METHODS FOR THE ISOLATION AND IDENTIFICATION OF POLYCYCLIC AROMATIC HYDROCARBONS FOUND IN COMPLEX MIXTURES AND THE DETERMINATION OF THEIR POSSIBLE TOXICITY BY MEANS OF BIOASSAY TECHNIQUES

Progress Report
for Period August 1, 1978 to August 1979

S.R. Lipsky and W.J. McMurray

Yale University School of Medicine
New Haven, Connecticut 06510

August 1979

Prepared for

The U.S. Energy Research and Development Administration
Under Contract No. C00/2958-3

DISCLAIMER

Progress Report

Introduction

In attempting to achieve our goals, this year has been both one of great frustration and some sense of accomplishment. In July 1978, our new Varian MAT 44 gas chromatography-mass spectrometer (GC/MS) was installed with the beginnings of what was considered to be the most versatile data acquisition system available for a low resolution mass spectrometer. After working properly for several weeks, major faults began to appear both in the electronic circuitry and in the data handling system. Over the ensuing months as one set of problems was resolved by the service engineers and ourselves, others began to emerge. As a consequence, about 50% of the system was replaced but to no avail. Most regretably, some eight months after installation, because of repeated failure in meeting overall specifications, we had no choice but to return the instrument to the manufacturer. In July 1979, a recently introduced computer controlled VG Model 16 GC/MS was delivered and has now become operational. The performance thus far, has been outstanding and has provided us with the opportunity to begin to gather data essential to our overall program. Nonetheless, because of the difficulties with our original device, we have significantly fallen behind schedule in our efforts to identify many of the noxious components thought to reside in the samples we have received from collaborating laboratories. In spite of this, it was possible to move ahead with extensive analyses of the sample fractions by means

of glass capillary column gas chromatography and the establishment of an elegant very high resolution liquid chromatographic system utilizing reversed phase microbore columns 1-10 meters in length. The interfacing of the latter device with the mass spectrometer was arbitrarily postponed until our basic investigations involving structural identification of important fractions by means of GC/MS was back on schedule. In addition, our studies exploring the possibility of utilizing adipose tissue (obtained via simple needle biopsy) as 'markers' for the repository of exogenous hydrocarbons was set into motion.

One final point about mass spectrometers. They are reasonably complicated devices that take 2-3 months to deliver after receipt of order and some 2-3 weeks + to fully install. An additional 1-2 months is often required to master the new device and the associated computer control and data acquisition systems. Accordingly, being in mass spectrometry for about 14 years, after viewing demonstrations by several manufacturers and carefully comparing specifications we exercised what we considered to be great care in the selection of an instrument. Because of all of this, when we discovered the magnitude of the inherent difficulties in the particular instrument delivered to us, and realizing the time frames involved in reordering and installing a replacement system, we were reluctant at the outset to follow this path until the company involved was given ample opportunity to rectify the situation. Unfortunately, in the final analysis, their attempts

proved to be futile and most regretably, this was the only rational solution to the problem.

Establishment of a Bioassay Laboratory

Through the efforts of Dr. A. Jonas, former chief of the Section of Comparative Medicine and myself, a laboratory was established in the Section of Comparative Medicine for the purpose of screening various chemicals for mutagenic and carcinogenic activity by means of the Ames bioassay system. The construction of the laboratory was made possible by a gift of $60,000 to the School of Medicine from the Olin Corporation of New Haven. The laboratory has recently become operational and has already processed samples for us. The laboratory is under the direction of Dr. Robert Jacoby and is supervised by Ms. B. Collett.

A. The Separation and Partial Identification of Individual Components Found in Shale Oils, Their Dimethyl Sulfoxide Extracts and Product Waters.

These studies were performed in collaboration with Dr. Dale Spall of the Los Alamos Scientific Laboratory. The materials under consideration here were as follows:

Crude Oils	DMSO Extracts
Paraho crude (PRHO)	(PRHO)
Occidental crude (OCCD)	(OCCD)
Louisiana sweet crude oil (LSNA)	(LSNA)
Kuwait crude (KUWT)	(KUWT)
Paraho refinery crude (PHRR)	
Paraho hydro treated crude (PHRR)	
ASE 90 (PTPD)	(PTPD)

Waters

Occidental Product Water
Paraho Product Water
Leach Water - Paraho Spent Shale
Leach Water - Paraho Raw Shale
Acid Waters from Coal Mine Dump Leachate

Analysis was performed by means of GC/MS. A OV-101 glass capillary column which was programmed from 80° to 260°C+ at 2°C/minute was interfaced with the mass spectrometer operated in the chemiionization mode. Retention times were obtained by the use of a standard mixture of polyaromatic hydrocarbons analyzed under similar conditions. Thus far, the following compounds have been tentatively identified:

(See Chart Next Page)

Approximate Levels of Polyaromatic Hydrocarbons (PAH'S)
in PPM (rounded off)

	SAMPLES : CRUDE OILS				
Components	KUWT	PRHO	OCCO	LSNA	SAE 90
naphthalene	400	500	300	600	900
acenaphthalene	150	525	325	325	600
phenanthrene	300	475	175	320	400
anthracene	225	425	100	200	350
fluorene	100	150	60	120	300
pyrene	200	120	2	180	475
fluoranthene	175	65	2	300	95
chrysene	20	30	tr.	25	20
napthacene	2	1	tr.	1	2
3 methylcolanthrene	1	1	tr.	1	1
7,12 dimethylbenzanthracene	1	1	tr.	1	1
benzo(e)pyrene	2	1	tr.	2	3
benzo(a)pyrene	3	3	tr.	3	6
benzo(f)quinolene	10	10	10	10	11
carbazole	10	10	10	10	20
acridine	3	2	5	4	8
1 azapyrene	tr.	tr.	tr.	tr.	tr.
12 azabenzo(a)pyrene	tr.	tr.	tr.	tr.	tr.
11 methyl 12 azabenzo(a)pyrene	tr.	tr.	tr.	tr.	tr.

trace = less than 1 ppm

Product Waters

Thus far, a preliminary screening analysis was performed on the Occidental and Paraho product waters. They were found to contain:

o,p,m cresols

phenol and alkylphenols

the xylenols

indoles, methyl, ethyl & C_4

primary and secondary alkyl amines

DMSO Extracts of Crude Oils (2-20% crude oils in extracts)

Preliminary analysis showed:

phenols

thiols

primary & secondary amines

thiophenes

acids

B. Biological Testing

The following samples were submitted for bioassay of mutagenic/carcinogenic activity (Ames test)

1. DMSO of Paraho Crude Oil
2. Paraho Crude Oil
3. Paraho Refinery Run Crude Oil
4. DMSO extract of tar
6. Leach Water from Paraho Raw Shale
7. Leach Water from Paraho Spent Shale
8. Anvil Pts. Burn Water
9. Coal Mine Water
10. Product Water Paraho
11. Product Water - Occidental
19. Paraho Refinery Hydrotreat

Since the biological testing of certain select samples was carried out in a recently established laboratory within the School of Medicine, we requested Dr. Robert Jacoby, head of this Mutagenesis Laborarory, as well as the Section of Comparative Medicine and his associate Ms. B. Collett to provide us with background information concerning the methods employed in the bioassay procedure. Their report is appended to this one.

Results

The 11 samples were examined for mutagenic activity with five strains of the bacterium Salmonella typhimurium (strains TA1535, TA1537, TA1538, TA98, and TA100) in the standard Ames - Salmonella microsome assay system. Each assay was performed in the presence and in the absence of a metabolic activation system. Sample #10 (Paraho Product Water) was mutagenic for strains TA1537, TA1538, and TA98 after metabolic activation with normal or Aroclor stimulated S-9 fractions. Sample Nos. 6 through 9 and No. 11 were nonmutagenic under all test conditions (Table 1).

Sample Nos. 1-4 and 19 were tested at 500 µg/plate after dilution in dioxane. The samples were nonmutagenic for strains TA1535 and TA100 under all test conditions. Mutagenic activity for strain TA98 was detected with samples Nos. 2 and 3 after metabolic activation with AROCLOR-stimulated S-9 fractions. Technical difficulties potentially related to sample toxicity were encountered with all 5 samples for strains TA1537 and TA1538. These tests are being repeated. (Table 2).

Exp. No. _____
Compound _____
Los Alamos _____
Samples

Table 2

Type of Assay: Standard Plate

Compound	Metabolic Activation S-9 NORMAL	Metabolic Activation S-9 AROCLOR	Micrograms of Compound Added per Plate	Histidine Revertants per Plate TA1535	TA1537	TA1538	TA98	TA100
Negative control	-	-		21	9	16	15	141
		+		20	32	33	44	159
Positive Controls								
Sodium azide	-	-	1.0	855				1232
9-Aminoacridine	-	-	100.0		3161			
2-Nitrofluorene	-	-	1.0			212	213	
2-Anthramine	-	-	2.0	22	9	25	37	160
	+		2.0	522	278	2262	5176	3726
		+	2.0	140	71	845	914	1015
No. 1	-	-	500.0	21	13	*	20	171
	+		500.0	23	16	18	31	170
		+	500.0	24	31	62	69	150
No. 2	-	-	500.0	21	*	*	17	151
	+		500.0	20	*	*	29	130
		+	500.0	22	40	114	247	216
No. 3	-	-	500.0	12	11	*	14	173
	+		500.0	19	*	1755	27	132
		+	500.0	25	31	143	290	237
No. 4	-	-	500.0	--	9	*	5	150
	+		500.0	26	15	*	35	154
		+	500.0	32	23	40	54	145
No. 19	-	-	500.0	17	5	*	10	127
	+		500.0	14	*	*	29	139
		+	500.0	20	25	40	52	196

* Abnormal Colonies

Exp. No. _____
Compound _____
Los Alamos _____
Water Samples

Table 1

Type of Assay: Standard Plate

Compound	Metabolic Activation S-9 NORMAL	Metabolic Activation S-9 AROCLOR	Micrograms of Compound Added per Plate	Histidine Revertants per Plate TA1535	TA1537	TA1538	TA98	TA100
Negative control	-	-		42	7	13	20	271
		+		44	26	38	40	330
Positive Controls								
Sodium azide	-	-	1.0	798				942
9-Aminoacridine	-	-	100.0		1972			
2-Nitrofluorene	-	-	1.0			247	281	
2-Anthramine	-	-	2.0	41	9	53	63	299
	+		2.0	609	178	1668	7932	8352
		+	2.0	210	90	1160	768	1116
No. 6	-	-	Undiluted	54	12	13	16	280
	+		"	52	23	17	46	256
		+	"	79	20	29	50	322
No. 7	-	-	Undiluted	24	7	16	18	317
	+		"	52	23	22	41	259
		+	"	49	26	30	51	295
No. 8	-	-	"	30	15	10	19	284
	+		"	60	10	31	38	259
		+	"	75	18	37	36	322
No. 9	-	-	"	28	8	11	20	255
	+		"	32	17	19	19	242
		+	"	60	17	32	37	253
No. 10	-	-	"	38	18	22	36	223
	+		"	56	94	559	685	308
		+	"	67	549	3610	3176	281
No. 11	-	-	"	22	11	16	13	262
	+		"	54	15	26	48	291
		+	"	66	24	52	43	333

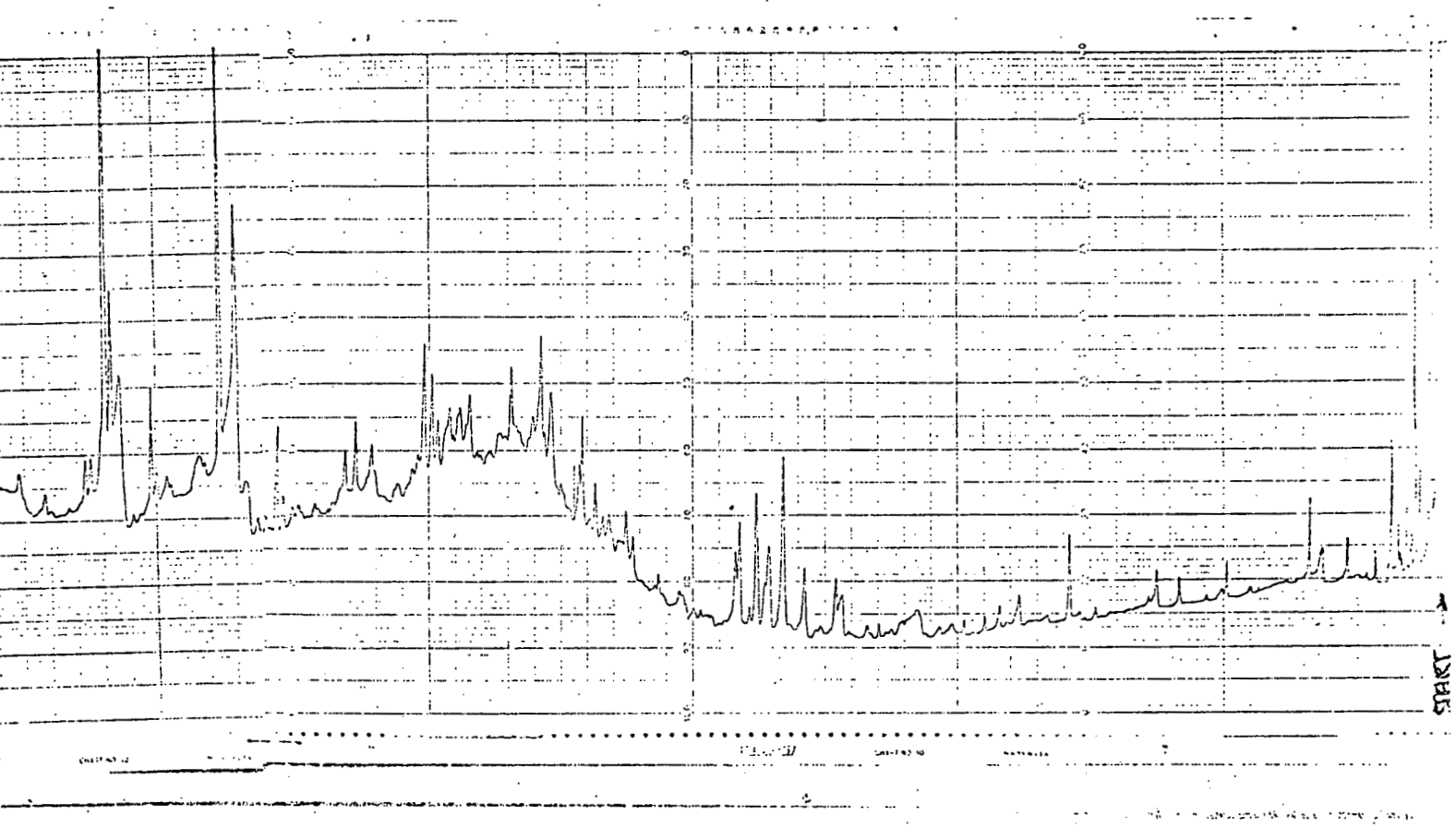
The Determination of 'Marker' Substances in the Adipose Tissue of Test Animals and Man

During the course of the year, we have collaborated with the Los Alamos Scientific Laboratory (Dr. M. Holland) in certain areas involving animal exposure studies. Thus far, our investigations have been limited to a study of the nature of the hydrocarbons found in the inguinal fat pads of a control group of hamsters. Preliminary efforts were directed to the isolation and separation of these substances by means of liquid chromatography and glass capillary column gas chromatography as follows:

Combined specimens of inguinal adipose tissue of the hamsters were weighed and then refluxed with NaOH (10-20 ml. of 2N NaOH in aqueous ethanol). They were then extracted with redistilled diethyl ether after dilution with 20-30 ml. of water. The ether extract was washed five times with dilute NaOH and then evaporated to dryness at room temperature. The residues were rehydrolyzed with 2N NaOH (30 min. at 100°C) and reextracted with diethyl ether. The extracts were then concentrated and subjected to lipid class analysis by means of silicic acid column chromatography. Here, the exit of a Pasteur pipette was blocked with a piece of silanized glass wool. Approximately 1-2 grams of 37-60 micron silica particles (LiChrosorb, E.M. Merck) were poured dry onto the top of the glass wool. The column was gently tapped to remove air bubbles and 'topped' with another piece of glass wool. The column was then placed in an oven at 200°C for 12-16 hours.

Figure 1

GC Profile of Hydrocarbon Fraction from the Fat Pads (Combined) of a Control Series of Hamsters



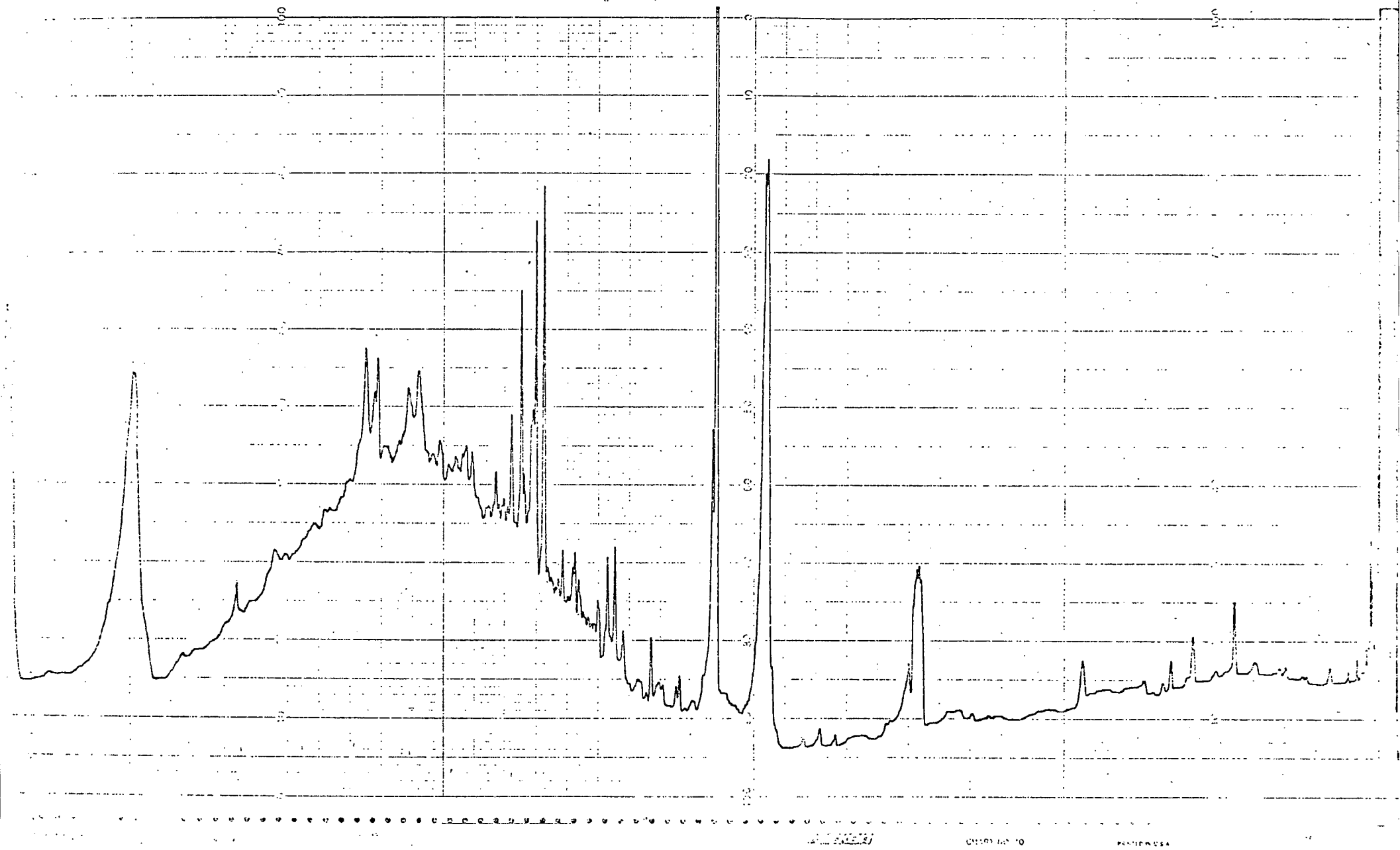
After cooling at room temperature, the column was washed with 2 column volumes of petroleum ether (30-60^{o} fraction). The sample was then placed on top of the column with a microsyringe and the hydrocarbon fraction was selectively separated from the other lipids by elution from the column with an additional 2-3 column volumes of petroleum ether. The petroleum ether band was concentrated and then injected into the gas chromatography containing an OV-101 coated capillary column which was programmed from 80^{o} to 250^{o} at 2^{o}C/minute. Figure 1 (same as #9 in Renewal Proposal) depicts a typical 'fingerprint' chromatogram of the nonsaponifiable, alkali stabilized and diethyl ether soluble hydrocarbon fraction of the inguinal fat pad of the hamster. Efforts are now underway to identify these components by means of gas chromatography-mass spectrometry.

The other test group of hamsters exposed to shale oil in various forms by various routes for various periods of time will be available to us in the near future. Because of the extraordinary solubility in lipids of many organic compounds that may enter the environment, the overriding objective here is to determine the presence of certain 'marker' substances or metabolites thereof, denoting a history of exposure (and possibly its extent) of the animal. Depending upon the nature of the final data, attempts will be made to answer questions regarding certain aspects of the transport, metabolism, deposition and turnover of these compounds in the fat depots.

Greatly encouraged by our preliminary findings in animals, we then solicitated the assistance of Professor Jules Hirsch of the Rockefeller University in providing us with specimens (simple needle biopsy) of gluteal fat obtained from a series of normal volunteers used for another study. Figure 2 (#10, in the Renewal Proposal) shows a typical gas chromatographic 'fingerprint' of the adipose tissue obtained from a needle biopsy and processed by the aforementioned methods. We are now in the process of collecting additional data in order that we may further define the 'normal' hydrocarbon patterns found in man. If studies of this type eventually prove to be rewarding, then selective investigations can begin on the use of a simple and safe needle biopsy method (the Rockefeller people have done thousands without incident) to procure samples of gluteal fat of workers exposed to certain classes of substances which are thought to contain noxious components. Hopefully long term, some correlation between the clinical status (the possible development of tumor formation or other toxic manifestations) of the individual (Man),the invitro bioassay results, and the appearance and/or disappearance (turnover) with time - of certain specific chemical substances in accessible tissue depots - could be effectively realized once and for all.

Figure 2

GC Profile of Hydrocarbon Fraction from Human Gluteal Fat Tissue (Needle Biopsy)



The Utilization of Microbore Columns in the High Resolution Liquid Chromatographic Analysis of Samples of Oils Derived From Shale or Coal

Because of the enormous complexity and diversity of many of the sample types we have had occasion to handle, we found that many of the conventional high performance liquid chromatographic systems employing 25-50 cm x 4 mm i.d. columns did not adequately fulfill our requirements which generally were:

a) very high resolution in order to separate the largest number of components possible by this technique

b) the convenience of readily sacrificing time for ultra high resolution so that any particular group of substances of interest could eventually be resolved

c) relatively low flow rates (5-80 ml/min.) so that the LC System can be interfaced with a mass spectrometer (a much more universal detector) for identification purposes (See Renewal Proposal for details here) without sample splitting

d) the ability to not only separate but also to detect very small quantities of material under circumstances where long elution times were involved.

Accordingly, we investigated two systems which we considered to hold promise in this regard. They were the open tubular capillary columns described by Ishi, J. of Chrom., 156, 173 (1978) and 157, 43 (1978), and the microbore column system of Scott (J. of Chrom. 169,

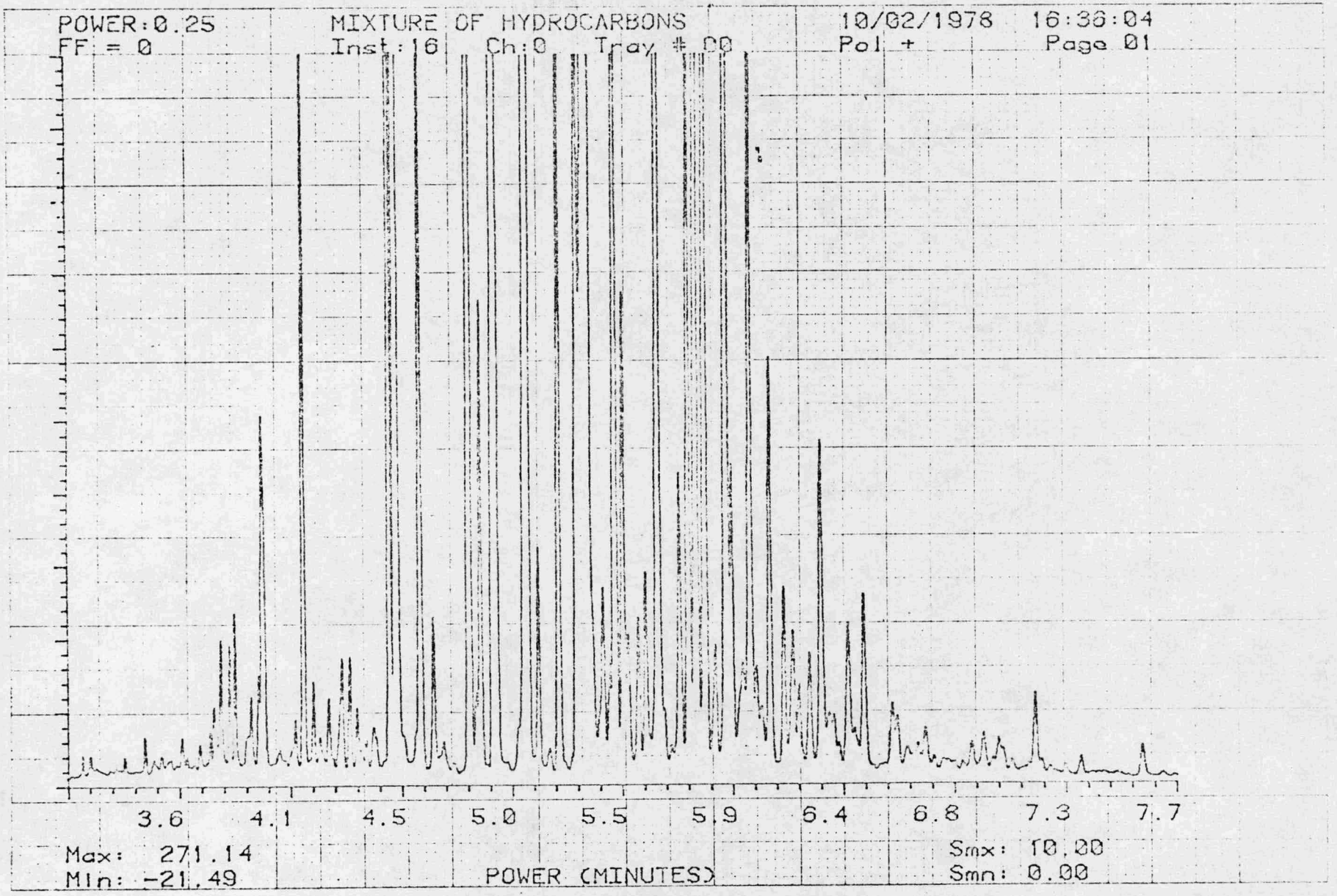
51, (1979). With reference to the former, we found that problems associated with the very stringent reduction of detector and injector volumes were so severe, that this system at this time appears highly impractical! This was not the case in point with the microbore column, and associated miniaturized UV flow cell. Here, we found extremely high mass sensitivity. For example, with conventional LC columns (25-50 cm x 4 mm i.d.) with a detector sensitivity of 10^{-8}gms/ml, a detector volume of 8 µl and associated tubing dead volume of at least 5 µl, one peak emerging in 1 ml. would have to contain about 10 nanograms to be detectable.

In contrast, using microbore columns in conjunction with a UV detector with same sensitivity (10^{-8} gms/ml) where the flow cell volume has been reduced to 0.8 µl and the dead volume of associated tubing - 0.2 µl, the peak width of an appropriately selected component band is usually in the order of 10-20 µl. Under these circumstances, the mass contained in the peak is in the range 0.1-0.2 nanograms, providing one with a gain of absolute mass sensitivity of approximately 50-100:1.

It was also apparent that the efficiency of such a column system increases linearly with length. Accordingly, it was found that 1 to 10 meter column lengths could be conveniently utilized without the use of extraordinarily high pressures. Moreover, peak broadening was minimal so that the high sensitivity could be maintained throughout the analysis despite the very long elution times.

Examples of the high resolution analysis of pentane extracts of coal derived materials are noted in Figures 3,4. Identification of the component bands will be carried out in the future (See Renewal Proposal) when this system is interfaced with a mass spectrometer.

Further work is also in progress to find suitable solvent systems for those oil fractions not soluble in the usual aqueous solvents used in reversed phase chromatography.



COLUMN 100 CM X 1 MM I.D.
SEPARATIONS OF HYDROCARBONS (PAH'S+)
ODS-2 REVERSE PHASE 10 UM
UV DETECTOR 254 NM
65% CH3CN/WATER

SAMPLE: Pentane Soluble Fraction Coal Derived Oil
W. Va. Energy Res. Center

Figure 3

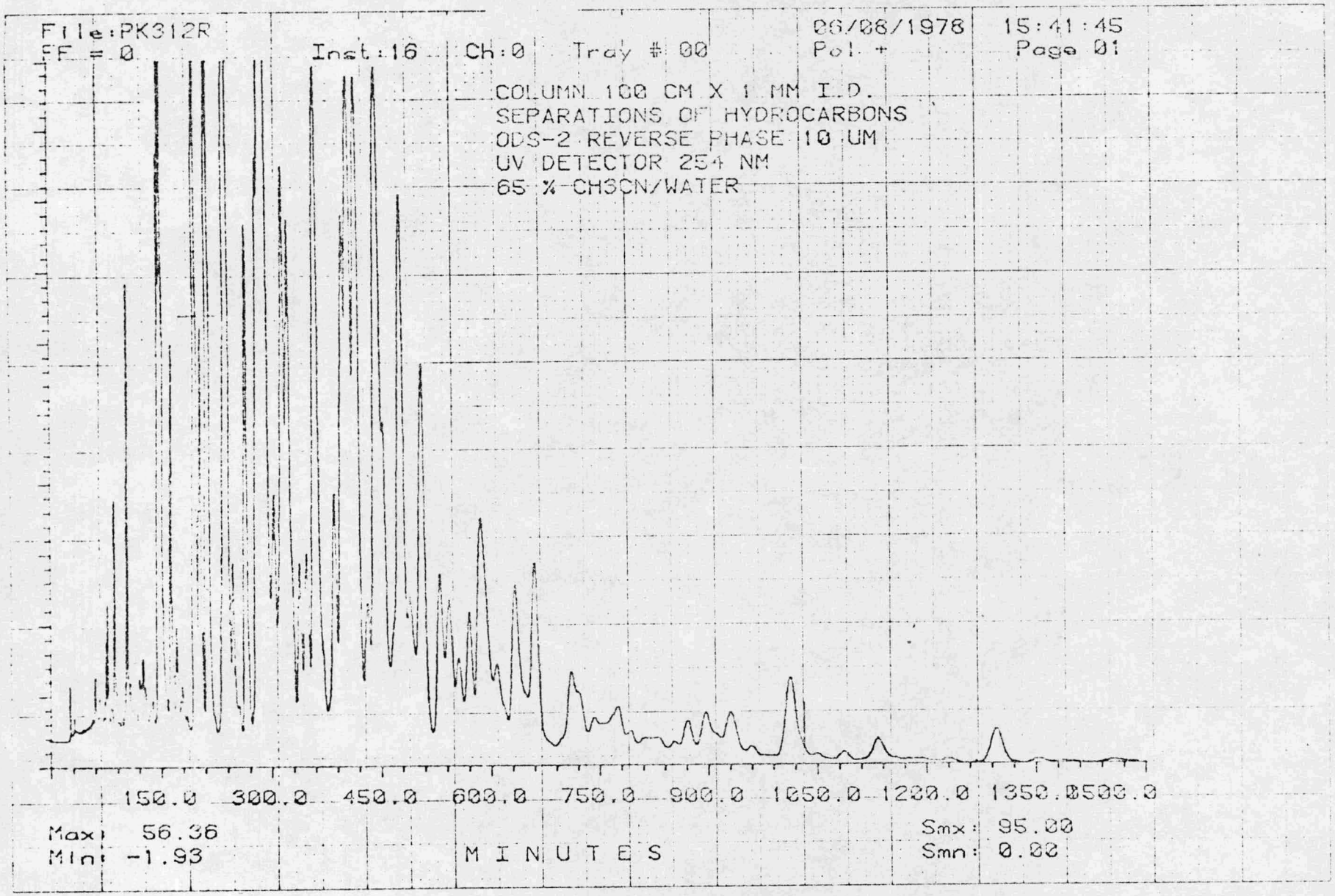


Figure 4

SAMPLE: Pentane Insoluble Fraction Coal Derived Oil
W. Va. Energy Res. Center

Appendix

Compiled by Dr. R. Jacoby and Ms. B. Collett

INTRODUCTION

The Mutagenesis Laboratory, Section of Comparative Medicine examined 11 samples for mutagenicity by *in vitro* microbiological assays with five strains of *Salmonella typhimurium* (TA1535, TA1537, TA1538, TA98, and TA100). Normal and Aroclor 1254-stimulated rat-liver homogenate metabolic activation systems were included in the assay procedures to provide metabolic steps that the bacteria either are incapable of conducting or do not carry out under the assay conditions.

The assay procedure with *S. typhimurium* has proven to be approximately 85 to 90% reliable in detecting carcinogens as mutagens, and it has about the same reliability in identifying chemicals that are not carcinogenic.[1] The assay does not always provide 100% correlation with animal carcinogenicity investigations, so neither a positive nor a negative response conclusively proves that a chemical is hazardous or nonhazardous to humans.

METHODS*

Salmonella typhimurium Strains TA1535, TA1537, TA1538, TA98, and TA100

The Salmonella typhimurium strains used in the Ames' test are histidine auxotrophs by virtue of mutations in the histidine operon. When these histidine-dependent bacteria are grown on a minimal media containing a trace of histidine, only those organisms that revert to histidine independence (his$^+$) are able to form colonies. A small amount of histidine is added to the medium to allow all the plated bacteria to undergo a few divisions because this growth is often essential for mutagenesis to occur. The his$^+$ revertants are scored as colonies against a background lawn. The spontaneous mutation frequency of each strain is relatively constant, but when a mutagen is added to the agar the mutation frequency is increased 2- to 100-fold.

The S. typhimurium strains were obtained from Dr. Bruce Ames of the University of California at Berkeley.[1-5] In addition to having mutations in the histidine operon, all the indicator strains have a mutation (rfa$^-$) that leads to a defective lipopolysaccharide coat; they also have a deletion that covers genes involved in the synthesis of vitamin biotin (bio$^-$) and in the repair of ultraviolet (uv)-induced DNA damage (uvrB$^-$). The rfa$^-$ mutation makes the strains more permeable to many large aromatic molecules, thereby, increasing the mutagenic effect of these

---

* Portions of the background text have been adapted and modified from the format of the Stanford Research Institute.

molecules. The *uvrB*$^-$ mutation decreases repair of some types of chemically or physically damaged DNA and thereby enhances the strains' sensitivity to some mutagenic agents. Strain TA1535 is reverted to *his*$^+$ by many mutagens that cause base-pair substitutions. TA100 is derived from TA1535 by the introduction of the resistance transfer factor plasmid pKM101. This plasmid is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens.[5] In addition, plasmid pKM101 confers resistance to the antibiotic ampicillin, which is a convenient marker to detect the presence of the plasmid in the cells. The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens. Strains TA1537 and TA1538 are reverted by many frameshift mutagens. TA1537 is more sensitive than TA1538 to mutation by some acridines and benzanthracenes, but the difference is quantitative rather than qualitative. Strain TA98 is derived from TA1538 by the addition of the plasmid pKM101, which makes it more sensitive to some mutagenic agents.

All indicator strains are kept at 4° C on minimal medium plates, supplemented with a trace of biotin, and an excess of histidine. The plates with the plasmid-carrying strains contain, in addition, ampicillin (25 µg/ml), to ensure stable maintenance of plasmid pKM101. New stock culture plates are made every two months from single colony reisolates that are checked for their genotypic characteristics (*his*, *rfa*, *uvrB*, *bio*) and for the presence of the plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37° C in nutrient broth (Oxoid, CM67). After stationary overnight growth, the cultures are shaken for 3 to 4 hours to ensure optimal growth.

Aroclor 1254-Stimulated Metabolic Activation System

Some carcinogenic chemicals, either of the aromatic amino type or polycyclic hydrocarbon type, are inactive unless they are metabolized to active forms. In animals and humans, an enzyme system in the liver or other organs (e.g., lung or kidney) is capable of metabolizing a large number of these chemicals to carcinogens.[4, 6-8] Some of these intermediate metabolites are very potent mutagens in the S. typhimurium test. Ames has described the liver metabolic activation system used in our laboratory.[6] Briefly, adult male rats (250 to 300 g) are given a single 500-mg/kg intraperitoneal injection of a polychlorinated biphenyl, Aroclor 1254. This treatment enhances the synthesis of enzymes involved in the metabolic conversion of chemicals. Four days after the injection the animals' food is removed but drinking water is provided ad libitum. On the fifth day, the rats are killed, and the liver homogenate is prepared as follows:

The livers are removed aseptically and placed in a preweighed sterile glass beaker. The organ weight is determined, and all subsequent operations are conducted in an ice bath. The livers are washed in an equal volume of cold, sterile 0.15 M KCl (1 ml/g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 M KCl, and homogenized with a Potter-Elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 9000 x g, and the supernatant, referred to as the S-9 fraction, is quickly frozen in dry ice and stored at -80° C.

The metabolic activation mixture for each experiment consists of, for 10 ml:

- 1.5 ml S-9
- 1.0 " $MgCl_2$ (80mM)
- 1.0 " KCl (330mM)
- 1.0 " G-6-P (50mM)
- 1.0 " NADP (40mM)
- 1.0 " Na_2HPO_4 (1000mM)
- 3.5 " H_2O

Method from Ames' Paper

In addition is Aroclor-activated S-9 fractions, S-9 fractions from normal rat livers are also used for our metabolic activation tests.

Assays in Agar

1. To a sterile 13 x 100 mm test tube containing 2.0 ml of 0.6% Top Agar* held in a 45° C waterbath, add in the following order:
2. 0.2 ml of indicator organisms (4 tubes/organism). After inoculation of organism place tube in 43° C heating block.
3. 0.1 ml of test compound.
4. 0.5 ml of metabolic activation mixture.

   Tube 1 - none

   Tube 2 - none

   Tube 3 - normal S-9 fraction

   Tube 4 - Aroclor-induced S-9 fraction

---

* 0.6% Top agar contains 0.05 mM histidine and 0.05 mM biotin.

Tube 1

1. 2.5 ml Top agar
2. 0.2 ml Bacteria
3. 0.1 ml Solvent

Tube 2

1. 2.5 ml Top agar
2. 0.2 ml Bacteria
3. 0.1 ml Test compound

Tube 3

1. 2.0 ml Top agar
2. 0.2 ml Bacteria
3. 0.1 ml Test compound
4. 0.5 ml Normal S-9

Tube 4

1. 2.0 ml Top agar
2. 0.2 ml Bacteria
3. 0.1 ml Test compound
4. 0.5 ml Aroclor induced S-9

For positive controls, we test each culture by specific mutagens known to revert each strain using steps (1), (2), (3), (optional) and (4).

This mixture is stirred gently and then poured onto minimal agar plates.† After the top agar has set, the plates are incubated at 37° C for 2 days. The number of *his*$^+$ revertant colonies is counted and recorded.

Each assay is carried out in duplicate with individual assays run on separate days. If results indicate that mutagenic activity is marginal, preincubation steps may be included. In this procedure, test compound is incubated with normal or Aroclor-activated S-9 fractions at 37° C for 20 minutes before plating.

---

† Minimal agar plates consist of, per liter, 15 g of agar, 50 g of glucose, 0.2 g of $MgSO_4.7H_2O$, 2 g of citric acid monohydrate, 10 g of K_2HPO_4 and 3.5 g of $NaHNH_4PO_4.4H_2O$.

REFERENCES

1. J. McCann, E. Choi, E. Yamasaki and B. N. Ames. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Nat. Acad. Sci. USA, 72, 5135-5139 (1975).

2. B. N. Ames, E. G. Gurney, J. A . Miller and H. Bartsch. Carcinogens as frameshift mutagens: Metabolites and derivatives of 2-acetylamino-fluorene and other aromatic amine carcinogens. Proc. Nat. Acad. Sci. USA, 69, 3128-3132 (1972).

3. B. N. Ames, F. D. Lee and W.E. Durston. An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc. Nat. Acad. Sci. USA, 70, 782-786 (1973).

4. B. N. Ames, W. E. Durston, E. Yamasaki and F. D. Lee. Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc. Nat. Acad. Sci. USA, 70, 2281-2285 (1973).

5. J. McCann, N. E. Spingarn, J. Kobori and B. N. Ames. Dectection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids. Proc. Nat. Acad. Sci. USA, 72, 979-983 (1975).

6. B. N. Ames, J. McCann, E. Yamasaki. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res., 31, 347-364 (1975).

7. L. D. Kier, E. Yamasaki and B. N. Ames. Detection of mutagenic activity in cigarette smoke condensates. Proc. Nat. Acad. Sci. USA, 71, 4159-4163 (1974).

8. L. A. Poirier and V. F. Simmon. Mutagenic-carcinogenic relationships and the role of mutagenic screening tests for carcinogenicity. Clin. Toxicol., 9, 761-771 (1976).

9. F. K. Zimmermann and R. Schwier. Induction of mitotic gene conversion with nitrous acid, 1-methyl-3-nitro-1-nitrosoguanidine and other alkylating agents in Saccharomyces cerevisiae. Mol. Gen. Genet., 100, 63-69 (1967).

10. D. J. Brusick and V. W. Mayer. New developments in mutagenicity screening techniques with yeast. Environ. Health Perspectives, 6, 83-96 (1973).