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Physical and Chemical Characterization of SRC Toxicology Program Samples

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Printed in the United States of America. Available from
National Technical Information Service
U.S. Department of Commerce
5285 Port Royal Road, Springfield, Virginia 22161
NTIS price codes—Printed Copy: A04 Microfiche A01

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ORNL/TM-10204
Dist. Category UC-90d

PHYSICAL AND CHEMICAL CHARACTERIZATION OF SRC TOXICOLOGY PROGRAM SAMPLES

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Date Published - November 1986

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EXECUTIVE SUMMARY

Coal-derived liquids and solids from the Wilsonville Solvent Refined Coal (SRC) pilot plant have received extensive biological assays at the International Research and Development Corporation (IRDC) as a part of the SRC Toxicology Program of the U. S. Department of Energy Office of Fossil Energy. The studies at IRDC were conducted under a subcontract from Air Products and Chemicals, Inc.

Three of the materials studied at IRDC required additional chemical and physical characterization to support interpretation of the bioassay results. A solid product from the Solvent Refined Coal process (SRC Solid) was observed to produce lung tumors in an inhalation bioassay using rats. The properties of the sample which might contribute to that response were not well characterized. Physical and chemical properties relevant to inhalation toxicity of the SRC Solid were determined in this laboratory. In a mouse skin painting assay of middle distillates derived from the SRC and two-stage liquefaction (TSL) processes, the TSL Middle Distillate was observed to produce much greater dermal irritation than did the SRC Middle Distillate. The hypothesis that the difference in skin irritation was a result of differences in their phenols contents was tested by comparative phenols analyses.

The SRC Solid was physically characterized by scanning and transmission electron microscopies and x-ray diffraction. The inorganic chemical composition was surveyed by both x-ray fluorescence and neutron activation analysis, and selected four to six-ring polycyclic aromatic hydrocarbon (PAH) dermal tumorigens were determined chromatographically. Some chemical comparisons were made with solid byproducts from other coal liquefaction processes. Potential bioavailability of the PAH in the SRC Solid was examined in leaching studies.

The SRC Solid was found to consist of ca. 3 μm and smaller rounded triangular and rectangular solid particles having an amorphous, noncrystalline organic composition. Very little inorganic matter was present. Iron (1,190 μg/g), sodium (1,450 μg/g), and zinc (730 μg/g)

were the major elements detected. With the possible exception of zinc, none appeared to be toxicologically important. These concentrations were much lower than those in samples of H-Coal vacuum bottoms flaked product.

Several PAH dermal tumorigens were determined in the SRC Solid, including benz(a)anthracene (12 μg/g), chrysene (31 μg/g), benzo(b+j)fluoranthenes (97 μg/g), benzo(k)fluoranthene (12 μg/g), benzo(e)pyrene (160 μg/g), benzo(a)pyrene (160 μg/g), indeno(1,2,3-cd)pyrene (110 μg/g), dibenz(a,c+a,h)anthracenes (98 μg/g), and benzo-(ghi)perylene (280 μg/g). These levels were one-half to one-third those in solid byproducts from the SRC-II, H-Coal, and Exxon Donor Solvent processes.

The leaching behavior of the PAH in the SRC Solid was studied in three media: a Tween-80 surfactant solution, minimum essential medium with fetal bovine serum (MEM/serum), and a dipalmitoylphosphatidylcholine (DPPC) vesicle suspension. The concentrations of the PAH leached into the media generally exceeded their water solubilities by factors of two to four or more. Concentrations of individual PAH ranged as high as 10 to 24 μg/l. The ratios of the PAH concentrations in the leachates were somewhat different from those in the SRC Solid itself. This probably reflects the different solubilities of the various PAH. It appeared that equilibrium concentrations of all but the largest PAH were reached in less than five days of leaching. The kinetics of dissolution of the larger PAH may be slower than for the smaller PAH. Analysis of DPPC vesicles separated from the suspension by ultracentrifugation suggested that the PAH concentrations in the vesicles were ca. 10^2 times greater than in the bulk leachate. Concentrations of some PAH in the vesicles ranged from one to two μg/g. These results suggest that there was considerable potential for a substantial leaching of PAH from the SRC Solid and accumulation of the leached PAH in cell membranes over the course of the rat inhalation exposure experiment.

The phenol and cresols concentrations in the SRC Middle Distillate and the TSL Middle Distillate were compared by a high performance liquid chromatography procedure. The SRC Middle Distillate was found

to contain phenol (2.4 mg/g), p+m-cresols (30.6 mg/g), o-cresol (4.2 mg/g), 3,4-dimethylphenol (4.3 mg/g), 2,5-dimethylphenol (14.2 mg/g) and at least five other C_2 or C_3-phenols. In contrast, none of these phenols were detected at a sensitivity of ca. 0.5 mg/g in the TSL Middle Distillate. Other compounds must be responsible for the greater dermal irritancy of the latter. Hydroaromatics such as tetralin and decalin are possible candidates.

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INTRODUCTION

Extensive biological assays of synthetic fuel materials derived from recent pilot plant operations have been conducted for the U.S. Department of Energy, Office of Fossil Energy, at the International Research and Development Corporation (IRDC) in Mattawan, MI, under a subcontract from Air Products and Chemicals, Inc. (APCI), Allentown, PA. Among the synthetic fuels subjected to bioassay at the IRDC were a solid product from the Solvent Refined Coal (SRC-I) process ("SRC Solid"), and two middle distillates from the SRC and Two-Stage Liquefaction (TSL) processes. The SRC Solid was subjected to a two-year inhalation bioassay with rats. The two middle distillates were examined in a two-year skin-painting bioassay using mice. Many of the chemical and physical properties of these materials have been determined (1) by APCI and by the Pacific Northwest Laboratory in Richland, WA.

Further characterization of the study materials was required to support the interpretation of several significant observations reported by IRDC investigators. The SRC Solid produced lung tumors in a large number of animals in the high dose group of rats exposed via inhalation. Pathologists observed tumors which almost certainly were squamous cell carcinomas in five animals after ca. 24 months of exposure. The causes of this response are not clear but could include chemical or solid state carcinogenesis. The IRDC investigators also reported that the high dose group of animals skin-painted with the TSL Middle Distillate exhibited much greater dermal irritation than did the animals exposed to the high dose of SRC Middle Distillate. The causes of this differential response are not known. One hypothesis was that the TSL Middle Distillate has a greater content of phenols than does the SRC Middle Distillate.

The Oak Ridge National Laboratory (ORNL) has been assisting the DOE Oak Ridge Operations Office in its oversight responsibilities for the APCI program by reviewing reports and participating in the program review meetings. The scope of work at ORNL was expanded to include a

determination of some of the chemical and physical factors needed to support the interpretation of the bioassay findings at IRDC. This report describes the characterization of selected chemical and physical properties of the SRC-I Solid Product which are relevant to its tumorigenic response in inhalation bioassay, and a determination of phenolics which might contribute to the different degrees of skin irritation observed in the skin-painting bioassay of SRC and TSL Middle Distillates.

MATERIAL HISTORY

The materials examined in this study were obtained from the stocks at IRDC which were subjected to bioassay. A brief description of the sample origin and collection procedures is given below to document the sample histories.

SRC-I Solid

The Hydrotreater Unit SRC-I Solid (SRC Solid) is identified as International Coal Refining Company (ICRC) ICRC/SCS sample no. 937-083(GL), Wilsonville sample no. 80962, from the Wilsonville Solvent Refined Coal plant. The material was collected on December 20-21, 1981, at sample location V-1082, during run no. 235, a demonstration plant run on Kentucky no. 9 coal. Three drums were shipped to Air Products and Chemicals, Inc. (APCI) Houdry Laboratories in Linwood PA, where they were stored before transfer to IRDC. A subcontractor, Sturtevant Mill company in Boston, MA, air-micronized the SRC-I Solid to a particle size range which produced an equivalent aerodynamic diameter of ca. 3 μm when aerosolized in the IRDC inhalation exposure generator. Documentation for the sample is reported elsewhere (1).

Several samples of the SRC-I Solid were obtained from IRDC. Three bulk samples were collected on May 16 (ca. 50 g) and August 2, 1985 (2x ca. 100 g) in glass bottles directly from the drum used to store the SRC-I Solid for inhalation studies. These samples were used in studies of elemental content and of leachable polycyclic aromatic hydrocarbons (PAH). Samples also were collected from the inhalation chambers at IRDC for examination of particle structure and morphology. These were collected with the help of IRDC personnel on May 16, 1985, from the high-dose group IV male rat inhalation exposure chamber, where the SRC-I Solid was being aerosolized at a nominal concentration of 50 mg/m^3. The chamber conditions at the time of sample collection were a temperature of 22°C, relative humidity of 63%, and a pressure of -2.5 cm of water relative to ambient. Particulate aerosol samples were

collected by filtration on 13 or 25 mm polycarbonate Nucleopore filters with 0.1 μm pore diameter (catalog no. 110405, lot CPR 02500), supported by P100 backup filters, and contained in 13 mm or 25 mm plastic Swinny filter holders. Filters with pore diameters of 0.05 and 0.5 μm also were used. The aerosol was sampled at a rate of ca. 500 cm^3/min. for 2.5 to 10 min. A small bulk sample (ca. 25 g) of the SRC-I Solid was collected from the sample feed hopper of the aerosolizing apparatus for the group IV inhalation chamber. This sample was used in some of the microscopic examinations where the mass of particulate sample collected from the inhalation chamber aerosol was insufficient.

Middle Distillates

Two samples of middle distillates which were subjected to mouse skin painting bioassay at IRDC were obtained for characterization of phenol and cresols. One was an SRC Middle Distillate, ICRC sample no. 2112CB. It was derived from SRC Light Oil (ICRC/SCS reference no. 940-086(A), Wilsonville sample no. 80960) collected on November 11, 1981 at sample location V-203 during Wilsonville run no. 235 on Kentucky no. 9 coal, and from SRC Process Solvent (ICRC/SCS reference no. 941-087(A), Wilsonville sample no. 80026) collected on February 8, 1982 at sample location V-131(A) during Wilsonville run no. 236 on Illinois no. 6 coal. A drum of each was supplied to APCI at Linwood, PA from the shipment obtained by the Hometown, PA facility. A blend consisting of 57.3 wt.% of the SRC Light Oil and 42.7 wt.% of the SRC Process Solvent was distilled by APCI in a 454 l (120 gallon) stainless steel pot equipped with a 10 cm (4 in.) diameter, 4.6 m (15 ft), 20 plate column. The distillate fraction with boiling range of 204-343°C (400-650°F) was taken as the no. 2112CB SRC Middle Distillate.

The Two Stage Liquefaction Middle Distillate (sample no. 2113CB) was derived from HTR Light Oil (ICRC/SCS reference no. 940-086(E), Wilsonville sample no. 80958) collected at sample location V-161 in the period of December 21, 1981 through January 8, 1982 during Wilsonville

run no 235, and from HTR Process Solvent (ICRC/SCS reference no. 940-086(F), Wilsonville sample no. 80959) collected at sample location V-1074 in the period of November 15-16, 1981, during Wilsonville run no. 235. A blend of 33.9 wt.% of the HTR Light Oil and 66.1 wt.% of HTR Process Solvent were distilled by APCI, and the 204-343°C (400-650°F) distillate was taken as TSL Middle Distillate. Approximately 100 g of each middle distillate sample in borosilicate glass was shipped under referigeration to ORNL on May 30, 1985 by IRDC. These samples are documented in greater detail in reference (1).

PHYSICAL AND CHEMICAL CHARACTERIZATION OF SRC SOLID

The SRC Solid was characterized for factors relevant to inhalation toxicity, which included a microscopic examination of its structure and morphology, elemental screening, and leachable PAH. The procedures and findings of these characterizations are discussed below.

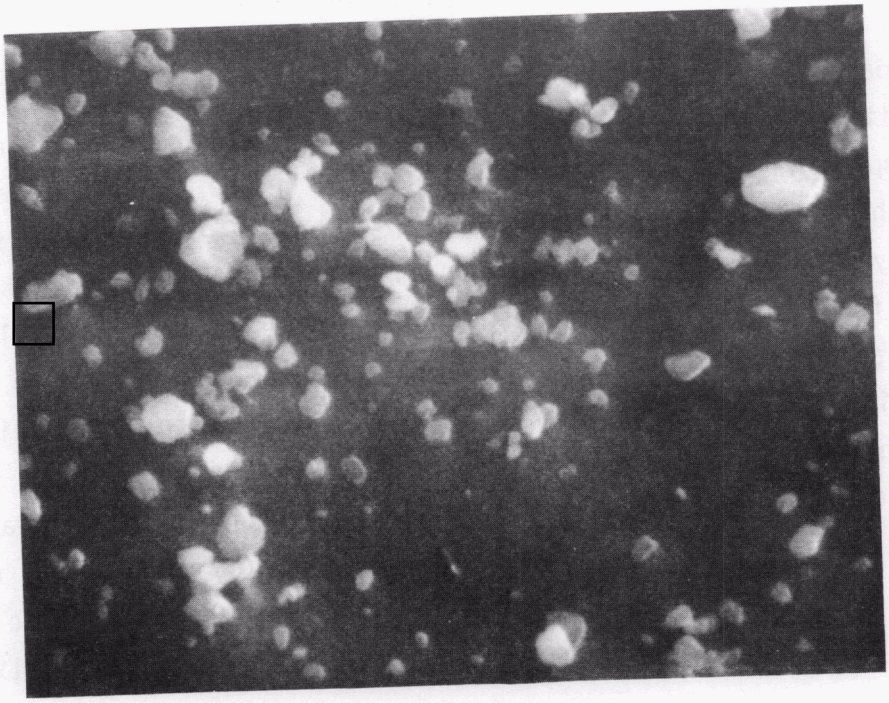
Particle Structure and Morphology

The particle structure and morphology of the aerosolized SRC Solid were examined using both scanning and transmission electron microscopy (SEM and TEM, respectively) and x-ray diffraction. The membrane filter samples of the SRC Solid aerosol collected from the inhalation exposure chamber at IRDC were mounted on carbon stubs using two-sided tape and were coated with carbon using a Vactronic Lab Equipment device (East Northport, NY). SEM was conducted using an ISI Super III SEM (Santa Clara, Ca) at magnifications of 3,000 to 12,000X. Energy-dispersive X-ray fluorescence (EDX) analysis was conducted with an Ortec (Oak Ridge, TN) Si(Li) detector over a range of 0-10 keV, although the range of 0-20 keV was examined with one sample.

Figure 1 shows the SEM microphotographs of a filter at magnifications of 3,000 and 6,000X. The field of view magnified in (B) is included in (A). The geometric particle size diameters appear to range from ca. 0.3 to 4 μm, which is consistent with the ca. 3 μm equivalent aerodynamic diameter determined for the aerosol by IRDC.

The aerodynamic diameter is affected by the particle size, shape, and density. The smallest particles are round, while the largest particles are triangular or rectangular in shape, with rounded corners presumably from the abrasion during the grinding and sizing of the SRC Solid. The particles appear to be platelike or flat in cross-section. The surface structure of the particles is not clearly visible in the microphotographs because the limit of resolution of this instrument is being approached. However, the surfaces do not appear to have appreciable roughness or prominent features.

A.



B.

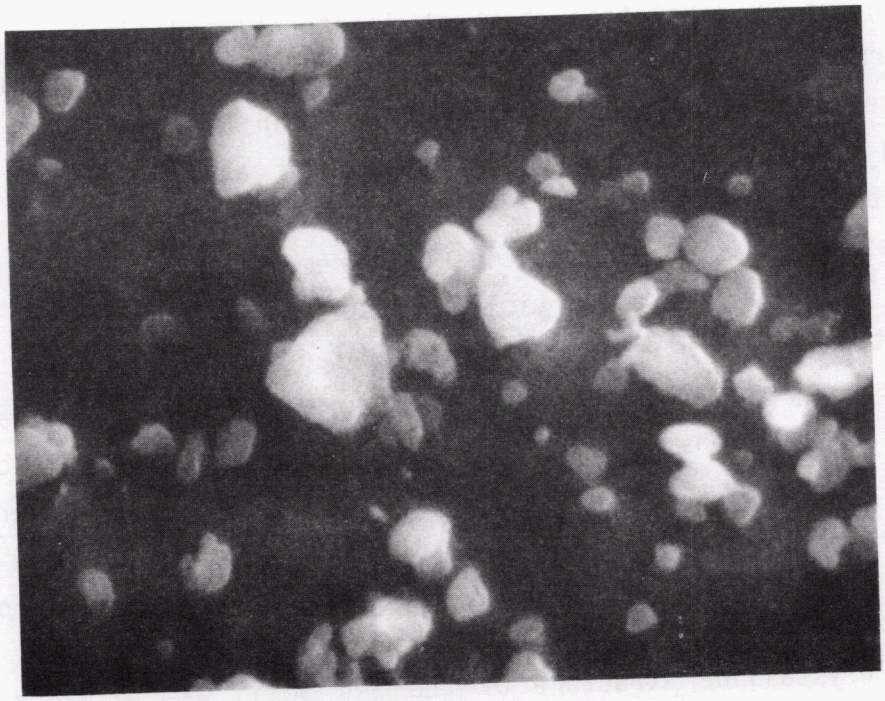
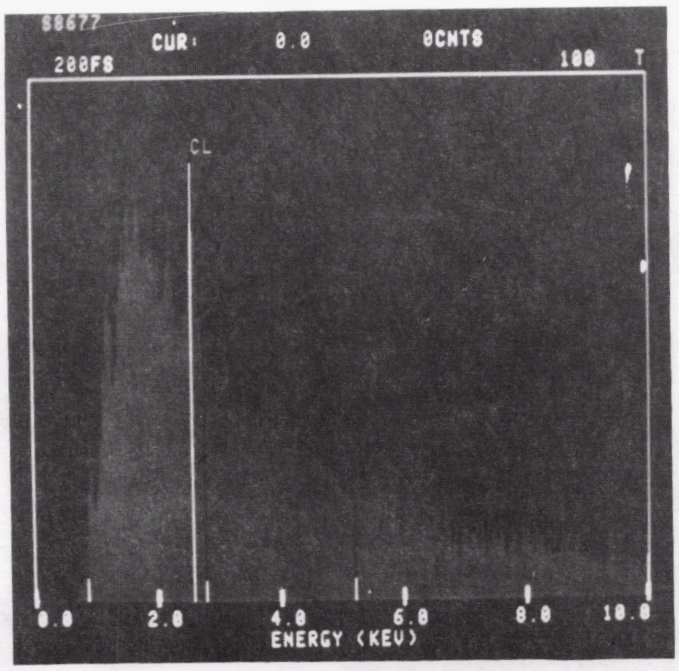


Figure 1. Scanning Electron Microphotographs of SRC Solid Particles Samples from Inhalation Exposure Chamber at IRDC.
A. 3,000 X (3 μm ⊢—⊣)
B. 6,000 X (3 μm ⊢———⊣)

EDX spectra of the blank filter and of a particle from Figure 1(A) are compared in Figure 2. The main feature of both spectra is the broad, diffuse background (Bremsstrahlung). The chlorine detected in both samples is attributed to the adhesive in the two-sided tape used to fix the filter onto the carbon stub. The only element detected with certainty in the SRC Solid sample is silicon, which undoubtedly is derived from a trace of mineral matter in the particles. Silicon is present at less than ca. 0.1% concentration. Magnesium also way be present in the SRC Solid, but the peak is difficult to distinguish from instrumental noise. A more detailed and sensitive analysis of the trace elemental content was achieved by neutron activation analysis (see next section), but it is evident from these results that the SRC Solid is predominantly an organic material, and that inorganic matter is not present at appreciable concentrations (< ca. 0.1% each element).

TEM of the samples was conducted by slurrying a few mg of SRC Solid (collected from the sample feeder of the aerosolization apparatus on the group IV inhalation chamber) in ethanol and placing a drop of the slurry on a copper stub coated with carbon. The ethanol was evaporated using the heat from a small tungsten lamp. The specimen was examined using a JEOL JEM-100CXII instrument at 100 keV and 17,400X magnification. Figure 3 shows a TEM microphotograph of the sample. Some dissolution of the particles occurred, judging from the more rounded edges of these particles versus those observed in the SEM microphotographs. However, the irregular shapes of most of the particles suggest that only the outer layers were dissolved, and that complete dissolution did not occur. The shadows of the particles are uniform, except where the image is obviously of two or more overlapping particles. There is no evidence of internal voids or cavaties, such as might be present if the particles were hollow or if they contained droplets of liquid.

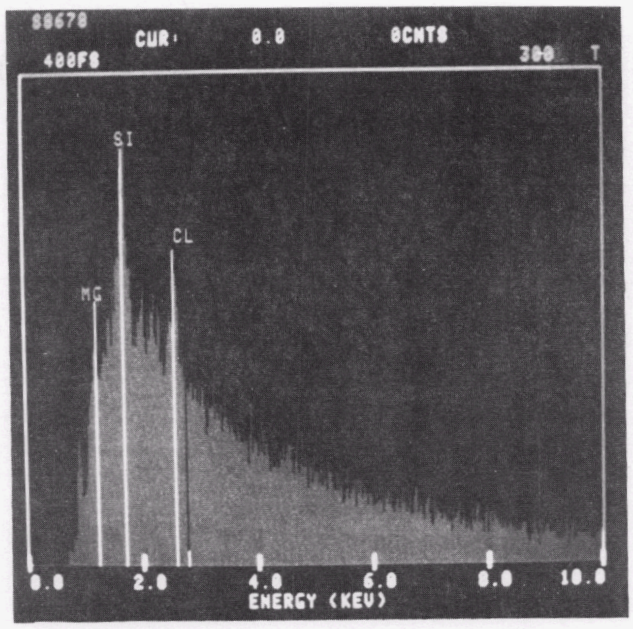
The crystalline structure of the SRC Solid was examined with a Norelco X-Ray Diffraction Unit (Phillips Electronics). The SRC Solid collected from the sample feed hopper of the group IV inhalation



A.

Figure 2. X-Ray Fluorescence Spectra of A. Blank Filter and B. SRC Solid Particle on Filter from Inhalation Exposure Chamber Sampling at IRDC.

B.



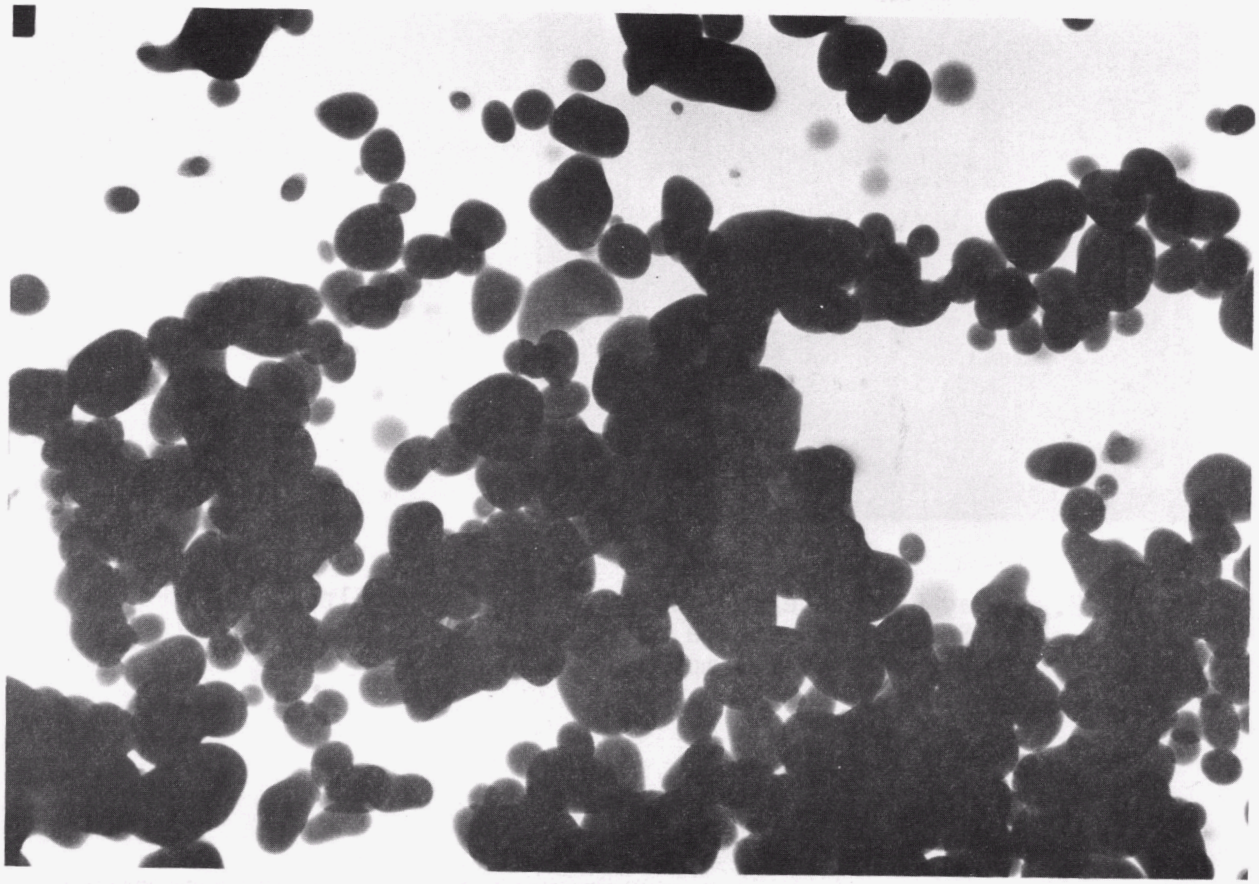


Figure 3. Transmission Electron Microphotograph of SRC Solid Particles Sampled from Dust Feeder on Aerosol Generator at IRDC (10,400 X, 1 μm ⊢——⊣)

chamber at IRDC was placed in a glass capillary tube and set in the center of the powder diffraction camera. The sample was rotated as the diffraction pattern was recorded. The X-ray diffraction of the SRC Solid was found to be diffuse, with no evident structure pattern. This indicates that the SRC Solid is an amorphous material, not a crystalline solid. This result is consistent with a predominantly organic carbon matrix.

Elemental Screening

To examine the SRC Solid in more detail for inorganic species which might contribute to its inhalation tumorigenicity, the elemental composition was screened by instrumental neutron activation analysis. This method is capable of determining more than 40 elements cost-effectively. The procedures used were similar to those reported elsewhere (12). Approximately one g of SRC Solid collected from the storage drum at IRDC was irradiated for 20 sec in the Oak Ridge Research Reactor. The radioactivity in the sample was allowed to decay a few minutes, and then a gamma ray spectrum was obtained on a Nuclear Data 6620 Data Acquisition System to identify and measure short-lived radionuclides. Elements that produce longer-lived radionuclides were determined by reirradiating the sample for 30 min and counting the sample after 2 to 3 days and again after 3 weeks.

Table 1 compares the results of the elemental screening of the SRC Solid with those reported previously (11) for the vacuum bottoms flaked product (VBFP) from the H-Coal pilot plant at Catlettsburg, KY. The data for the latter are for samples collected during a run on the same type of feed coal as used at the Wilsonville SRC plant (Kentucky no. 9 coal), and during a run under better, lined-out operation on a different coal type (Illinois no. 6 coal). The main observation from these data is that the SRC Solid has much lower concentrations of inorganic matter than does the H-Coal VBFP. This result is consistent with the processing histories of the samples. The SRC Solid was produced as a deashed, cleaned coal solid product, while the H-Coal

Table 1. Comparison of NAA trace element screening of SRC solid product and H-Coal vacuum bottoms flaked product

Element	Concentration,[a] μg/g		
	SRC solid product[b]	No. 595 H-Coal VBFP[c]	No. 889 H-Coal VBFP[d]
Na	1,450	286	1,120
Al	194	14,000	23,500
Cl	29.8	65	1,020
Sc	0.06	3.5	5.2
Ti	125	779	1,110
V	5.9	60	60.3
Cr	15.9	20	37.5
Mn	5.7	33.3	141
Fe	1,190	23,000	30,900
Co	3.5	6.0	7.3
Zn	730	≤80	136
Ga	14.2	4.2	6.1
As	9.9	4.0	2.4
Br	28.1	1.7	5.8
Mo	42.6	6.4	42.3
Sb	0.3	2.5	9.9
La	3.2	9.2	10.4
W	56.9	1.0	0.8
U	2.3	3.2	5.8
Mg	ND	679	987
K	ND	3,100	3,790
Ca	ND	2,200	12,800
Se	ND	2.5	3.7
Rb	ND	20	42.3
I	ND	≤0.9	0.88
Cs	ND	1.2	2.1
Ba	ND	60	93.2
Ce	ND	17	24.0
Nd	ND	1.9	9.1
Sm	ND	ND	2.5
Eu	ND	ND	0.5
Tb	ND	0.2	0.2
Yb	ND	0.5	1.0
Hf	ND	0.7	1.1
Ta	ND	0.2	0.2
Au	ND	<0.007	ND
Th	ND	2.1	3.7

[a]ND = not detected.
[b]Solid product derived from KY No. 9 coal.
[c]Vacuum bottoms flaked product (VBFP) from Catlettsburg, KY run No. 7 on KY No. 9 coal (data from reference No. 11).
[d]VBFP from Catlettsburg, KY run No. 8 on IL No. 6 coal (data from reference No. 11).

VBFP is a byproduct containing the ash and inorganic matter from the feed coal in the H-Coal process. The major elements determined in the SRC Solid were sodium (1,450 μg/g), iron (1,190 μg/g), and zinc (730 μg/g). All other metals detected were found at concentrations of less than 200 μg/g, and most were present at less than ca. 60 μg/g. Toxicologically, only zinc (a priority pollutant metal) might be of concern.

PAH Dermal Tumorigens

The concentrations of selected four to six-ring PAH dermal tumorigens were determined in the SRC Solid because of the strong association (2-4) of certain PAH with skin tumorigenicity, particularly those PAH localized in high-boiling coal liquid distillate fractions (> ca. 350-400°C, > ca. 650-700°F) (4-6) which include many known carcinogenic PAH (7,8). The comparison of the PAH dermal tumorigen content of this sample with those of other solid coal-derived samples which have not been subjected to such an extensive inhalation bioassay would suggest the need (if any) for bioassay of those other samples.

The analytical procedure involved sequential high performance liquid chromatography (HPLC) and capillary column gas chromatography (GC) (8,9). Briefly, a one g sample of bulk solid was dissolved in a minimum volume of tetrahydrofuran, and a known activity of carbon-14 labeled benzo(a)pyrene was added. High molecular weight material was precipitated from the solution by addition of pentane. The filtrate was reduced in volume, and made up to 10 mℓ in methylene chloride. A 0.25 mℓ aliquot was separated on a semipreparative scale, normal phase HPLC column (9.4 mm ID X 25 cm, Partisil PAC 10 Magnum 9 10/25 bonded phase from Whatman, Inc., Clifton, NJ). The volume of 10% (vol./vol.) methylene chloride in hexane eluate starting at four min before the elution time of benz(a)anthracene and extending through 10 min after the elution of benzo(ghi)perylene was collected and concentrated to 0.3 or 0.1 ml along with a known mass of 1,1'-biphenyl internal standard. The recovery of the procedure was determined by measuring the carbon-14

labeled benzo(a)pyrene with liquid scintillation spectroscopy. The PAH were tentatively identified and measured by capillary column GC using the method of internal standards. The HP 5880 GC (Hewlett-Packard, Palo Alto, CA) was equipped with a 30 m x 0.25 mm ID 0.25 μm film thickness DB-5 bonded phase fused silica column and a flame ionization detector. The column oven was held at 100°C for 3 min and was programmed to 180°C at 25°C/min, and then to 280°C at 2°C/min and held at 280°C for 20 min. The hydrogen carrier gas flow rate was 1.5 mL/min. The identifications of the PAH were confirmed by gas chromatography-mass spectroscopy (GC-MS) under similar chromatographic conditions.

Comparisons of the concentrations of selected four- to six-ring PAH dermal tumorigens in the SRC Solid with those in solid byproducts from the SRC-II, H-Coal, and EDS processes are presented in Table 2. All four samples are distillation residues, but the SRC Solid is a deashed product whereas the others are ash-containing byproducts. The distributions of the PAH isomers are similar, but not the same. Benzo(ghi)perylene, benzo(a)pyrene, and benzo(e)pyrene are major species in all the samples. However, the ratios of benzo(a)pyrene to benzo(e)pyrene and benz(a)anthracene to chrysene are quite different in all the samples. The SRC Solid also has the lowest concentrations of benz(a)anthracene and chrysene relative to the other PAH. The main observation is that the concentrations of these PAH in the SRC Solid are much lower than those in the other samples. The sum of these PAH are one-half to one-third those of the other samples, and for some of the individual PAH, the differences are even more pronounced. The lower PAH content of the SRC Solid may be attributed in part to the removal of asphaltenes during critical solvent deashing. This finding suggests that those other materials have a potential for a stronger tumorigenic response than the SRC solid. Their inhalation toxicity should be investigated. Certainly, other factors such as particle retention in the lungs, bioavailability of tumorigenic agents, presence of promoters, etc. would influence the responses, and a sound prediction based on PAH content alone cannot be made.

Table 2. Comparison of selected four- to six-ring PAH dermal tumorigens in SRC Solid and coal-derived solid byproducts

PAH	Relative tumorigenicity[a]	Concentration, μg/g			
		SRC solid[b]	No. 1704 SRC-II vac. btm.[c]	No. 889 H-Coal VBFP[d]	No. 1659 EDS VTB[e]
Benz(a)anthracene	+	12	140	190	280
Chrysene	±	31	180	240	270
Benzo(b/j)fluoranthenes	++ to +++	97	270	300	170
Benzo(k)fluoranthene	++ to +++	12	ca. 80	ca. 90	90
Benzo(e)pyrene	±	160	360	330	180
Benzo(a)pyrene	++++	160	290	360	360
Indeno(1,2,3-cd)pyrene	+	110	450	350	150
Dibenz(a,c/a,h)anthracenes	+++/±	98	35	91	50
Benzo(ghi)perylene	±	280	1,100	830	310
Total		960	2,905	2,781	1,860

[a]From references (7) and (8).
[b]From Wilsonville, AL run no. 235 on KY no. 9 coal in SRC-I mode. Two replicates.
[c]From Fort Lewis, WA run in March-April, 1980 on Powhatan no. 6 coal in SRC-II mode. One determination only.
[d]Vacuum bottoms flaked product (VBFP) from Catlettsburg, KY run no. 8 on IL no. 6 coal in fuel oil mode. Two replicates.
[e]Vacuum tower bottoms (VTB) from ECLP, Baytown, TX in June-August, 1982 run on TX Lignite in Bottoms Recycle mode. Two replicates.

POLYCYCLIC AROMATIC HYDROCARBON LEACHING STUDIES OF SRC SOLID

It has been reported (13-16) that carcinogenic PAH adsorbed on inhaled particulates must first be transported into the target membranes before biological effects can be exerted. They are subsequently metabolised by membrane-bound enzymes. Because of their nonpolar, lipophilic nature, PAH are likely to partition from particles into lipid bilayers of cell membranes. Phospholipids are one of the major components of lung surfactants. One of the in-vitro models for the membrane bilayer consists of phospholipid vesicles. It is felt that leaching of particulate PAH by surfactants such as phospholipids can provide useful information on their bioavailability.

In this study, three media were selected as leaching materials to assess the potential rates and extraction efficiencies of selected PAH dermal tumorigens from the SRC Solid. A nonionic surfactant, Tween 80 (polyoxyethylene sorbitan monooleate) was used to evaluate the leaching procedure as well as the analytical methodology. This surfactant previously has been employed (17) to increase the compatibility of oil samples with bacterial mutagenicity testing media. Included in this study was a tissue culture medium (a mixture of Eagle's minimum essential medium with fetal bovine serum) which has been used (18) as a leaching medium in studies of the bioavailibility of PAH coated on coal fly ash samples. Dipalmitoylphosphatidylcholine (DPPC), a major component of pulmonary surfactant (19), also was used as a leaching medium. DPPC vesicles have been employed in studies of membrane uptake of PAH from particulate matter (13-16) and of the physical and chemical interactions of lung surfactants with air pollutants and fly ash (20,21).

Leaching Media

Tween 80: A 0.13% solution of Tween 80 in Tris buffer (0.01 M Tris-amine, 0.05 M KCl, pH 7.50) was prepared by sonication of a known quantity of Tween 80 in a buffer solution for 10 min at room temperature.

Minimum essental medium: a mixture of 10% (vol/vol) fetal bovine serum in Eagle's minimum essential medium (MEM) was prepared by mixing the two freshly thawed reagents at room temperature. The MEM/serum was stored at -4°C. A wetting agent of 2% Tween 80 in Hank's buffer solution was prepared by sonication for 10 min at room temperature (18).

Dipalmitoylphosphatidylcholine: DPPC vesicles were prepared by sonication of a suspension of DPPC at a concentration of 10 mg/ml in a buffer (0.01 M Tris-amine, 0.05 M KCl, pH 7.50). The milky suspension was heated for one hr at 50°C (14,15).

Leaching of SRC Solid

The bulk SRC Solid sample was leached at a concentration of approximately 1 mg of solid per ml of solution. The leaching medium was gradually added to the weighed solid with intermittent shaking or ultrasonication to form a homogenous suspension. In the leaching with MEM/serum, the solid particles were first wetted in the wetting agent prior to addition of the MEM/serum mixture. A 1.5 ml volume of wetting agent was used per 100 ml of MEM/serum. Generally, the leaching was conducted in the dark with volumes of 50, 100, or 150 ml in 100, 150, or 250 ml Erlenmeyer flasks placed in a 37°C water bath shaker. These conditions were selected to model animal lung conditions.

At predetermined time intervals (5 days, 2, 4, or 6 weeks) a 50 ml aliquot of the SRC Solid leaching suspension was removed from the water bath and processed for analysis. In a separate Tween 80 sequential leaching study, on every fifth day of leaching, the suspension was centrifuged with a clinical benchtop centrifuge (model CL International Equipment Co., Needham, MA) and the supernatant was removed for analysis. Fresh Tween 80 solution was added to the precipitate to continue the leaching.

Blanks for all three leaching media were prepared as above (except that the SRC Solid was omitted). Leaching was run for five days before analysis.

Collection, Isolation, and Analysis of Leached PAH

After the SRC Solid leaching suspension was removed from the shaking water bath, either the entire sample or a 50 ml portion was centrifuged in a clinical benchtop centrifuge to precipitate the solid particulate matter. The supernates from the Tween 80 and MEM/serum leachates were then filtered through a 1 μm Teflon filter which had been prewashed with isopropanol to reduce its hydrophobic characteristics. The supernate from the DPPC leachate was further centrifuged in a Beckman model L-2 ultracentrifuge (Beckman Instruments, Berkely, CA) for 1 hr at 27,000 rpm (ca. 90,000 G) in a type 30 rotor.

Nonpolar fractions of the leachates were collected by pumping measured volumes of the centrifuged and filtered leachates through a 4.6 mm ID x 3.7 cm, RP-18 Sphere-10 reverse phase guard column cartridge (Brownlee Labs, Inc., Santa Clara, CA) at a flow rate of 1 ml/min and pressure of 600 psig. The column was then washed with an equal volume of water, and the nonpolar fraction was eluted with an equal volume of methanol. The methanol eluate was evaporated to dryness in a rotary evaporator at 50°C, transferred with methylene chloride to a tared vial, the solvent was evaporated again in a stream of nitrogen, and the nonpolar fraction residue was weighed.

A nonpolar fraction was separated from the DPPC precipitate. The top layer of the DPPC precipitate from the ultracentrifugation was carefully pipetted off of the underlying SRC Solid which had not been removed by the low speed benchtop clinical centrifugation. The DPPC precipitate was suspended in methanol and dried in a rotary evaporator. The last traces of water were removed azeotropically by evaporating with ethanol and benzene. The residue was dissolved in ca. 50 ml of benzene, washed with eight 50 ml volumes of Tris buffer, and combined with the subsequent benzene backwashes of the Tris buffer. The combined benzene solution was dried over calcium chloride, filtered through a fine porosity sintered glass funnel, and the solvent was removed.

A subfraction enriched with four to six-ring PAH dermal tumorigens was separated from the nonpolar fractions using the same semipreparative scale, normal phase HPLC procedure as described in the previous section. The PAH analyses were conducted by capillary column GC using the method of internal standards, and were corrected for any interferences from the blank. Recoveries were estimated from separate experiments utilizing carbon-14 labeled benzo(a)pyrene. PAH identifications were confirmed by GC-MS.

Results

An evaluation of the efficiency of the PAH collection and fractionation procedures was conducted by measuring the recovery of carbon-14 labeled benzo(a)pyrene added to SRC Solid leachate samples. The data in Table 3 indicate an overall recovery of ca. 65% for the entire procedure. The collection efficiency ranged from 70 to 94%, and the fractionation efficiency varied from 69 to 92%. The PAH from the DPPC leaching were contained in both precipitate (DCCP vesicles) and suspension (DPPC vesicles not removed by ultracentrifugation, plus aqueous phase) phases from ultracentrifugation of the supernatant from the benchtop centrifugation of the DPPC leachate. In spite of this split, the overall recovery of the PAH from the DPPC medium was the same as those for the other two media. Although this level of radiotracer recovery is not optimum, it is within a range which can be used reasonably to correct the measured PAH concentrations.

The leaching behavior of selected four to six-ring PAH dermal tumorigens from the SRC Solid is presented in Tables 4-6 for the Tween 80, MEM/serum, and DPPC vesicles media (respectively). Because of the relative ease in its analytical preparation and its low blank, the Tween 80 surfactant medium was used the most extensively to study PAH leaching behavior.

In the continuous leaching experiment, the SRC Solid was leached continuously but statically with the Tween 80 medium, and samples were taken for analysis at preselected time intervals to determine the time

Table 3. Recovery of carbon-14 labeled benzo(a)pyrene from leaching media

Step	Tween 80	MEM[a]	DPPC[b]		
			Suspension	Ppt.	Total
Collection	86.4	70.2	9.4	84.8	94.2
Fractionation	74.4	92.1	68.8	68.8	
Overall	64.3	64.7	64.8		

[a]Minimum essential medium with fetal bovine serum.
[b]Dipalmitoylphosphatidylcholine vesicles.

Table 4. Comparison of continuous and sequential leaching of PAH from SRC Solid with Tween 80 surfactant

PAH	Concentration,[a] µg/L							
	Continuous leaching				Sequential leaching[b]			
	5d	2 wk	4 wk	6 wk	1st	2nd	3rd	4th
Benz(a)anthracene	5.0	I	2.2	2.7	5.0	8.0	2.5	1.1
Chrysene	6.2	4.1	5.1	5.4	6.2	6.5	4.0	3.2
Benzo(b/j)fluoranthenes	7.8	7.3	12	9.6	7.8	18	15	9.5
Benzo(k)fluoranthene	-	2.1	2.8	0.3	-	3.1	3.5	2.3
Benzo(e)pyrene	15	9.5	13	10	15	17	16	10
Benzo(a)pyrene	14	8.8	12	9.1	14	15	15	9.7
Indeno(1,2,3-cd)pyrene	10	11	11	5.6	10	12	14	8.2
Dibenz(a,c/a,h)anthracenes	3.9	4.5	4.9	2.3	3.9	5.5	5.6	3.1
Benzo(ghi)perylene	13	18	18	8.6	13	19	22	13

[a]Corrected for blank and recovery. I = interference prevented quantitation. d = days, wk = weeks. Single determination for each data point.
[b]Five days of leaching for each step.

Table 5. Leaching of PAH from SRC solid with minimum essential medium/serum mixture

PAH	Concentration,[a] μg/L			
	5d	2 wk	4 wk	6 wk
Benz(a)anthracene	2.7	1.7	1.9	0.44
Chrysene	2.7	2.1	2.1	1.3
Benzo(b/j)fluoranthenes	6.7	4.7	4.5	2.2
Benzo(k)fluoranthene	0.32	0.48	0.64	-
Benzo(e)pyrene	6.3	5.5	4.5	2.8
Benzo(a)pyrene	5.5	4.6	3.0	1.6
Indeno(1,2,3-cd)pyrene	3.3	3.0	1.0	0.88
Dibenz(a,c/a,h)anthracenes	1.7	1.3	0.63	0.84
Benzo(ghi)perylene	6.2	5.2	3.1	1.6

[a]Corrected for blank and recovery. d = days, wk = weeks. Single determination for each data point.

Table 6. Leaching of PAH from SRC Solid by dipalmitoyl-L-α-phosphatidylcholine (DPPC)

PAH	Total leachate,[a] μg/L		Concentration in phase					
			DPPC Suspension,[b] μg/L				DPPC Ppt.,[c] μg/g	
	5 d	6 wk	5 d	2 wk	4 wk	6 wk	5 d	6 wk
Benz(a)anthracene	5.6	6.1	-	1.8	0.64	0.55	0.56	0.55
Chrysene	-	-	-	-	-	-	-	-
Benzo(b/j)fluoranthenes	12	14	-	3.5	1.3	1.4	1.2	1.3
Benzo(k)fluoranthene	-	-	-	-	-	-	-	-
Benzo(e)pyrene	11	14	-	3.2	1.3	1.2	1.1	1.2
Benzo(a)pyrene	11	15	-	2.0	0.90	0.81	1.1	1.4
Indeno(1,2,3-cd)pyrene	13	24	-	0.93	0.62	0.33	1.3	2.3
Dibenz(a,c/a,h)anthracenes	5.4	11	0.74	3.7	4.5	1.6	0.47	0.90
Benzo(ghi)perylene	-	-	-	-	-	-	-	-

[a]Corrected for blank in both phases and recovery in DPPC precipitate phase. d = days, wk = weeks. Single determination for each data point.
[b]Corrected for blank only.
[c]Corrected for blank and recovery.

required to reach leaching equilibrium and the equilibrium PAH concentrations. The PAH concentrations generally appeared to reach an equilibrium before the first time point of 5 days (Table 4). This is very rapid in comparison with the length of the inhalation exposure experiment (ca. 2 years), and it suggests the potential for considerable PAH leaching during the bioassay. If the lung clearance times of the particles and fluids in the animal experiments can be determined, it may be possible to estimate the maximum PAH concentrations to which the lung tissues were exposed.

The PAH concentrations leached by the Tween 80 medium (Table 4) appeared to exceed the water solubilities (Table 7) by factors of two to four or greater. There is a lack of PAH solubility data at the temperature used for leaching (37°), and for some PAH only upper limits to the solubility enhancement could be calculated. The ratios of the PAH concentrations leached from the SRC Solid were somewhat different from the ratios of the PAH in the solid (Table 8). This observation probably reflects the generally decreasing water solubility and increasing lipophilicity of the PAH as the number of rings is increased.

A sequential leaching protocol (Table 4) also was performed with the Tween 80 medium to examine the PAH leaching behavior in a quasi-dynamic leaching model. The SRC Solid was leached for 20 days in which the leaching medium was changed every 5 days. The PAH concentrations leached in the different steps were similar to those in the continuous leaching experiment over three leachings, indicating a considerable "capacity" of the PAH available for leaching from the SRC Solid. By the fourth leaching, the PAH concentrations began to fall off, suggesting that the PAH in the outer layers of the particles were becoming depleted. It also may indicate a decrease in the kinetics of leaching.

Data for continuous leaching experiments using the MEM/serum mixture are presented in Table 5. The concentrations of leached PAH were slightly lower than those observed with the Tween 80 (see also Table 7). Except for the benzo(b/j)fluoranthenes, the ratios of the PAH

Table 7. Apparent enhancement of PAH aqueous solubility by media in five-day leaching of SRC Solid

PAH	Aqueous solubility @ 37°C,[a] µg/L	Solubility Enhancement[b]		
		Tween 80	MEM/Serum	DPPC
Benz(a)anthracene	21[c]	0.24	0.13	0.27
Chrysene	4.3[d]	1.4	0.63	-
Benzo(e)pyrene	7.8[e]	1.9	0.81	1.4
Benzo(a)pyrene	3.5[f]	4.0	1.6	3.1
Dibenz(a,c/a,h)anthracene	>0.5[g]	<7.8	<3.4	<11
Benzo(ghi)perylene	>2.6[h]	<5.0	<2.4	-

[a]Data for 37°C unless otherwise noted.
[b]Concentration in leachate divided by solubility in pure water.
[c]Calculated from data in references (22) and (23).
[d]Calculated from data in reference (23).
[e]Calculated from data in reference (24).
[f]Calculated from data in reference (22).
[g]Solubility data for (a,h) isomer at 25°C from reference (25).
[h]Solubility data for 25°C from reference (26).

Table 8. Comparison of the PAH concentration ratios in the SRC Solid and those leached from the SRC Solid by different media

PAH	Concentration relative to BaP			
	SRC solid	Tween 80[a]	MEM/serum[a]	DPPC[a]
Benz(a)anthracene	0.08	0.36	0.49	0.51
Chrysene	0.19	0.44	0.49	-
Benzo(b/j)fluoranthenes	0.61	0.56	1.2	1.1
Benzo(k)fluoranthene	0.08	-	0.06	-
Benzo(e)pyrene	1.0	1.1	1.1	1.0
Benzo(a)pyrene	1.0	1.0	1.0	1.0
Indeno(1,2,3-cd)pyrene	0.69	0.71	0.60	1.2
Dibenz(a,c/a,h)anthracenes	0.61	0.28	0.31	0.49
Benzo(ghi)perylene	1.75	0.93	1.1	-

[a]After five days of leaching.

concentrations were similar (Table 8). The main difference in the leaching behavior in the two media was the decline in PAH concentrations with time. Some decreases in PAH concentrations were apparent at two weeks of leaching, but six weeks were required for the effect to become pronounced. It is possible that proteins in the serum were binding some of the PAH from solution.

PAH leaching behavior with the DPPC vesicle system is shown in Table 6. This was a much more difficult media to prepare for analysis. Consequently, the data are not as extensive. The leaching media could not be completely separated from the SRC Solid by the low rpm centrifuging with the benchtop clinical centrifuge, and ultracentrifugation was required. However, under these more severe conditions, some of the DPPC vesicles also were precipitated and it was extremely tedious to cleanly pipette them off of the underlying SRC Solid precipitate. This biphasic sample required that PAH concentrations be measured in both the ultracentrifugation suspension and DPPC precipitates. Because of this more complex procedure for the analysis of the DPPC leachates, PAH leaching data are presented only for 5 days and 6 weeks. Data are included for the suspension phase at two other time points. The enhancement of PAH solubility for the larger PAH (dibenz-[a,c/a,h]anthracenes and indeno[1,2,3-cd]pyrene was greater than that of the Tween 80 and MEM/serum media. Except for the benzo(b/j)fluoranthenes, the concentrations of other PAH in the leachates were very similar to those for the Tween 80 medium. Comparison of the data calculated for the total leachate (from the suspension and precipitate results) with those measured for the precipitate (corresponding to DPPC vesicles) illustrates the affinity of the PAH for the DPPC vesicles. The PAH concentrations in the vesicles were ca. 10^2 times more concentrated than in the bulk leachate. Individual PAH concentrations in the vesicles exceeded 1 μg/g for some PAH. This finding shows that the PAH leached from the SRC Solid are present mainly in the vesicles rather than in the aqueous phase which constitutes the bulk of the leaching media. It suggests that cell membranes can efficiently absorb dissolved PAH or efficiently participate directly in the PAH leaching/dissolution process.

The general results of these leaching studies suggest that in the inhalation bioassay of the SRC Solid, the PAH were quite bioavailable and concentrations far in excess of their water solubilities may have accumulated in cell membranes. The bioavailability of these tumorigenic PAH in the SRC Solid may have contributed to the tumorigenic response, given the high aerosol concentrations (50 mg/l for the high dose group) of the SRC Solid and the long exposure protocol (6 hrs per day, 5 days per week, for 20-24 months).

PHENOLS DETERMINATIONS IN MIDDLE DISTILLATES

One hypothesis for the greater degree of dermal irritation caused by the skin painting of the TSL Middle Distillate versus the SRC Middle Distillate was that the former contained higher concentrations of phenols than did the latter. This hypothesis was tested by subjecting the two samples to a comparative phenols analysis by HPLC (27). Four replicate 25-35 mg portions of the middle distillates were dissolved in three ml of methylene chloride, and a known activity of carbon-14 labeled phenol was added. The sample was stirred overnight with three ml of 0.1 N aqueous sodium hydroxide. The aqueous layer was carefully pipetted off and mixed with 20 µl of glacial acetic acid before the volume was adjusted to five ml with the HPLC mobile phase. A 20 µl aliquot was analyzed on a 25 cm x 6.3mm OD RSIL-C18-HL column (Alltech Associates Houston, TX) eluted with 40% (vol/vol) of acetonitrile in water ion-modified by the addition of one ml of 1 M acetic acid - sodium acetate buffer (pH 4.6) per l of solution. The mobile phase flow rate was 1 ml/min. Detection was by fluorescence at an excitation wavelength of 266 nm and an emission wavelength of 300 nm. Recoveries were estimated by measuring the radiolabeled phenol tracer using liquid scintillation counting. Quantitation in HPLC was by the method of external standards.

The results are presented in Table 9. Phenol, the cresols, and several dimethylphenols were not detected in the TSL Middle Distillate within the sensitivity of the procedure, which was 0.5 mg/g for each phenol. As the HPLC chromatogram in Figure 4 shows, there was no evidence for these or other phenols in the TSL Middle Distillate. In contrast, at least ten phenols were detected in the SRC Middle Distillate, with p-/m-cresol being the most concentrated species at 30.6 mg/g (calculated as p-cresol). Five unidentified phenols also were detected. Their retention on the HPLC column suggests that they correspond to C2- and C3-phenols. The total of the five phenols determined in the SRC Middle Distillate is 5.65 wt.%; the additional five species probably would add another 1 to 3 wt.%, depending on their

Table 9. Comparison of selected phenols in SRC and TSL middle distillates

Phenolic compound	Concentration in middle distillate,[a] mg/g	
	SRC	TSL
Resorcinol	<0.5	<0.5
Phenol	2.4 ± 0.17	<0.5
p-/m-Cresols[b]	30.6 ± 8.8	<0.5
o-Cresol	4.2 ± 0.66	<0.5
3,4-Dimethylphenol	4.3 ± 0.55	<0.5
2,5-Dimethylphenol	14.2 ± 5.2	<0.5
2,3-Dimethylphenol[c]	-	-
Total	56.5	<3

[a]Average and standard deviation of four determinations.
[b]Calculated as p-Cresol.
[c]Not resolved from interference.

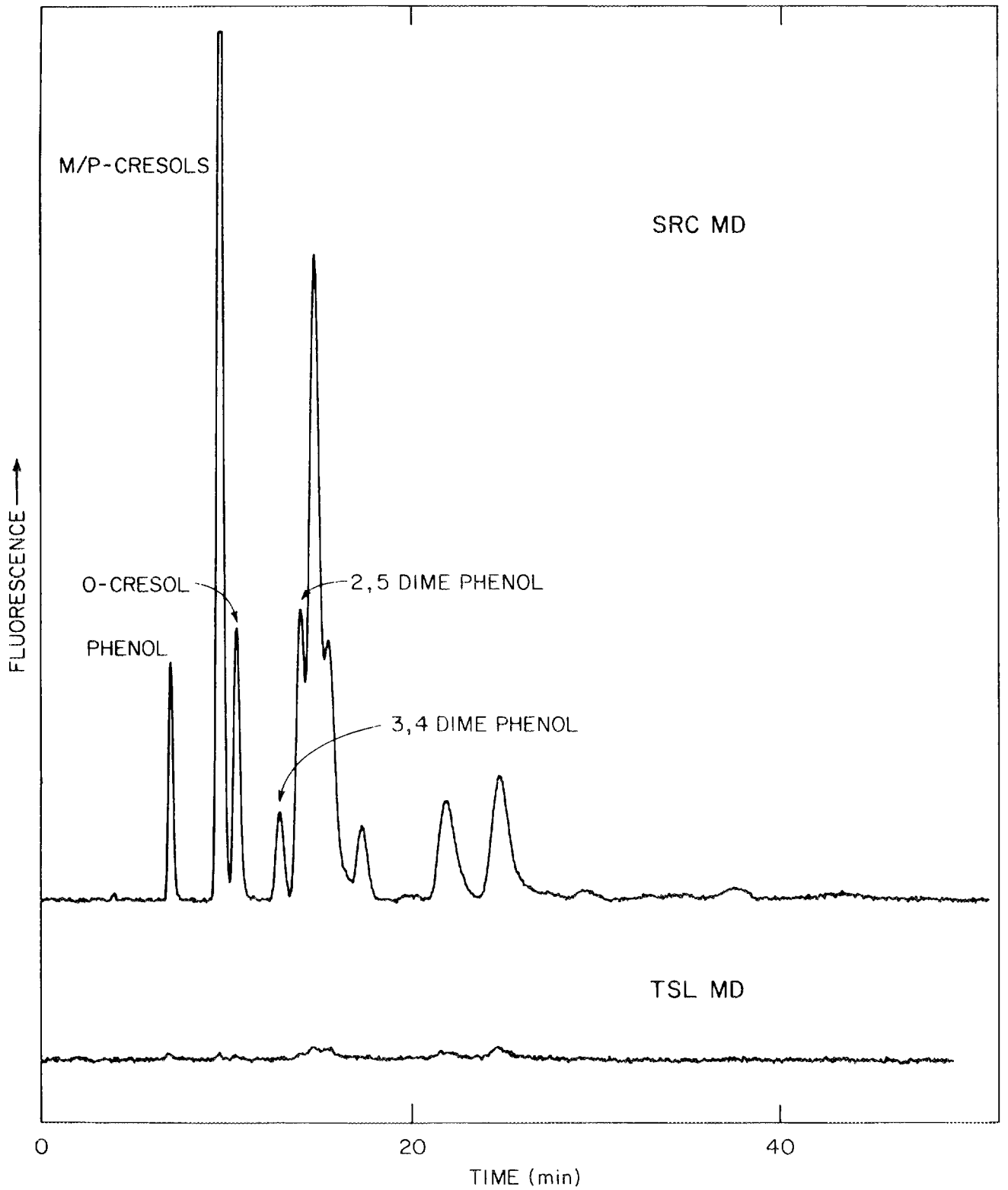


Figure 4. Comparison of HPLC Profiles of Phenol and Cresols in SRC Middle Distillate and TSL Middle Distillate.

specific fluorescence. These total phenols determined in the SRC Middle Distillate would amount to 6-8 wt.% versus <0.3 wt.% for the TSL middle Distillate.

These findings suggest that the greater dermal irritation caused by the TSL Middle Distillate was not attributable to the phenols determined in this study, but rather other compounds. Hydroaromatics, such as decalin are classified by NIOSH (28) as irritants. They are logical candidates for the greater skin irritancy of the TSL Middle Distillate. The proof of this hypothesis would require further study.

CONCLUSIONS

The components traditionally thought to contribute to the tumorigenic response of the SRC-I Solid in the inhalation bioassay are PAH which are found at concentrations up to 280 μg/g each. These compounds are readily leached from the solid and can accumulate in phospholipid vesicles at μg/g concentrations.

The greater skin irritation caused by the TSL Middle Distillate versus the SRC Middle Distillate does not appear to be associated with their content of phenol and C_1- or C_2-phenols. Other compounds, such as hydroaromatics, may be responsible for the irritation.

ACKNOWLEDGEMENT

Several staff members of the Analytical Chemistry Division are acknowledged for their assistance in this work. C. S. MacDougall performed the SEM-EDX and X-ray diffraction analyses and G. Olerich ran the GC-MS analyses. J. W. Russell, a summer student, conducted the comparison of selected 4-6 ring PAHs in the coal liquids, solids, and tars. Neutron activation analyses were performed under J. F. Emery's supervision.

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