocarbon

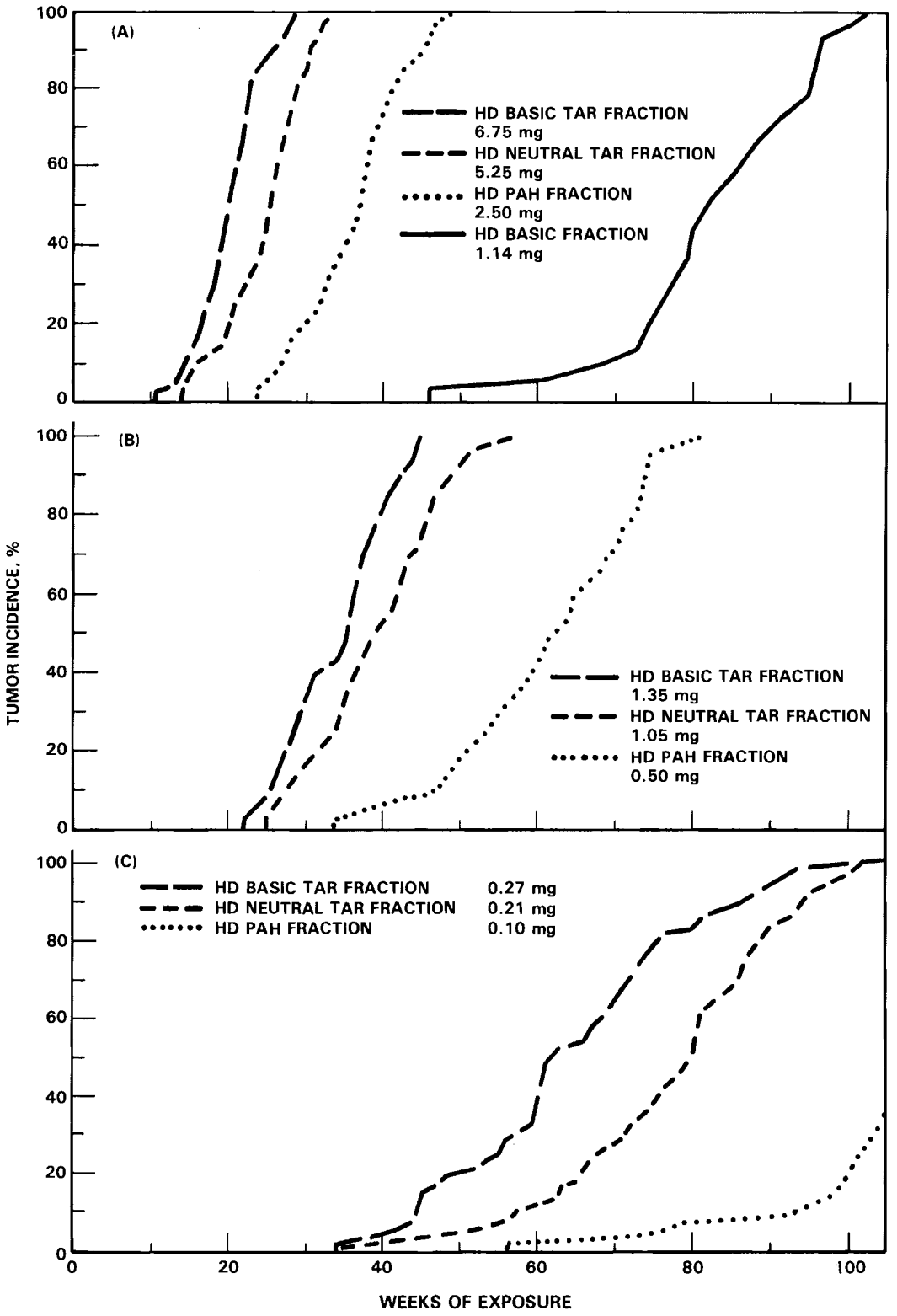

FIGURE 6.3. Tumor Incidence Among Mice Exposed Twice Weekly to HD or its Fractions. A) High Dose, B) Medium Dose, C) Low Dose.

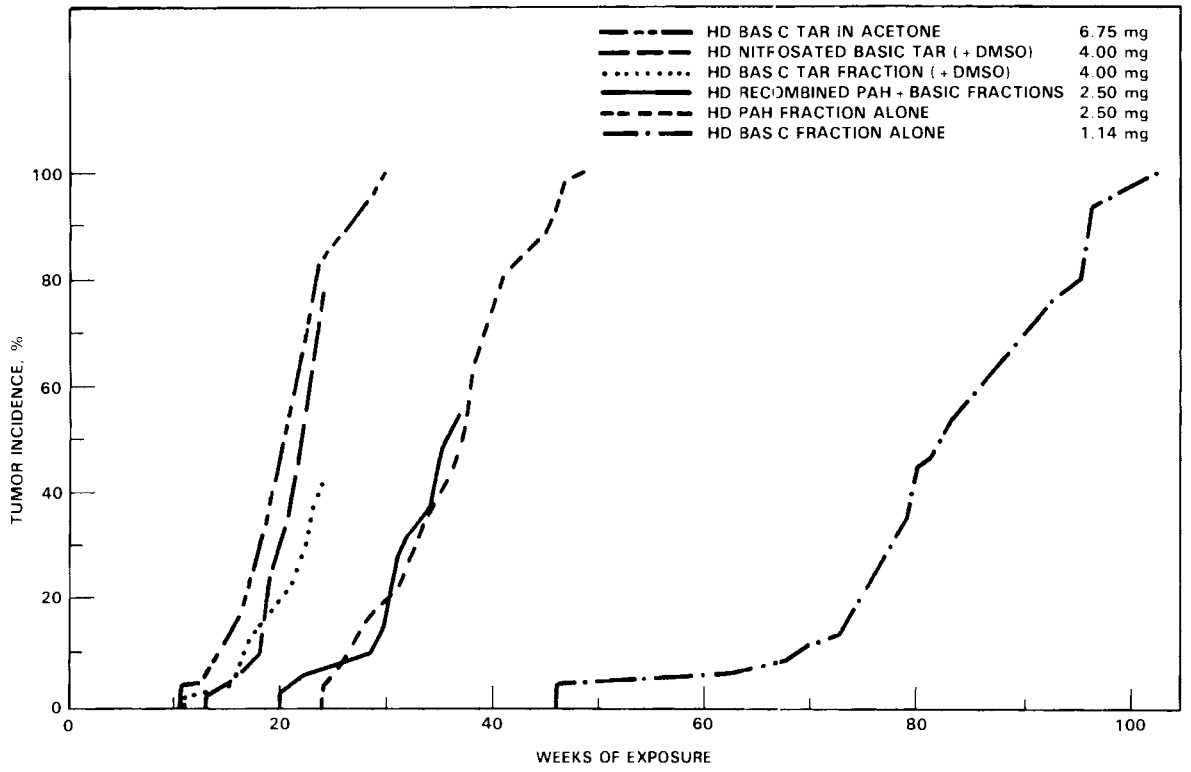

FIGURE 6.4. Tumor Response After Nitrosation of the BTF and After Combining the PAH and BF.

of PAH compounds with APAH and/or other NPAC compounds. We therefore assayed recombined PAH and basic fractions to further investigate this concept.

The highest concentration of recombined PAH and BF applied was 2.50 mg of total material, consisting of 1.72 mg of PAH fraction and 0.78 mg of BF, but not the same properties as found in the whole material. This is the same proportion as the doses of each fraction applied in the earlier study (2.5 mg of PAH fraction to 1.14 mg of BF), but not the same proportion as found in the whole material. The highest dose of the combined fractions applied was based on the highest dose of either fraction applied in the earlier study (2.5 mg of PAH fraction). This total dose was used so that if an increased carcinogenic potency of the recombined fractions was observed, the effect would reflect synergism, rather than a higher dose. The middle and low doses were 5- and 25-fold dilutions of the high dose, as in earlier assays of individual HD fractions. Acetone was used as the vehicle.

These studies are still in progress. Results to date (Table 6.5, Figure 6.4) indicate a carcinogenic response in the highest dose groups similar to that observed previously for the highest dose of PAH fraction alone. This suggests that the components of the BF do not have a synergistic role with the PAH fraction in the epidermal carcinogenicity of HD.

Studies by Pelroy and Stewart (1981) demonstrated that destruction of the APAH by nitrosation removed the most of the microbial mutagenicity of the BF,

TABLE 6.5. Incidence of Grossly Observed Skin Tumors in Mice in Ongoing Studies of Recombined or Nitrosated Solvent Fractions of SRC-II HD

Material	Concentration (mg/50 µl)	Days on Study	Mice at Risk[a]	Mice with Skin Tumors	Latency (No. Days) Minimum	Median	Maximum
SRC-II HD PAH + BF	0.10	351	47	0	--	--	--
	0.50	351	50	5	260	--	--
	2.50	351	49	49	143	253	325
HD BTF + DMSO	4.00	267	50	50	78	176	225
Nitrosated HD BTF + DMSO	4.00	267	50	50	94	157	218

[a]Excludes mice that died without developing a tumor at the application site

BTF, and NTF. We therefore assayed nitrosated basic tar to determine if carcinogenicity was destroyed. Nitrosation of the BTF required its dissolution in DMSO, a strong organic solvent that enhances penetration of large molecules through skin. The nitrosated basic tar samples we used contained 49% tar and 51% DMSO (by weight). To determine the effects of DMSO on the carcinogenic response of our mouse-skin assay system to the basic tar fraction, we reassayed the non-nitrosated basic tar in DMSO at a concentration (49% BTF, 51% DMSO) equal to that of the nitrosated BTF. Both nitrosated and non-nitrosated BTF/DMSO mixtures were then diluted with acetone to a concentration of 4.0 mg tar per 50 µl. We also reassayed the mutagenicity of the nitrosated and non-nitrosated BTF samples in the Ames/Salmonella assay system to confirm the decrease in mutagenicity induced by nitrosation.

These studies are still in progress. Results to date (Table 6.5, Figure 6.4) indicate that nitrosation of HD basic tar does not decrease its carcinogenicity. Therefore, the dermal carcinogenicity of basic tar is unlikely to be primarily due to the presence of APAH.

6.3 PHASE III: BOILING-POINT DISTILLATES OF SRC-II WIDE-BOILING-RANGE LIQUID

The most recently begun studies on this project are examining the carcinogenic potential of a wide-boiling-range (>300-850°F) SRC-II material and bp cuts as described in Section 2.0. The studies reported in the previous sections have indicated that most of the microbial mutagenicity, mammalian-cell genotoxicity and tumor-initiating activities are confined to cuts boiling above 700°F. We are attempting to determine if the response in the chronic skin-painting assay is also limited to the higher-boiling components.

The boiling ranges of the materials assayed and the doses of each are shown in Table 6.6. The doses were determined by acute (14-day) epidermal exposures which determined: 1) the maximum concentration tolerated by mice without acute toxicity, and 2) the maximum concentration of each material that could be accurately and reproducibly applied to the skin in a suitable organic solvent.

Results to date are shown in Table 6.6 and Figure 6.5. These tumor latency data indicate that the 750 to 800°F, 800 to 850°F and >850°F cuts have a greater carcinogenic potential than the wide-boiling-range material. This difference in carcinogenic potency is more evident in groups exposed to the middle doses (Figure 6.5B) of these materials than in the groups exposed to the highest doses (Figure 6.5A). The response to the 700 to 750°F is, at this point in the assay, somewhat less than to the whole-boiling-range material at both the high and middle doses. No tumors have been observed to date in mice exposed to the 300 to 700°F cut.

As a further comparison of the carcinogenic potential of various SRC process materials, PS from the SRC-I mode operation at the Ft. Lewis pilot plant was assayed. Doses of this material applied and results to date are shown in Figure 6.6. To date, the tumor latency curve for SRC-I PS at the highest dose (10.4 mg/application) parallels very closely that for the >300-850°F SRC-II liquid at the same dose (Figure 6.5). At the middle dose (1.0 mg/application), the minimum tumor latency was considerably shorter for the SRC-I PS, but the latency curves are now nearly parallel as the tumor incidence increases in response to the SRC-II liquid.

TABLE 6.6. Incidence of Grossly Observed Skin Tumors in Mice Exposed to SRC Crude Materials or bp Cuts

Material	Concentration (mg/50 ml)	Days on Study	Mice at Risk[a]	Mice with Skin Tumors	Latency (No. Days) Mimimum	Median	Maximum
SRC-I PS[b]	0.10	742	22	10	553	--	--
	1.00	638	47	47	251	392	553
	10.40	407	50	50	98	188	364
SRC-II HPS[c]	0.10	404	49	0	--	--	--
	0.10	496	47	0	--	--	--
	1.00	496	49	33	323	461	--
	10.40	428	50	50	118	188	343
300-700°F	0.10	484	44	0	--	--	--
	1.00	484	42	0	--	--	--
	10.40	484	45	0	--	--	--
700-750°F	0.10	461	45	0	--	--	--
	1.00	461	47	10	245	--	--
	10.40	400	44	44	98	190	398
750-800°F	0.10	442	49	1	379	--	--
	1.00	442	50	49	142	324	--
	10.40	442	49	49	79	171	351
800-850°F	0.05	414	46	2	351	--	--
	0.50	414	50	49	162	267	--
	5.00	414	48	48	67	150	254
>850°F	0.02	391	47	3	300	--	--
	0.25	365	49	49	176	274	361
	2.50	330	50	50	92	177	225

[a]Excludes mice that died without developing a tumor at the application site

[b]Process solvent

[c]Harmarville PS whole-boiling-range material (300->850°F)

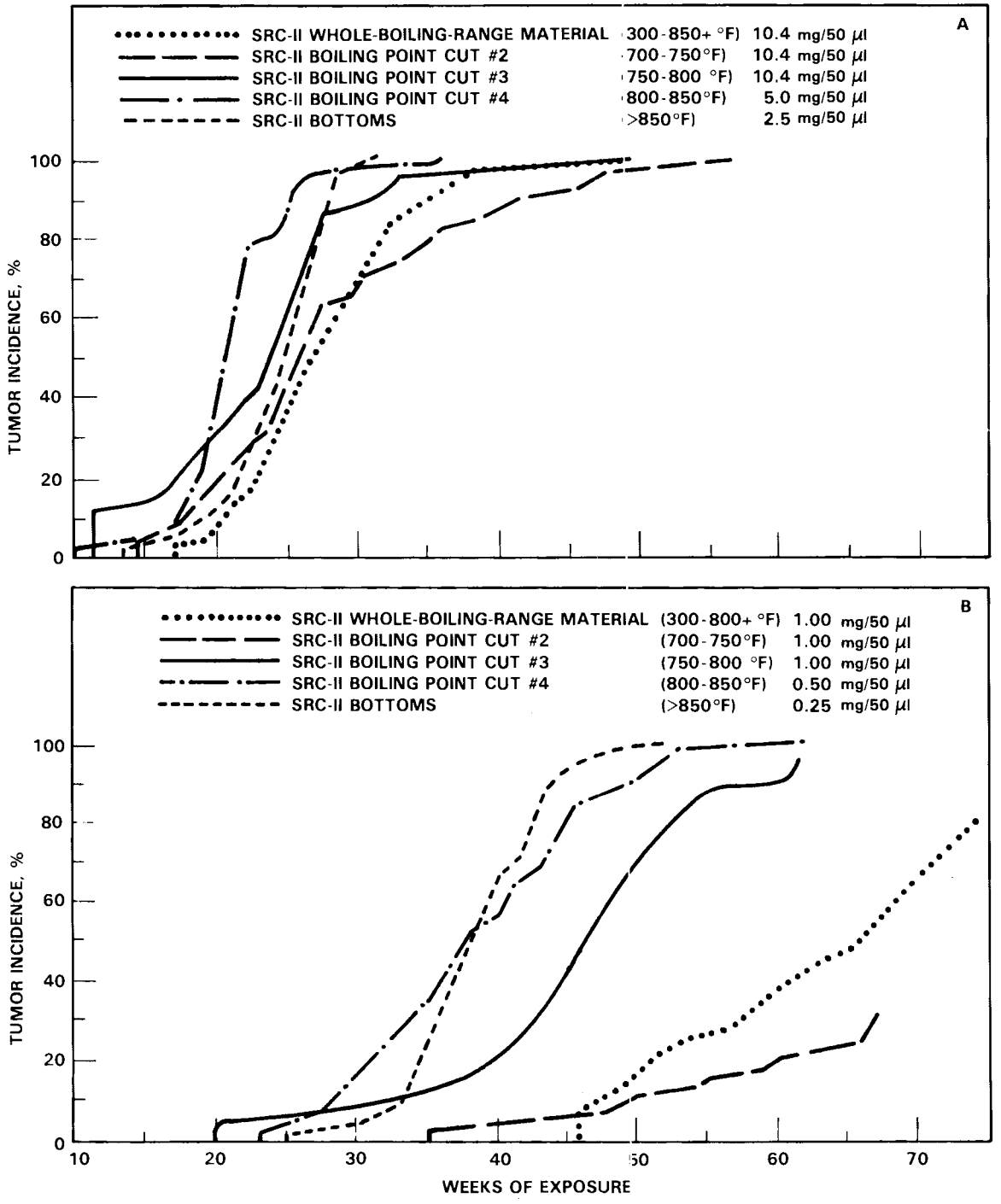

FIGURE 6.5. Tumor Response to a Wide-Boiling-Range (>300-850°F) Coal Liquid and its bp Cuts. A. High Dose, B. Medium Dose.

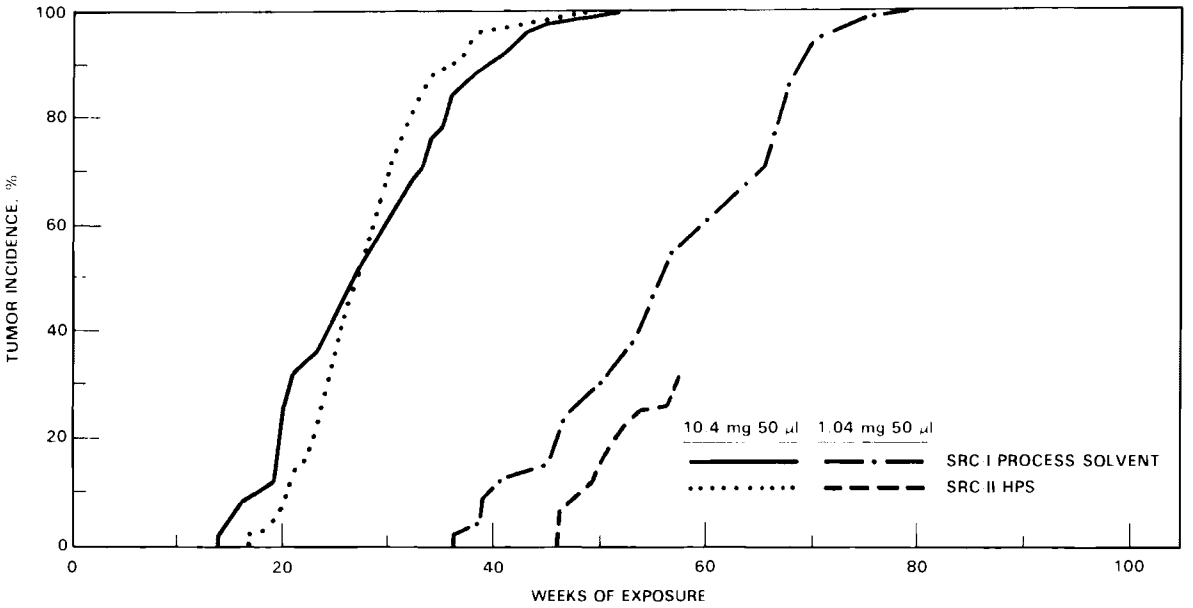

FIGURE 6.6. Comparison of the Tumor Response to SRC-I PS and a Wide-Boiling-Range (>300-850°F) SRC-II Coal Liquid.

7.0 CORRELATIONS AMONG BIOASSAYS

As stated previously, one of the purposes of these studies was to examine the agreement among the various assays used at PNL. With the number of complex materials examined thus far, it should be possible to determine how well the in vitro tests correlate with the skin-painting assays, and also to determine the correspondence between the results of the two skin-painting assays. In addition, where assays do not agree qualitatively or quantitatively, it may be possible to suggest reasons for the differences.

Comparing results from many experiments using several different biological endpoints is, at best, difficult. For quantitative comparisons, we have used HD as the reference material and calculated the effect produced by other materials relative to that obtained with HD. Values from the Ames and CHO assays were obtained from linear regression analysis dose-response curves. For SHE cell assay, the reference point is the percentage of cells transformed by 10 μg of test material.

Comparisons of initiating activities were performed by comparing total tumor yield per group of 30 mice or by comparing the percentage of mice showing tumors. The response with a test material at a given dosage, since one dose is usually used, is compared with the response to HD at the same dosage by referring to a dose-response curve for HD (Figure 5.1). Table 7.1 shows values derived in this manner for several materials.

TABLE 7.1. Potency in I/P Assays of Several Synthetic Fuels Relative to SRC-II HD[a]

Material	Potency
HD	100
BaP	144,000
2-AA	50,000
Wilmington petroleum crude	22
BTF[b]	110
NTF[b]	135
BF[b]	53
PAH fraction[b]	75
SRC-II >300-850°F	24
700-750°[c]	18
750-800°[c]	14
800-850°[c]	49
>850°[c]	314

[a]Based on total tumor yield group of 30 mice initiated with test material/tumor yield per group of 30 mice initiated with the same dose of HD x 100.

[b]Derived from HD

[c]Derived from the >300-850°F material.

In chronic skin-painting experiments, the test materials are applied at two or three different doses, with each dose varying by 5- or 10-fold in concentration. Since the actual doses used, particularly for studies of fractions, were not always similar to those used for HD, we have plotted dose against response on semi-logarithmic paper using median latency as the dependent variable for HD (Figure 7.1). The response produced by a test material at any dose can then be compared by obtaining the response to HD at the same dose. The response to the test material relative to HD can then be calculated by dividing the median latency for HD by that for the test material. Table 7.2 shows the range of values obtained by this method for materials reported in Section 6.0. For most materials, relative potencies are similar at all doses tested, indicating a dose-reponse curve parallel to that for HD. In a few instances, the values for a material at low and high doses are quite different from each other, indicating that the slope of the dose-response curve is significantly different from that for HD.

Most of the materials for which results are shown in Tables 7.1 and 7.2 have also been assayed in the in vitro systems. Table 7.3 shows the biological activity of these materials relative to HD in the Ames, SHE, CHO, I/P, and chronic skin-painting assays. We also rank-ordered these materials with BaP and 2-AA to show semi-quantitatively how results from different assays compare (Table 7.4). In general, materials shown to be carcinogenic in the chronic skin-painting assay are also positive in the other assays. The major exception to this finding is that the PAH fraction of HD was negative in the Ames assay. Quantitatively, there is less agreement among the results obtained in the various assays. The Ames assay responded well to materials enriched in nitrogen-containing compounds and poorly to the neutral PAH. In contrast, the mammalian systems, both in vitro and in vivo, responded better to neutral PAH materials.

It is noteworthy that when crude bp cuts were examined, the highest activity was found in the >850°F cut in all the assays. In general, all the assays indicated increasing biologic activity with increasing distillation temperature.

The mammalian assays rank the materials examined in similar, although not identical, orders. The major exception to this statement is that the SHE cell response to 2-AA is very low compared with that in the other assays.

There were also notable quantitative differences between responses obtained in the I/P and chronic skin-painting assays, particularly in results obtained with the distillate cuts. Thus, while both assays indicated that the higher bp cuts are more active, the I/P assay indicated a nominal 7- to 22-fold difference in response among the 750-800°, 800-850°, and the >850°F cuts, while the chronic skin-painting assay indicated only about a 2-fold difference. However, it should be pointed out that the majority of tumor latency data from the chronic skin-painting assay in progress on the distillate cuts are from the highest dose groups. Latency data from the middle and low dose groups (Figure 6.5) indicate a wider difference in neoplastic response among the various bp cuts. Nevertheless, these data suggest that there is a marked suppression of initiating activity in some of the bp cuts in the I/P assay. This suppression may be minimized in the chronic assay, where multiple dosing allows optimal initiation of the cells capable of responding. These data also

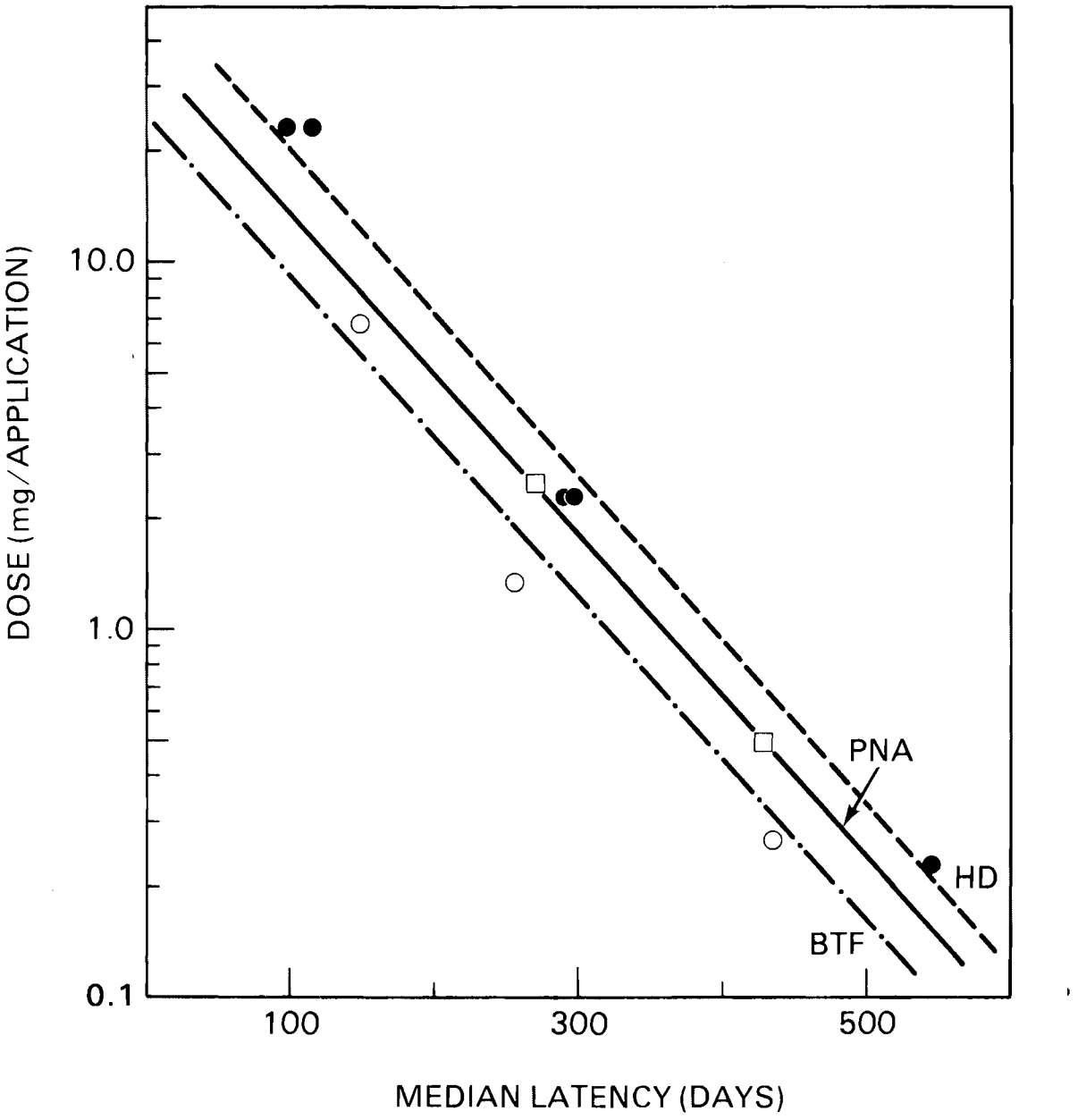

FIGURE 7.1. Semi-Logarithm Plot of Dose Versus Response for HD, NTF and BTF in the Chronic Skin-Painting Assay.

suggest that all of the distillate cuts boiling above 700°F possess significant promoting activity since they are complete carcinogens.

Several test materials have been examined only in Ames and CHO assays. An example is the >850°F distillate cut, which was fractionated by HPLC to yield eight fractions ranging from nonpolar to polar. Response of the two systems to these fractions are shown in Figure 7.2, which demonstrates the extreme sensitivity of the CHO assay to PAH, with some sensitivity to azaa-

TABLE 7.2. Potency[a] in Chronic SKin-Painting Assays of Several Synthetic Fuels Relative to SRC-II HD

Material	Dose Ranges of Test Material (mg/Application) 0.25-0.50	1.0-2.5	5.0-10.5	15-25
Wilmington petroleum crude		68		26
Parahoe oil shale		89		48
BTF[b]	120	144	140	
NTF[b]	97	128	126	
BF[b]		65		
Polynuclear aromatic fraction[b]	107	113		
SRC-II >300-850°F			88	
700-750°F[c]			87	
750-800°F[c]		120		
800-850°F[c]	142		157	
>850°F[c]	243	171		

[a]Based on median latency for test material/median latency for the same dose HD x 100.

[b]Derived from HD

[c]Derived from the >300-850°F material

TABLE 7.3. Comparison of the Biological Activity of Several Complex Mixtures Relative to SRC-II HD (HD = 100)

Test Material	Assay Ames	SHE	CHO	I/P	Chronic
Wilmington petroleum crude	0	+	ND[a]	22	68
Parahoe shale oil	2	32	38	ND	89
HD solvent fractions					
Basic tar	58	76	ND	110	144
Neutral tar	88	52	ND	135	128
Basic	480	75	ND	53	65
PAH	0	92	ND	75	113
Boiling point cuts					
300-850°+	200	ND	ND	24	88
300-700°	70	7	ND	0	0
700-750°	95	10	ND	18	87
750-800°	138	63	ND	14	120
800-850°	148	65	86	49	157
>850°	450	105	200	314	243

[a]Not determined

TABLE 7.4. Relative Ranking of Biological Activity of Several Organic Materials in Five Assay Systems

Material	Ranking				
	Ames	SHE	CHO	I/P	Chronic
Wilmington petroleum crude	13-14	12	ND[a]	12	12-13
Parahoe shale oil	12	10	5	ND	9-11
BF of HD	2	4-5	ND	7-8	12-13
BTF of HD	11	4-5	ND	5	5
NTF of HD	9	8	ND	4	6
PAH fraction of HD	13-14	3	ND	6	8
300-700°bp cut	10	13	ND		14
700-750°	8	11	ND	10-11	9-11
750-800°	7	6	ND	10-11	7
800-850°	6	7	ND	10-11	7
>850°	3	2	3	3	3
>300-850°F	5	ND	ND	9	9-11
BaP	4	1	1	1	1
2-AA	1	9	2	2	2

[a]Not determined

renes and much smaller response to other chemical classes. In contrast, the Ames assay does not respond very well to neutral PAH carbazoles, or to azaarenes, but is exquisitely sensitive to the APAH.

Chemical class fractions prepared from the 800-850 and the >850° boiling point cuts were assayed in the Ames, SHE, CHO, and I/P assays. Table 7.5 shows the biological activity of these fractions relative to that for HD. There was good agreement between results of the mammalian-cell and I/P assays, although quantitatively, there was considerable divergence between assays for certain materials. For example, the I/P assay was more responsive to both the PAH and NPAC fractions than was the SHE system. On the other hand, the CHO results for the >850°F cut and its fractions agreed well with the I/P results for the PAH and NPAC fractions. Again, it can be seen that the major part of the Ames response is due to the NPAC fraction.

Inconsistent results were obtained for the aliphatic and HPAH fractions, particularly for the >850°F cut. Little activity was found for these fractions in the SHE assay, but substantial activity was obtained in the CHO system. The I/P assay showed a low level of response to the HPAH for the >850°F material, but an anomalously high response to the aliphatic fraction from this cut. This high value must be viewed with extreme caution since it is based on a low response obtained with application of a small dose (approximately 0.34 mg). This low response became magnified as we extrapolated the data for comparison with HD. We do not know which component is responsible for the initiating activity in the aliphatic fraction; however, there may be a small amount of contamination with neutral PAH which could account for its activity.

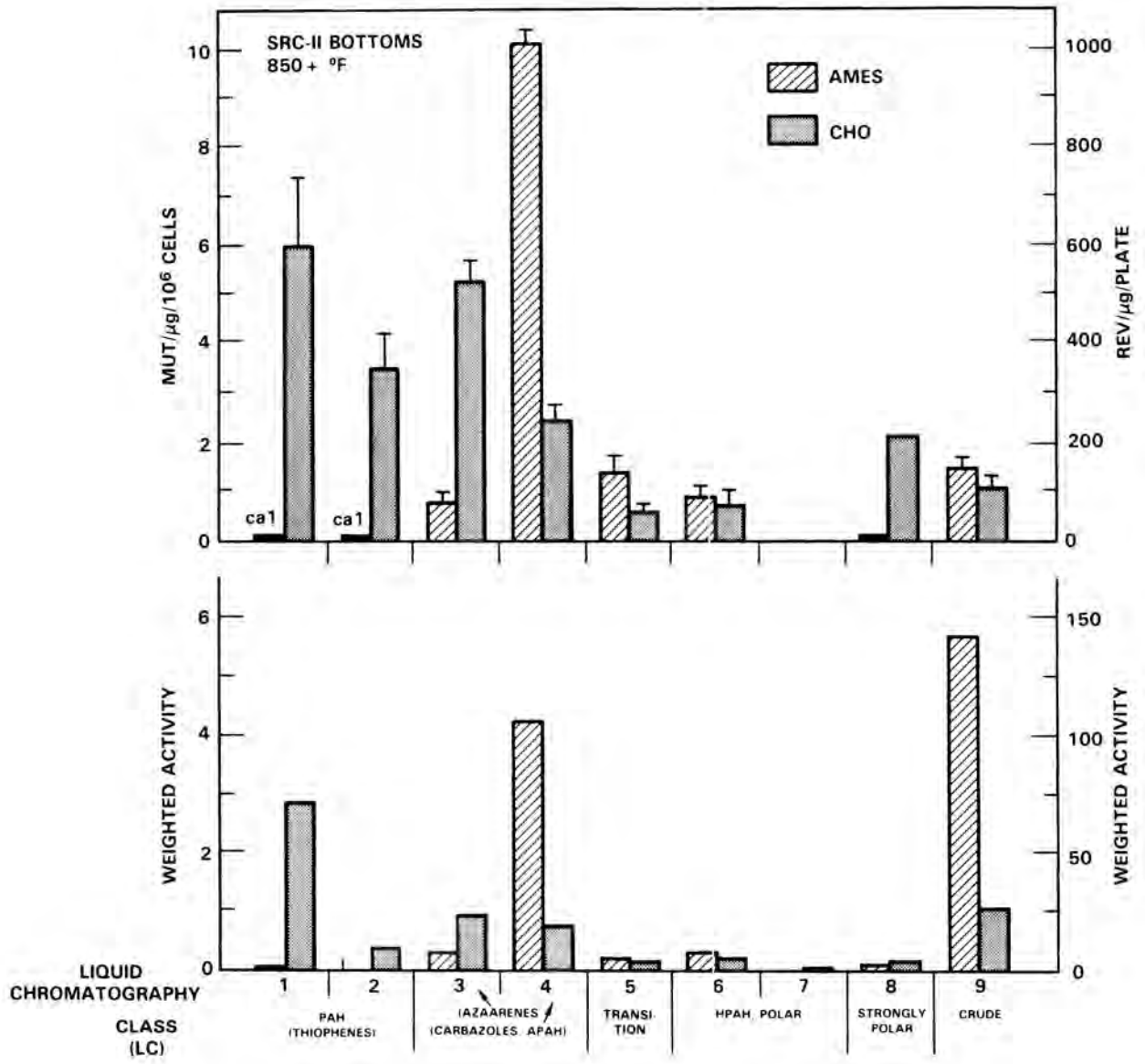

FIGURE 7.2. Comparison of Response in the Ames and CHO Cell Assays to Fractions Obtained by HPLC of the >850°F bp Cut.

The relative ranking of these materials in the four assays is shown in Table 7.6. The results suggest that the assays employed in these studies often show substantial qualitative agreement; however, there are exceptions, as demonstrated by the failure of the Ames assay to respond to the PAH fraction from HD. Since the PAH fraction from HD was assayed (2 or 3 years ago), changes made in the Ames assay make it more responsive to mixtures of PAH, as shown by the response with the PAH fractions prepared by alumina chromatography. A positive response might be obtained if the PAH fraction of HD were assayed now.

TABLE 7.5. Activity of Chemical Class Fractions in the Ames, SHE, CHO, and I/P Assays Relative to SRC-II HD

Test Material	Assay			
	Ames	SHE	CHO	I/P
800-850°F Crude	148	25	86[a]	49[b]
Aliphatic Fraction	0.0	3	ND	10
Neutral PAH	0.0	36	ND	158
NPAC	700	15	ND	79
HPAH	0.0	0.0	ND	---[b]
>850°F Crude	450	105	200	314
Aliphatic Fraction	0.0	10	129	(1000)[c]
Neutral PAH[d]	5	100	571	588
NPAC[e]	375	52	257	245
HPAH[f]	50	3	243	---[b]

[a]Not determined

[b]Not a significant response

[c]Obtained by a large extrapolation of a small response as a result of the small amount of fraction tested.

[d]Polyaromatic hydrocarbon

[e]Nitrogen-containing polycyclic aromatic compounds

[f]Hydroxy-containing polycyclic aromatic hydrocarbons

Quantitatively, there is less concordance among the results from the various assays. In some cases, there may be good agreement between sets of assays; in other instances, there may be almost no quantitative agreement. This may not be surprising since the endpoints differ and result from many different mechanisms. Moreover, the scientific community does not fully agree as to how to calculate carcinogenic potency. The antagonisms that we demonstrated among components of some of these complex mixtures further complicate the situation. Quantitative agreement among assays may, therefore, be an unreasonable expectation and the occurrence of such agreement may be fortuitous.

Despite the lack of quantitative agreement among the various assays, we can now make some suggestions about the deployment of the in vitro assays. For general screening of a large number of materials, the Ames assay should be used because it is rapid, inexpensive and uses small amounts of material. If a positive response is obtained, it is highly likely that the material will prove to be carcinogenic in mouse skin-painting assays. A negative response in the Ames assay should not lead to the conclusion that the material has no carcinogenic potential. Rather, the material should be subjected to further testing, using the CHO cell, the I/P, or chronic skin-painting assays. For practical reasons, chronic skin-painting assays testing would be reserved for

TABLE 7.6. Relative Ranking of the Biological Activity of Chemical Class Fractions Prepared from Selected Distillate Cuts

Test Material	Assay			
	Ames	SHE	CHO	I/P
800-850° Crude	4	5	6	5
Aliphatic fraction	7-10	8	ND[a]	9-10
Neutral PAH[b] fraction	7-10	4	ND	3-4
NPAC[c]	1	6	ND	6
HPAH[d]	7-10	10	ND	9-10
>850° Crude	2	1	4	2
Aliphatic fraction	7-10	7	5	7
Neutral PAH[b] fraction	6	2	1	1
NPAC	3	3	2	3-4
HPAH	5	9	3	8

[a]Not determined

[b]Polyaromatic hydrocarbon

[c]Nitrogen-containing polycylic aromatic compound

[d]Hydroxy polycyclic aromatic hydrocarbon

materials which are deemed to be approaching commerical production and for which values for an integrated carcinogenic response are desired. However, for purposes of broadening the data base for comparing results from various assays, it is desirable to have chronic skin-painting assay results for additional materials.

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