OAK RIDGE NATIONAL LABORATORY LIBRARIES

3 4456 0570393 1

ornl

OAK
RIDGE
NATIONAL
LABORATORY

UNION
CARBIDE

Semi-Annual Report for July 1 to December 31, 1980 Environmental Protection Agency Pass-Through Program

J. L. Epler
R. J. M. Fry
J. M. Holland
A. W. Hsie
L. B. Russell
W. L. Russell
J. K. Selkirk
H. R. Witschi

Biology Division
Oak Ridge National Laboratory
Oak Ridge, Tennessee 37830

OPERATED BY
UNION CARBIDE CORPORATION
FOR THE UNITED STATES
DEPARTMENT OF ENERGY

SEMI-ANNUAL REPORT FOR

July 1 to December 31, 1980

ENVIRONMENTAL PROTECTION AGENCY PASS-THROUGH PROGRAM

J. L. Epler
R. J. M. Fry
J. M. Holland
A. W. Hsie
L. B. Russell
W. L. Russell
J. K. Selkirk
H. R. Witschi

Interagency Agreements
EPA-IAG-D5-EG81-AN
DOE-40-516-75

Biology Division
Oak Ridge National Laboratory
Oak Ridge, Tennessee 37830
Operated by
Union Carbide Corporation
for the
Department of Energy
Contract No. W-7405-ENG.-26

Some of the data reported in this Semi-Annual Report for July 1 to December 31, 1980, Environmental Protection Agency (EPA) Pass-Through Program, was taken with funding from the Department of Energy (DOE) Office of Health and Environmental Research. This is a logical result of an integrated program of biological research.

The samples and data taken from gasifier studies funded by the Department of Energy expand and validate the results of the projects funded by the EPA Pass-Through Program. These are acknowledged accordingly and are included for the sake of completeness.

CONTENTS

	Page
Introduction	ii
List of Major Tasks	iii
Budget Resources	1
Major Tasks	4

INTRODUCTION

This semi-annual report of the Pass-Through projects of the Environmental Protection Agency (EPA) covers the period July 1 to December 31, 1980. All of the projects reported here are being carried out in the Biology Division of the Oak Ridge National Laboratory. The titles of the individual projects and the names of the principal investigators are given on the following pages. These projects comprise an integrated effort by a number of individuals in addition to those listed as principal investigators. The names of the senior staff members carrying out the work are listed on the reports of the individual tasks.

The purpose of these projects is to investigate potential adverse health effects of chemical substances (mixtures and isolated compounds) associated with emerging processes for producing energy from fossil fuels. Materials are first tested with short term biological assays, primarily tests for mutagenesis and early toxicity. Based on the results of those tests some materials are investigated further for longer term toxicity including carcinogenesis, teratogenesis and heritable genetic effects. Results of these studies are made available for guiding decisions about choices of energy-producing processes or process modifications.

LIST OF MAJOR TASKS

		Page
A.	Biological Screening Study of Industrial Gasifiers - J. L. Epler	4
B.	Biological Screening Study of Liquefaction and Shale Oil Operations - J. L. Epler	23
C.	Mutagenicity Assays of Fractionated Coal Conversion Products, Screening - J. L. Epler	56
D.	Mutagenicity and Carcinogenicity of Energy Related Pollutants in Cultured Mammalian Cell Systems - A. W. Hsie	87
E.	*In vivo* Screening for Gene Mutations in Mouse Germ Cells and Somatic Cells - L. B. Russell	89
F.	Use of the Mouse Specific-Locus Method to Quantify the Gene Mutation Hazard from Mutagens Associated with Non-Nuclear Energy Technologies - W. L. Russell and L. B. Russell	93
G.	Animal Toxicology Studies of Effluents from Low BTU Gasifiers - R. J. M. Fry, J. M. Holland, H. R. Witschi	95
H.	Animal Toxicology Studies of Shale Oil and H-Coal Liquefaction Operations - R. J. M. Fry, J. M. Holland, H. R. Witschi	108
I.	Determine Formation Kinetics of Activation and Detoxification Products in Skin Cells - J. K. Selkirk	187

EPA SEMI-ANNUAL REPORT

January 1981

BUDGET RESOURCES

Subagreement No.: D5-E681-AN

Title: SCREENING

Major Tasks:

A. Biological Screening Study of Industrial Gasifiers

- J. L. Epler

B. Biological Screening Study of Liquefaction and Shale Oil Operations

- J. L. Epler

C. Mutagenicity Assays of Fractionated Coal Conversion Products, Screening

- J. L. Epler

D. Mutagenicity and Carcinogenicity of Energy Related Pollutants in Cultured Mammalian Cell Systems

- A. W. Hsie

E. *In vivo* Screening for Gene Mutations in Mouse Germ Cells and Somatic Cells

- L. B. Russell

Resources:

July - Dec. 1980 Costs	Total Cost To Date	Total Funds Authorized
321,450	2,386,940	3,150,000

EPA SEMI-ANNUAL REPORT

January 1981

BUDGET RESOURCES

Subagreement No.: D5-E681-AQ

Title: MUTAGENICITY AND TERATOGENICITY

Major Tasks:

F. Use of Mouse Specific-Locus Method to Quantify the Gene Mutation Hazard from Mutagens Associated with Non-Nuclear Energy Technologies

- W. L. Russell and L. B. Russell

G. Animal Toxicology Studies of Effluents from Low BTU Gasifiers

- R. J. M. Fry, J. M. Holland, H. R. Witschi

H. Animal Toxicology Studies of Shale Oil and H-Coal Liquefaction Operations

- R. J. M. Fry, J. M. Holland, H. R. Witschi

Resources:

July - Dec. 1980 Costs	Total Cost To Date	Total Funds Authorized
233,693	1,329,154	1,691,000

EPA SEMI-ANNUAL REPORT

January 1981

BUDGET RESOURCES

Subagreement No.: D5-E681-AT

Title: MOLECULAR DAMAGE AND REPAIR

Major Tasks:

I. Determine Formation Kinetics of Activation and Detoxification Products in Skin Cells

- J. K. Selkirk

Resources:

July - Sept. 1980 Costs	Total Cost To Date	Total Funds Authorized
14,081	313,452	315,000

BIOLOGICAL SCREENING STUDY OF INDUSTRIAL GASIFIERS

J. L. Epler, Principal Investigator

Oak Ridge National Laboratory
Biology Division, Post Office Box Y
Oak Ridge, Tennessee 37830

EPA-IAG-D5-E681-AN
DOE-40-516-75

Project Contact: DOE/Biomedical Programs
EPA Coordination: EPA/ORD

Office of Research and Development
U.S.E.P.A.
Washington, D.C. 20460

ABSTRACT

The principal objective of the cellular bioassay of low Btu gasifier processes is the testing of primary effluents and fugitive emissions for potential effects on man. The specific issues include (1) potential effects on man of process discharges to air or water (2) availability of environmentally acceptable methods for disposal of solid waste produced from small low Btu gasifiers, and (3) association of hazards with the handling and combustion of products by the gasifier.

Our proposed approach to answering these questions involves a parallel, two-level program. Level one (supported by EPA/DOE) is cellular bioassays. These assays will accumulate baseline data on typical effluents and emissions and ascertain how the relative toxicity of major effluents and fractions thereof vary as a function of changes in process conditions. In addition, biological effects studies using cellular assays will provide essential data base for eventual determination of correlation with whole animal studies.

A battery of short-term bioassays will be applied to selected process samples including product tars, aqueous discharges, and solid wastes. The battery includes mutagenicity tests in bacteria, fungi, mammalian cells, and whole animals; cytotoxicity and embryotoxic tests; and teratogenicity tests.

Level two consists of the mammalian toxicity bioassays (supported by DOE). As data become available through analytical chemistry, area monitoring, and cellular bioassay programs, this information can be included in guiding decisions concerning whether other materials or tests are indicated for additional evaluation of the process in further mammalian work.

INTRODUCTION

Specific Aims

The feasibility of using short-term assays to predict the potential biohazard of various low Btu gasifier test materials will be examined in a coupled chemical and biological approach. The primary focus of the research will be the use of preliminary chemical characterizations and preparation for bioassay, followed by testing in short-term assays in order to rapidly ascertain the biohazard. (This work is site specific).

Tests in the cellular bioassay category will (1) act as predictors of profound long-range health effects such as mutagenesis and/or carcinogenesis, (2) act as a mechanism to rapidly isolate and identify a hazardous biological agent in a complex mixture, and (3) function as a measure of biological activity correlating baseline data with changes in process conditions. Assuming that the complex mixtures can be fractionated and approached in these short-term assays, we will accumulate information reflecting on the actual compounds responsible for the biological effect and/or biohazard. Thus, tests in this category will (4) aid in identifying the specific hazardous compounds involved and relegate priorities for (a) further validative testing, (b) testing in whole animals, (c) more definitive chemical analysis and monitoring.

Two coal gasification sites are under test or under construction: University of Minnesota-Duluth and Pike County (KY). The latter may be changed.

Approach - Low Btu Gasification: The principal focus of the cellular bioassays is the testing of primary effluents and fugitive emissions for potential effects on man (Gasifiers in Industry Program/DOE). This portion of the evaluation is concerned with questions of (1) relative toxicities of process by-products (e.g., tars) and effluents (e.g., ash, particulates),

(2) toxicity variation as a function of process conditions (e.g., different coals, start-up, steady state), and (3) fugitive emissions which present the greatest potential for toxicity in the workplace.

Testing with only one microbial system has been shown to be faulty in a number of cases with pure compounds. We propose to confront this objection by using a battery of short-term genetic and/or DNA assays on the primary effluents and potential fugitive emissions. Thus, potential hazards from both the mutagenic and carcinogenic battery of assays would be used only on selected active compounds determined by the coupled effort of chemical and initial biological screens. The actual components can then be characterized either as highly purified fractions or actual pure chemicals. Feedback to chemical screening then becomes a feasible monitoring method.

The battery of tests will be composed of the following: (1) Salmonella histidine reversion, (2) Mammalian cell and/or yeast gene mutation and recombination/conversion assays, (3) DNA repair, and (4) mammalian (whole animal) assays. Only selected samples would be tested in the full battery of assays dependent upon largely the preliminary results on the "Ames" screening. Furthermore, depending on the chemical analyses, the identified potential hazardous compound might enter into the overall evaluation. Cytotoxicity will be considered a normal component of the genetic/DNA screens; however, other assays to look rapidly and specifically at toxic effects of "cellular" systems will also be carried out with appropriate samples and selected active compounds.

The various assay systems and their application to appropriate test material or selected active compounds (representative of the biohazard present) are divided into two categories: (1) <u>testing</u> specifically applicable

to low Btu gasification and to the other coal conversion facilities included in site-specific assessments and (2) research or validation applicable to the ongoing generic approach of EPA/DOE and other agencies in health-effects studies of synthetic fuel technologies. The two approaches must interrelate and reinforce one another in order to evaluate the facilities and processes effectively. Since even the cellular bioassays are only predictive and, at that, still developmental in nature, comparisons made in the program can function only as a practical use of limited state-of-the-art assays. Considerable basic research must parallel these screening efforts in order to relect accurately on the question of the environmental acceptability of various liquefaction processes.

In considering the potential biohazards of the developing coal conversion technologies, the previous comparisons of petroleum- versus shale-derived materials must be extended to coal-derived liquids and tars. The newly available techniques in short-term testing should aid in answering these questions.

The studies will also parallel our projected work with other syncrude oils, shale oils, and petroleum equivalents and complement the ongoing generic studies.

The selection of samples (see Tables) emphasizes work on particulates and tars. In addition to the standard Ames assay, cytotoxicity and teratogenesis assays are also being carried out with selected samples depending on the initial results with microbial assays. Choice of samples (and/or fractions thereof) will be extended to validative tests for mutagenesis, e.g., mammalian cell gene mutation and Drosophila and for cytogenetic damage, e.g., sister-chromatid exchange.

An approximate time schedule is given below:

Milestones

Year	Milestone
1 (FY79)	Present proposal and conduct feasibility studies.
2, 3 & 4	Conduct comparative cellular bioassays with test run samples from gasifier #1*
3, 4 & 5	Conduct comparative cellular bioassays with test run samples from gasifier #2**

*University of Minnesota-Duluth
**Pike County, KY or acceptable substitute

TECHNICAL PROGRESS

Microbial Mutagenesis (T. K. Rao & F. W. Larimer)

Short-term mutagenesis assays have been utilized to characterize complex environmental mixtures for possible health effects. These bioassays are intended to serve as predictors of long-range health effects, to guide chemical separation procedures for the isolation and concentration of biologically active materials, to identify chemical agents responsible for the biological activity, and to determine priorities for further, extensive testing. Organic extraction, coupled with chemical class fractionation, is a prerequisite for most of these assays.

Our emphasis has been mainly on evaluating various test materials from the newly emerging synfuel technologies. The bacterial mutagenicity assay developed by Ames utilized certain well-characterized histidine mutants of *Salmonella typhimurium*. We have previously reported the application of a class separation procedure developed by Swain-Stedman for evaluating certain

coal- and shale-derived oils. In an effort to minimize chemical reactivity during the fractionation procedure, separation with Sephadex LH-20 gel chromatography was used with several synfuels. Mutagenicity results obtained have indicated that the alkaline fractions containing azaarenes, primary amines, and nitro polyaromatics are mutagenic fractions, along with the neutral fraction containing PAHs and nitrogen-containing PAHs. Specificity to revert the frameshift strains TA1538 and TA98 and dependence on specific activation systems correlate with the biological activities of these organic constituents.

(a) Samples - Samples utilized in these studies were collected during various runs at the UMD facilities. Chemical characterization and preparation for bioassay was followed by various biological assays (see Tables).

(b) Chemical Fractionation - The chemical fractionation procedure has been described in our publications. The procedure involves separation of acidic and basic fractions by using liquid/liquid partitioning into ether-soluble and -insoluble acids and bases (water-soluble fractions were generally found inactive in the mutagenicity assays). The neutral fraction was separated, using Sephadex LH-20, into aliphatics and aromatic, polyaromatic, and polar fractions by isopropanol elution. Solvents were removed by rotary evaporation, and the residue was dissolved in dimethyl sulfoxide for mutagenicity assays.

(c) Results - ESP tars from the gasifier at UMD were analyzed for mutagenic activity in the Ames salmonella histidine reversion assay. The tar sample was chemically separated into chemically defined fractions (see Tables). and assayed for mutagenic activity. The strain designated to detect frameshift mutations (TA98) was used in these assays and metabolic activation

with Aroclor induced rat liver microsomal enzymes (S-9 mix) was included. Specific mutagenic activity was determined by linear regression of the dose response curves. The results are given in the table that follows.

Conclusions

i) The ESP tar samples collected at different sampling dates were mutagenic in the salmonella histidine reversion assay. The mutagenic constituents required metabolic activation (achieved by the addition of rat liver S-9 mix).

ii) The mutagenic activity varied at different sampling dates. Samples collected on 12/12/79, 2/4-14/79 and 2/25-28/80 were more mutagenic than other samples.

iii) The mutagenic activity remained mainly in the basic fraction indicating aromatic amines as the suspect agents.

iv) ESP tars obtained from 2/4-14/79 exhibited significant mutagenic activity in the neutral (PAH fractions) and basic fractions.

Both the ether-soluble base (ESB) fraction and the polyaromatic fraction of the "May" (UMD) electrostatic precipitator tar were also mutagenic and toxic to yeast as the initial extension or validation of the bacterial data. The ESB fraction was direct-acting while the PAH fraction required metabolic activation. 5- to 10-fold mutation induction and 50 to 90% cell inactivation were observed in the 0.1 - 1 mg/ml dose range. The overall message - the Ames test results have meaning when extended to other genetic systems.

Mutagenic activity of gasifier tars in the *Salmonella* assay

Code #	Sample description (Date of sample collection)	% Distribution ACID	BASE	NEUTRAL	Sp. Activity (Rev/mg) TOTAL
05	ESP tar (11/27,29/79)	-	-	-	0
06	Tar, holding tank (11/19,24/79)	-	-	-	0
16	ESP tar (12/2/79)	0	100	0	143
17	" (12/5/79)	0	100	0	88
18	" (12/11/79)	0	100	0	142
19	" (12/12/79)	0	100	0	736
23	ESP tars (2/4--14/79)	<1	99	0	523
24	"	0	97	<3	180
25	"	-	-	-	0
26	"	0	96	4	120
27	"	<1	100	0	1855
28	"	0	63	37	706
29	"	0	93	7	41344
30	"	0	28	72	11756
36	ESP tars (2/15--20/80)	0	100	0	9464
37	"	0	100	0	162
38	"	0	100	0	150
39	"	0	100	0	232
83.2	ESP tar (2/6--7/80)	0	100	0	232
87	Vent gas cyclone tar (3/3/80)	0	100	0	68
88	Tar from dirty top gas	0	100	0	35
89	ESP tars (2/25--28/80)	0	99	<1	864
90	"	0	99	<1	8188
91	"	0	100	0	261
92	"	0	100	0	259
		0	100	0	339

Mammalian Mutagenesis (L. B. Russell, W. M. Generoso, W. L. Russell)

This project is designed to evaluate the potential of certain process mixtures from the UMD coal gasification project to induce genetic effects in mice.

I. UMD Materials:

Last time we reported the completed result of the dominant-lethal study in males and the early result of the total reproductive capacity test in females with the electrostatic precipitator tar (ESP) No. 13. We now report on the completion of the total reproductive capacity test in females with ESP No. 13. During this reporting period we also started work on a second sample called ESP No. 83.

1. ESP No. 13 - The total reproductive capacity study with ESP tar No. 13 was conducted using doses of 2000 or 2400 mg/kg (maximum tolerated dose) to (SEC x C57BL)F_1 females. Average litter sizes of experimental females in the early posttreatment period (within 62 days) were clearly lower than those in controls, but subsequent litters were of normal size. It is not clear whether the reproductive effect observed is due to dominant-lethal mutations, cell killing or to nongenetic effect associated with alterations in female physiology.

2. ESP No. 83 - This tar was selected for testing in mice because it was found by the Epler's group to be highly mutagenic in certain submammalian systems.

Preparatory to any of the genetic studies planned, we conducted an acute toxicity study to determine the maximum tolerated dose (MTD) for single intraperitoneal injection. The MTD was found to be 2500 mg/kg.

Using this dose, we started a dominant-lethal study in (101 x C3H)F_1 males. Experimental and control males were mated to (C3H x 101)F_1 females. This study is still in progress but data on matings that occurred up to 20 days posttreatment are now available. This period corresponded to treated postmeiotic stages. Data, so far, show no detectable increase in induced dominant-lethal mutations.

We have just started a total reproductive capacity test in females with ESP No. 83.

Cytotoxicity and Embryotoxicity (J. N. Dumont & T. W. Schultz)

Cytotoxicity of selected UMD gasifier samples.* Experiments have been completed in which neutralized distilled-water leachates of cyclone ash No. 12 and wet ash No. 9 as well as aqueous extracts of electrostatic precipitator tar (ESPT) No. 83 have been screened under short-term static conditions for cytotoxic effects to the common ciliate *Tetrahymena*.

For leachate testing, cells were grown in axenic culture prior to harvesting by centrifugation. Cells were resuspended in a 1:250 dilution ($\sim$ 1,500 cells/ml) in 5 ml aliquots of various percent concentrations of leachate. At representative time points 10 μl aliquots were removed and the cells counted with the aid of dark-field microscopy. Through 96 hours of exposure 100% of neither the cyclone ash nor the wet ash leachate significantly alter cell viability or population growth.

---

*Leachate waters provided by D. K. Brown, Environmental Sciences Division, ORNL.

Aqueous (culture medium extracts of ESPT were prepared by coating the lower inner surface of a 125 ml Erlenmyer flask with 8 ml of warm ESPT prior to addition of 60 ml of sterile medium and slow stirring for 16 hours. Cells exposed to various concentrations of extracts were cultured for 48 hours. Population growth impairment was monitored spectrophotometrically at 540 nm. The 48-hr IGC50 (inhibitory growth concentration for 50% of the cells) was determined by linear regression of probit percent 48-hr control absorbance vs. percent concentration. The toxicity regression model for these studies is: Absorbance (probit) = 8.4594 - 5.4367) (% concentration), with a correlation coefficient of -0.989. The IGC50 and IGC100 values are 0.64 and 1.07%, respectively. The no-observed effects concentration is 0.08%.

Embryotoxicity and teratogenicity of selected UMD gasifier samples. Preliminary experiments have been conducted to determine the embryo-toxicity and teratogenicity of distilled-water extracts of UMD gasifier cyclose ash No. 12, wet ash No. 9 and electrostatic precipitator tar No. 83 to early embryos of the frog *Xenopus laevis*. Tar extracts were prepared by slow-stirring 1 part tar with 8 parts water for 16 hours. Ash leachates were prepared by the Environmental Sciences Division, ORNL. Groups of 50 embryos each were placed in 200 ml aliquots of graded concentrations of each extract and tested under acute static conditions. Following 96 hours of exposure data concerning number of dead, numbers of abnormals, stage of development, and size as well as motility and pigmentation were collected. Mortality and abnormality data were analysed by linear regression.

Aqueous extracts of tar No. 83 are highly toxic with 1.5% causing 100% mortality within 96 hours of exposure. The 96-hr LC50 value is estimated

to be 0.7% extract. Eighteen percent of the survivors at 0.25%, the lowest concentration tested have gross abnormalities. The cyclone ash No. 12 leachate is non-toxic with 100% leachate having no adverse effects on embryos exposed for 96 hours. Leachate of wet ash No. 9, on the other hand, while causing no mortality does cause abnormal development as well as affecting size, motility and pigmentation (Tab. 1). The no-observable effects concentration is 55%.

Table 1. Effects of neutralized distilled-water leachate of wet ash No. 9 on early embryos of *Xenopus laevis*.

Concentration (%)	0	70	80	90	100
% abnormal/survivor	3.8	11.3	26.0	30.5	40.3
developmental stage	46	44-46	44-46	44-46	44-46
length (mm sd)	9.3 ± .4	7.6 ± .4	7.7 ± .7	7.4 ± .5	7.8 ± .4
motility	++++	++	++	++	++
pigmentation	++++	++	++	++	++

Summary of Screening

1) Short-term mutagenesis with Salmonella and yeast have shown mutagenic activity for ESP tars.

2) Mutagenic activity concerned with basic and neutral fractions.

3) Prelminary identification of compounds is in progress.

4) Battery of short-term tests is being applied to predicted classes of constituents.

5) Cytotoxicity of tar-aqueous extract aqueous effluents/streams, and leachates suggest high toxicity with the materials and little toxicity with aqueous sample. Initial mutagenicity screening parallels the toxicity results.

6) Mammalian mutagenesis underway with two tar samples.

Key Publications - October 1978 - Present

Cowser, K. E., ed. Proposed Environmental and Health Program for University of Minnesota Gasification Facility, Oak Ridge National Laboratory, January 23, 1978.

Cowser, K. E., ed. Environmental and Health Program for Pike County Coal Gasification Facility, Oak Ridge National Laboratory, August, 1979.

Cowser, K. E., ed. Briefing for Environmental Working Group Gasifiers in Industry on University of Minnesota-Duluth Project/Pike County, Kentucky Project, Oak Ridge National Laboratory, June 13, 1980.

Cowser, K. E., ed. University of Minnesota-Duluth Coal Gasification Project. Quarterly Progress Report

- Period Ending Sept. 30, 1979	ORNL/TM-7272
- Period Ending Dec. 31, 1979	ORNL/TM-7268
- Period Ending Mar. 31, 1980	ORNL/TM-7354
- Period Ending June 30, 1980	In Press
- Period Ending Dec. 31, 1980	In preparation

Epler, J. L., et al. Analytical and biological analyses of test materials from the synthetic fuel technologies. I. Mutagenicity of crude oils determined by the *Salmonella typhimurium*/microsomal activation system, Mutat. Res. 57: 264-76 (1978).

Epler, J. L., T. K. Rao, M. R. Guerin. Evaluation of feasibility of mutagenic testing of shale oil products and effluents. Environ. Health. Persp. 30: 179-184 (1979).

Generoso, W. M., K. T. Cain, M. Krishna and S. W. Huff. Genetic lesions induced by chemicals in spermatozoa and spermatids of mice are repaired in the egg. Proc. Natl. Acad. Sci. USA 76: 435-437 (1979).

Guerin, M. R., I. B. Rubin, T. K. Rao, B. R. Clark, J. L. Epler. Distribution of mutagenic activity in petroleum and petroleum substitutes. Fuel, in press (1980).

Guerin, M. R., C.-h Ho, T. K. Rao, B. R. Clark, J. L. Epler. Polycyclic aromatic primary amines as determinant chemical mutagens in petroleum substitutes. Environ. Res., in press (1980).

Ho, C. -h. M. R. Guerin, B. R. Clark, T. K. Rao, J. L. Epler. Preparation-scale isolation of alkaline mutagens from complex mixtures. Environ. Sci. and Technol., in press, 1980.

Rao, T. K., J. A. Young, A. A. Hardigree, W. Winton, J. L. Epler. Analytical and biological analyses of test materials from the synthetic fuel technologies. II. Mutagenicity of organic constituents from the fractionated synthetic fuels. Mutat. Res. 54: 185-191 (1978).

Rao, T. K., B. E. Allen, D. W. Ramey, J. L. Epler, I. B. Rubin, M. R. Guerin, B. R. Clark. Analytical and biological analyses of test materials from the synthetic fuel technologies. III. Fractionation with LH-20 gel for the bioassay of crude synthetic fuels. Mutat. Res., in press (1980).

Rao, T. K., J. L. Epler, M. R. Guerin, B. R. Clark and C. -h. Ho. Mutagenicity of nitrogen compounds from synthetic crude oils; collection, separation and biological testing. Proceedings of the Second Symposium on the Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures, Williamsburg, Va., March 4-7, 1980.

Epler, J. L. et al. Toxicity of Leachates, EPA-600/2-80-057 (March) 1980.

Specific cellular bioassay tests

TEST
Cellular screening bioassays
CS1. Salmonella
CS2. Yeast
CS3. DNA repair
CT1. Mammalian (CHO) cell cytotoxicity
CT2. RAM
CT3. Invertebrate cytotoxicity
CT4. Amphibian and mammalian teratogenesis
Screening/validating assays
VT1. Drosophila
VT2. Mammalian cell gene
VT3a Mammalian cell chromosomal (CHO)
VT3b Mammalian cell chromosomal (leukocyte)
VT4. In vitro cell transformation
Mammalian mutagenesis assays
M1. Dominant lethals
M2. Heritable translocations
M3. Specific locus
M4. "Spot test" for somatic mutation
M5. Total reproductive capacity

UMD

Specific cellular bioassay tests on process samples

Sample point	Type of test	Sample type	Chemical preparation	Application
9 Gasifier ash	CS1,CS2,CS3 CT1,CT2	Particulate -leachate (3)[b]	Fractionation	
	CS1,CT3	Particulate (3)	None	
10 Top gas particulates	CS1,CT3	Particulate (3)	None	
11 Bottom gas tars and particulates	CS1,CT3	Particulate (2)	None	
12 Cyclone ash	CS1,CS2,CS3 CT1,CT2	Particulate -extract (3)	Fractionation	Low Btu gasifier comparison
	CS1,CT3	Particulate (3)	None	
15 Tar from ESP	CS1,CS2,CS3 CT1,CT2	Liquid (3)	Fractionation	
	M1,M4	Liquid (2) (Composite)	None or minimal	
18 Gas boiler particulates	CS1	Particulate -extract (3)	Fractionation	
	CS1,CT3	Particulate (3)	None	
19 Storage tank tar	CS1,M1,M4	Liquid (1) (Composite)	Fractionation	
20 Tar boiler particulates	CS1	Particulate -extract (3)	Fractionation	
	CS1,CT3	Particulate (3)	None	
Auxiliary fuel	CS1	Liquid (1) (Composite)	Fractionation	Ongoing base research program
Selected active compounds (SAC)	CS1,CS2,CS3,CS4 CT1,CT2,CT3 VT1,VT2a,VT2b M1,M5 M2,M3,M4	Identified chemical compounds and selected active process samples		Ongoing base research program and low Btu gasifier comparison

[b]Indicates number of samplings.

Table. Specific cellular bioassay tests on process samples — Pike County Phase I[a]

Sample point	Sample type and preparation	Type of bioassay	No. of samples	Sample size	Fractions per sample	Total tests (maximum) per assay	Application
5. Gasifier ash	Solid						Low-Btu gasifier comparison
	crude		3	250 g			
	organic extract	CS1, CS2, CS3, CT2[b]			4	12	
	aqueous extract	CS1, CS2, CS3, CT1, CT3			4	12	
9. Water-jacket overflow	Liquid						Low-Btu gasifier comparison
	crude	CS1, CT3	1	10 liters		1	
	resin/organic conc.	CS1, CS2, CS3, CT4		conc.	4	4	
11. Tars/dirty gas	"Liquid"[c]						Low-Btu gasifier comparison
	crude	M1, M4	1	200 g		1	
	organic extract from:		(3)	(?)			
	(a) particulate solids	CS1, CS2, CS3, VT2 CT4, CT2,[b] VT3A, VT3B	3	10 g	4	12	
	(b) condensate (aqueous)		3	100 ml	4	12	
	(c) condensate (organic)		3	100 ml	4	12	
15. Primary cyclone ash	Solid						Low-Btu gasifier comparison
	crude		3	250 g			
	organic extract	CS1, CS2, CS3, CT2			4	12	
	aqueous extract	CS1, CS2, CS3, CT1, CT3			4	12	
17. Tars/hot raw gas	"Liquid"[c]						Low-Btu gasifier comparison
	crude						
	organic extract from:		(3)	(?)			
	(a) particulate solids	CS1, CS2, CS3, VT2 CT4, CT2,[b] VT3A, VT3B	3	10 g	4	12	
	(b) condensate (aqueous)		3	100 ml	4	12	
	(c) condensate (organic)		3	100 ml	4	12	
19. Boiler fuel	Liquid						Low-Btu gasifier comparison
	crude		1	200 g			
	organic extract	CS1			4	4	
20. Boiler ash	Solid						Low-Btu gasifier comparison
	crude		3	250 g			
	organic extract	CS1, CS2, CS3, CT2			4	12	
	aqueous extract	CS1, CS2, CS3, CT1, CT3			4	12	

Table (cont.)

Sample point	Sample type and preparation	Type of bioassay	No. of samples	Sample size	Fractions per sample	Total tests (maximum) per assay	Application
22. Secondary cyclone ash	Solid						Low-Btu gasifier comparison
	crude		3	250 g			
	organic extract	CS1, CS2, CS3, CT1, CT3			4	12	
	aqueous extract	CS1, CS2, CS3, CT1, CT3			4	12	
24. Stack gas	"Solid" (Airborne particulates)						Low-Btu gasifier comparison
	organic extract from:		3 (3)	250 g (?)			
	(a) particulate solids	CS1, CS2, CS3, VT2 CT2,[b] VT3A, VT3B	3	10 g	4	12	
	(b) condensate (aqueous)		3	100 ml	4	12	
	(c) condensate (organic)		3	100 ml	4	12	
26. Silo ash	Solid						Low-Btu gasifier comparison
	crude		1	250 g			
	aqueous extract	CS1, CS2, CS3, CT1, CT1, CT2,[b] CT3			4	4	
Selected active compounds	Dependent on chemical data and bioassays	All assays				Multiple	Ongoing base research program and low-Btu gasifier comparison

[a]The listings here are specific site samples to be identified and characterized. Other work with the same or similar samples parallels these studies as part of the base research program.

[b]CT2 – RAM test applicable to particulate solids only.

[c]"Liquid" = composite of condensates as crude material.

BIOLOGICAL SCREENING STUDY OF LIQUEFACTION AND SHALE OIL OPERATIONS

J. L. Epler, Principal Investigator

Oak Ridge National Laboratory
Biology Division, Post Office Box Y
Oak Ridge, Tennessee 37830

EPA-IAG-D5-E681-AN
DOE-40-516-75

Project Contact: DOE/Biomedical Programs
EPA Coordination: EPA/ORD

Office of Research and Development
U.S.E.P.A.
Washington, D.C. 20460

ABSTRACT

The objective of this biological screening is to provide early information on the potential biohazards unique to liquefaction or shale oil operations. A battery of short-term bioassays will be applied to selected process samples including product oils, aqueous discharges, and solid wastes. The battery includes mutagenicity tests in bacteria, fungi, mammalian cells, and whole animals; cytotoxicity and embryotoxicity tests; and teratogenicity tests.

The principal focus of the biological assays is the testing of primary effluents and fugitive emissions for potential effects on man. This portion of the evaluation is concerned with questions of (1) relative toxicities of process materials, products, aqueous wastes, and solid wastes; (2) toxicity variation as a function of process conditions (e.g., 3 different coals, 2 different modes - fuel oil and syncrude); and (3) possible fugitive emissions which present the greatest potential for toxicity in the workplace. Our approach to answering these questions involves a parallel, two-level program. Level one (supported by EPA/DOE) is cellular bioassays. These assays will accumulate baseline data on typical effluents and emissions and ascertain how the relative toxicity of major effluents and fractions thereof vary as a function of changes in process conditions.

Level two (supported by DOE) consists of the mammalian toxicity bioassays. These assays will involve characterization of the acute, subacute, and chronic toxicity of primary process by-products and effluents. As data become available through the analytical chemistry, area monitoring, and cellular bioassay programs, this information can be included in guiding decisions concerning whether other materials or tests are indicated for additional evaluation of the process.

INTRODUCTION

Specific Aims:

The feasibility of using short-term assays to predict the potential biohazard of various Shale Oil and H-Coal test materials will be examined in a coupled chemical and biological approach. The primary focus of the research will be the use of preliminary chemical characterizations and preparation for bioassay, followed by testing in short-term assays in order to rapidly ascertain the biohazard. (This work is site specific).

Tests in the cellular bioassay category will (1) act as predictors of profound long-range health effects such as mutagenesis and/or carcinogenesis, (2) act as a mechanism to rapidly isolate and identify a hazardous biological agent in a complex mixture, and (3) function as a measure of biological activity correlating baseline data with changes in process conditions. Assuming that the complex mixtures can be fractionated and approached in these short-term assays, we will accumulate information reflecting on the actual compounds responsible for the biological effect and/or biohazard. Thus, tests in this category will (4) aid in identifying the specific hazardous compounds involved and determine priorities for (a) further validative testing, (b) testing in whole animals, and (c) more definitive chemical analysis and monitoring.

The H-Coal program will be carried out in two phases: Phase I will address samples that are currently available from pilot-demonstration scale operations; short-term mutagenesis, cytotoxicity along with mammalian toxicity and skin carcinogenesis assays will be carried out with these materials. Phase II will address samples developed when the H-Coal plant is underway. Phase I tests (already underway) will

include the H-Coal raw distillate and various stages of upgrading along with H-Coal products. These preliminary assays will parallel our existing efforts with other syncrudes. The information received should aid in selection of actual process samples for evaluating the Catlettsburg, Kentucky H-Coal pilot plant now under construction. It will also provide useful comparison of the changes which occur in the biological characteristics of specific process liquids as a function of scale-up.

The principal focus of the Paraho/SOHIO Shale Oil project is the testing of primary effluents and products for potential effects on man. This portion of the evaluation of Paraho Samples is concerned with questions of relative toxicities of process materials and refinery products.

Approach - H-Coal Liquefaction: The H-coal program is being carried out in two phases: Phase I evaluates samples (see Appendix) that are currently available from process-development unit-scale operations. Microbial mutagenesis and some mammalian toxicity assays will be carried out with these materials. Phase II will evaluate samples collected when plant operation is underway. Phase I samples (already underway) include the H-coal raw distillate and various stages of upgrading along with H-coal product components and their combination (process-development unit run at Trenton plant). The information received should aid in selection of actual process samples and relevance in Phase II (samples available approximately early 1981-March 1981).

The various assay systems and their application to appropriate test materials or selected active compounds (representative of the

biohazard present) are divided into two categories: (1) testing specifically applicable to H-coal and to the other liquefaction facilities included in site-specific assessments and (2) research or validation applicable to the ongoing generic approach of EPA/DOE and other agencies in health-effects studies of synthetic fuel technologies. The two approaches must interrelate and reinforce one another in order to evaluate the facilities and processes effectively. Since even the cellular bioassays are only predictive and, at that, still developmental in nature, comparisons made in the program can function only as a practical use of limited state-of-the-art assays. Considerable basic research must parallel these screening efforts in order to reflect accurately on the question of the environmental acceptability of various liquefaction processes.

In considering the potential biohazards of the developing coal conversion technologies, the previous comparisons of petroleum- versus shale-derived materials must be extended to coal-derived liquids and tars. Clarification of the question of decreased activity in upgraded oils also needs to be reevaluated. The newly available techniques in short-term testing should aid in answering these questions.

The studies will also parallel our projected work with other syncrude oils, shale oils, and petroleum equivalents and complement the ongoing generic studies.

The selection of samples (see Appendix) emphasizes work on product oils and precursors. In addition to the standard Ames assay, cytotoxicity and teratogenesis assays are also being carried out with selected samples depending on the initial results with microbial assays. Choice of samples (and/or fractions thereof) will be extended to

validative tests for mutagenesis, e.g., mammalian cell gene mutation and *Drosophila* and for cytogenetic damage, e.g., sister-chromatid exchange.

Approach - Shale Oil (Paraho/SOHIO Upgrading Study): The principal focus of the Shale Oil project is the testing of primary residues and products for potential effects on man. This portion of the evaluation of Paraho Samples is concerned with questions of relative toxicities of process materials and refinery products. The samples are available through the refining of Paraho retort crude oil at the SOHIO plant. Products and precursors and their conventional petroleum analogs are being compared. The sample list and the bioassays that are considered are given in the Appendix.

Sample description and source - Samples utilized in this study were supplied by the U.S. Environmental Protection Agency/U.S. Department of Energy (USEPA/USDOE) repository for research materials. Process operating conditions at the time of sampling, sampling conditions, and sample histories are not sufficiently defined to provide process-specific conclusions. Sample listings (with repositiory numbers) and sources are given in the Appendix and Tables.

TECHNICAL PROGRESS

MUTAGENESIS

Microbial Mutagenesis (T. K. Rao & F. W. Larimer)

Short-term mutagenesis assays have been utilized to characterize complex environmental mixtures for possible health effects. These bioassays are intended to serve as predictors of long-range health

effects, to guide chemical separation procedures for the isolation and concentration of biologically active materials, to identify chemical agents responsible for the biological activity, and to determine priorities for further, extensive testing. Organic extraction, coupled with chemical class fractionation, is a prerequisite for most of these assays.

Mutagenicity testing coupled with chemical separation and characterization was utilized to isolate and identify chemical agents with prolonged health hazards such as mutagenicity/carcinogenity in the hydrogenated coal samples (H-coal). Organic extraction and liquid/liquid partitioning (see Appendix) was used to separate acidic and basic fractions. The neutral material was separated using Sephadex LH-20 into aliphatic fraction and aromatic fractions of various ring sizes. The basic fraction was subfractionated eluting with benzene and ethanol on a basic Allumina Column and then isopropanol and acetone using a Sephadex LH-20 column (see Appendix). The neutral fraction was subfractionated using Sephadex LH-20 column and sequential elution with isopropanol and acetone into aliphatic and aromatic fractions. The aromatic fraction was separated into PAH, N-PAH and Polar fractions by elution with benzene/hexane (1/3), benzene/hexane (2/1) and ethanol respectively using a salicic acid column (see Appendix). The frameshift strain TA-98 (developed by B. N. Ames) was used for the mutagenicity analysis and metabolic activation with rat liver homogenate (S-9 mix) was incorporated into the assays.

Results

Table 1 illustrates mutagenicity results obtained with a series of H-coal samples. Mutagenic activity* was distributed between the

*Ames, B. N., et al. Mutation Res. 31, 347-364 (1975)

basic and neutral fractions indicating nitrogen containing aromatic hydrocarbons along with fused ring aromatic hydrocarbons as the suspect agents. Hydrogenation (samples 1604, 1603) has either eliminated or reduced the amount of mutagenic activity of the raw sample (sample 1601).

Since the basic and neutral fractions contained most of the mutagenic activity, they have been subfractionated and subjected to mutagenesis analysis and chemical characterization.

Results obtained with the basic subfractions (ESB) are given in Table 2. Most of the mutagenic activity was recovered in the acetone subfraction (ESB-A) which contains approximately <10% weight of the ESB fraction. Aza arenes aromatic amines are the suspect agents from this fraction.

Results obtained with the neutral subfraction are given in Table 3. Two H-coal samples (samples 1309 & 1310) were subjected to mutagenesis analysis. The aliphatic fractions were inactive while the PAH and PAH-N fractions contained significant mutagenic activities. The polar subfraction exhibited significantly more (>35%) activity than the PAH fractions. Thus the mutagenic activity of the neutral fraction, which was previously ascribed to the PAHs may be due to the PAHs containing polar substitutions.

Table 1. Distribution of mutagenic activity[a] in fractionated H-coal samples.

ORNL Repository No.	Description	% Distribution Acid	Base	Neutral	Total Mutagenic Activity (Rev/mg)
1309	H-Coal ASB (syn)	1	46	53	970
1310	H-Coal VSOH (syn)	0	42	58	2400
1311	H-Coal VSB (syn)	0	66	17 (16)	2000
1312	H-Coal ASOH (Fo)	—	—	—	0
1313	H-Coal ASB (Fo)	0	100	0	140
1314	H-Coal VSOH (Fo)	0	25	75	4200
1315	H-Coal VSB (Fo)	0	89	9 (2)	6300
1601	H-Coal (raw)	0	37	63	350
1602	H-Coal HDT/L	0	0	100	540
1603	H-Coal HDT/M	0	0	100	210
1604	H-Coal HDT/H	0	0	0	0

[a]Determined from the dose response curves obtained with the strain TA-98 (Ar S-9 included).

Table 2. Distribution of mutagenic activity[a] in the ether soluble basic fraction (ESB).

Sample Description (ORNL Rep. #)	(Rev/mg fraction) ESB	ESB-B[1]	ESB-I[2]	ESB-A[3]
H-Coal ASOH (1312)	2,400	0	600	32,000
H-Coal ASB (1313)	30,000	0	880	55,000
H-Coal VSOH (1314)	64,000	0	0	220,000
H-Coal VSB (1315)	128,000	0	8,000	208,000

[a]as in Table 1.

[1]B = Benzene; 2 I = Isopropanol; 3 A = Acetone.

Table 3. Mutagenicity of neutral subfractions from salicic acid column.

Subfractions	Mutagenicity H Coal ASB (1309)	Mutagenicity H Coal VSOH (1310)
Neutral	+	+ +
Aliphatic	—	—
Polyaromatic (PAH)	±	±
Polyaromatic-nitrogen (PAH-N)	±	±
Polyaromatic-polar (Polar)	+ +	+ +

[a]1000-2000 Rev/mg (+ +); 200-1000 Rev/mg (+), 10-200 Rev/mg (I), <10 Rev/mg (-).

COAL LIQUEFACTION AND GASIFICATION WORKING GROUP ON DNA REPAIR

Semi-annual Progress Report for Period
of July 1 - December 31, 1980

James D. Regan and W. C. Dunn

During the above period, we assayed several compounds from the selected chemical lists for both the H-coal and UMD processes. Compounds selected included those from a list of organic constituents of the acetone fraction from ESB (dated Jan. 16, 1980). These compounds as well as other predicted or known products of both processes were assayed for their ability to cause DNA damage and subsequent repair in human fibroblast cultures by use of the bromodeoxyuridine photolysis assay[1]. A variety of effects were observed, including the presence of repaired regions and the apparent formation of DNA cross-links by some agents (Table I).

In addition, we have observed UV-like excision repair due to DNA damage by derivatives and metabolites of compounds known to be coal conversion derivatives[2]. The parent compounds, metabolites and/or derivatives are listed in Table II.

In summary, we are continuing to find a wide range of active (DNA damaging) compounds associated with the H-coal and UMD coal conversion processes.

[1]Regan, James D., R. B. Setlow and R. D. Ley, Proc. Nat'l. Acad. Sci. U.S.A., 68, 708-712 (1971).

[2]Francis, A. A., R. D. Snyder, W. C. Dunn and James D. Regan, Classification of chemical agents as to their ability to induce long or short patch DNA repair in human cells. (Submitted to Mutation Research).

Table I. Materials assayed from selected chemicals lists of H-coal and UMD processes: organic constituents of the acetone fraction of ESB and other compounds.

Compound	Concentration and Exposure Time	Effect
Dibenzacridine	10u<u>M</u>, 90min.	Apparent DNA Cross-linking[a]
8-aminoquinoline	100u<u>M</u>, 90min.	No effect
4-methylchrysene	10u<u>M</u>, 60min.	Apparent DNA Cross-linking[a]
5-methylchrysene	10u<u>M</u>, 60min.	Apparent DNA Cross-linking[a]
4-aminobiphenyl	50u<u>M</u>, 90min.	No effect
1-naphthylamine	100u<u>M</u>, 90min.	Photodynamic Breakage of DNA[b]
2-naphthylamine	100u<u>M</u>, 90min.	No effect

[a]DNA molecular weight (M_w) increases with increasing doses of 313nm light.

[b]0.34 DNA single-strand breaks/10^8daltons at a 313nm dose of $10^4 J/m^2$.

Table II. DNA repair following exposure of human fibroblasts to metabolites or derivatives of compounds associated with H-coal and UMD processes.

Compound	Parent Compound	Concentration and Exposure Time	#Repaired sites per 10^8daltons DNA
ICR-170[a]	Acridine	1.0uM, 60min.	1.4
7-bromomethyl benzanthracene	Benzanthracene	10uM, 90min.	2.4
Dimethylbenz-anthracene-5,6-epoxide	Benzanthracene	10uM, 90min.	3.1
BP-diol-epoxide	Benzo(a)pyrene	1.0uM, 90min.	2.8 /4.6

[a]2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)aminopropylamino] acridine dihydrochloride

[b]7β,8α-diol-9β,10β-epoxy-benzo(a)pyrene

[c]7β,8α-diol-9α,10α-epoxy-benzo(a)pyrene

MUTAGENICITY OF MODEL COMPOUNDS AND ORGANIC MIXTURES OF COAL LIQUIFACTION AND GASIFICATION TECHNOLOGIES: A SUMMARY OF STUDIES WITH THE CHO/HGPRT SYSTEM

A. W. Hsie

Previous studies have shown that the Chinese hamster ovary cell/hypoxanthine-guanine phosphoribosyl transferase (CHO/HGPRT) system is useful for determination of mutagenicity of various physical and chemical agents. Employing this assay we have studied the mutagenic activity of several classes of model chemical mutagens and three subfractions of organic mixtures of coal liquifaction and gasification technologies, and summarize the findings as follow.

Polycyclic Hydrocarbons. We have studied the mutagenicity of benzo(a)pyrene [B(a)P] and its 19 metabolites, including 11 phenols, 3 epoxides, 3 diols, and 2 diolepoxides. For comparison, benzo(e)pyrene [B(e)P] and pyrene were added to this study. Also included were benz(a)-anthracene (BA) and 4 related compounds (7,12-dimethyl BA, anthracene, and two phenolic derivatives of BA). The carcinogenic polycyclic hydrocarbons B(a)P, BA, and 7,12-dimethyl BA require metabolic activation to be mutagenic. The noncarcinogenic polycyclic hydrocarbons, pyrene and anthracene, are nonmutagenic even with metabolic activation. B(a)P-4, 5-epoxide and B(a)P-7,8-diol,9-10-epoxide(syn) are mutagenic. Since CHO cells cannot activate procarcinogens such as B(a)P, these cells appear to be most useful in screening for the mutagenicity of metabolites such as those of B(a)P. Because of the limited availability of B(a)P derivatives, some of the experiments remain to be pursued in detail.

Metallic Compounds. We found that $MnCl_2.4H_2O$, $FeSO_4.7H_2O$ and $CoCl_2.6H_2O$ are mutagenic, while $NiCl_2.6H_2O$, $BeSO_4.4H_2O$, and $CdCl_2$ are weakly mutagenic. Determination of metal mutagenicity is apparently complicated by the ionic composition of the medium. For example, we found that the mutagenicity and cytotoxicity of $MnCl_2$ were abolished by the excess of $MgCl_2$. The unusual environment required for demonstration of mutagenicity of $MnCl_2$ makes assessment of its biological hazard difficult. This too may account in part for varying results obtained in studying the mutagenicity of $AgNO_3$, $CaCl_2$, $Pb(CH_3COO)_2.3H_2O$, RbCl, H_2SeO_3, $TiCl_4$, and $ZnSO_4.7H_2O$.

Nitrosamines and Related Compounds. Nitrosamines generally require metabolic activation to be cytotoxic and/or mutagenic. In addition to investigating two common aliphatic nitrosamines, dimethylnitrosamine (DMN) and diethylnitrosamine (DEN), we have studied the mutagenicity of 11 cyclic nitrosamines, including 3 nitrosopiperidines, 3 nitrosopyrrolidines, 3 nitrosopiperazines, and 2 nitrosomorpholines. Our studies also include the nitrosamine-related chemicals dimethylamine, formaldehyde, and sodium nitrite. We have found that all 9 carcinogenic nitrosamines (DMN, DEN, 2-methyl-1-nitrosopiperidine, 3,4-dichloro-1-nitrosopiperidine, nitrosopyrrolidine, 3,4-dichloronitrosopyrrolidine, 1,4-dinitrosopiperazine, 1,5-dinitrosohomopiperazine, nitrosomorpholine) are mutagenic and all 4 noncarcinogenic nitrosamines (2,5-dimethylnitrosopiperidine, 2,5-dimethylnitrosopyrrolidine, 1-nitrosopiperazine, nitrosophenmetrazine) are non-mutagenic. Formaldehyde and sodium nitrite are non-mutagenic, and dimethylamine is mutagenic at high concentrations. Variable carcinogenicity data on the latter three chemicals exist in the literature.

Quinoline Compounds (Total of 5). Quinoline, a known carcinogen, is mutagenic with metabolic activation. Another carcinogen, 4-nitroquinoline-1-oxide, is highly mutagenic; its mutagenicity decreases when assayed in the presence of the activation system. The carcinogenicity of 8-hydroxy-, 8-amino-, and 8-nitroquinoline is not known, but these compounds exhibit variably weak mutagenicity in preliminary experiments.

Fractionated Synthetic Fuel. In addition to studying the mutagenicity of individual environmental agents such as polycyclic hydrocarbons, quinolines, nitrosamines, metallic compounds, etc., we have found that the CHO/HGPRT assay can detect the cytotoxicity and mutagenicity of a crude organic mixture, in this case three subfractions of a crude synthetic oil (fractionated by M. R. Guerin of the Analytical Chemistry Division, ORNL) supplied by the Pittsburgh Energy Research Center. The acetone effluent which contains heterocyclic nitrogen compounds derived from the basic fraction is most mutagenic in the presence of a metabolic activation system. Earlier, it appeared that the extreme toxicity of the unfractionated crude fuel prevented meaningful mutagenicity studies in the CHO/HGPRT system.

Mammalian Mutagenesis and Teratogenesis

(L. B. Russell, R. Filler and W. M. Generoso)

This project is designed to evaluate the potential of certain proven mixtures from the H-Coal project to induce chromosome aberration effects in mice germ cells. Studies include induction of total reproductive capacity effects, dominant-lethal mutations and, when needed, heritable translocation.

So far, no PDU samples have been tested; these assays will be used only with the pilot plant materials. However, parallel efforts with a syncrude from PETC and the SCR process are completed or in progress. The crude oils do induce genetic effects in the mouse system.

CYTOTOXICITY AND EMBRYOTOXICITY

(J. N. Dumont and T. W. Schultz)

Cytotoxicity of aqueous extracts of selected H-coal liquids.

Investigations have been completed in which the cell population growth impairment of the ciliate *Tetrahymena pyriformis* exposed to aqueous extracts (AEs) of four H-coal organic liquids were monitored. Aqueous (culture-medium) extracts were prepared by slow stirring from each of the coal-derived liquids and then diluting appropriately with fresh medium prior to testing to determine extract concentration levels that adversely affect population growth. The 48-hr IGC50 (inhibitory growth concentration for 50% of the cells) was calculated by the least-squares method of linear regression, where Y is the probit of the percent 48-hr control absorbance at 540 nm and X is the percent concentration of the extract.

The results of these studies are presented in Table 1. The no-observed effects concentration for the AEs are 1.98%, 0.45%, 0.10%, and 0.85% for oils 1309, 1310, 1312, and 1313, respectively. The corresponding complete growth inhibition concentrations are 5.73%, 5.47%, 4.84%, and 6.94%, respectively.

Table 1. Cytotoxicity of selected H-Coal samples.

Sample	ORNL Repository No.	Toxicity Regression Equation	Correlation Coefficient	ICG50 (%)
Atmospheric Still Bottoms Synthetic Crude Mode	1309	Y = -0.8276X + 8.2708	-0.982	3.95
Atmospheric Still Overhead Synthetic Crude Mode	1310	Y = -0.6009X + 6.7549	-0.978	2.92
Atmospheric Still Bottoms Fuel Oil Mode	1312	Y = -0.6348X + 6.6352	-0.936	2.58
Atmospheric Still Overhead Fuel Oil Mode	1313	Y = -0.5400X + 7.1275	-0.986	3.94

Cytotoxicity of aqueous extracts of three crude oils. Studies have been undertaken in which the cytotoxic effects of three organic liquid comparative research materials (CRMs) on *Tetrahymena pyriformis* have been monitored. The three CRMs are a coal-derived liquid (CRM-1), a crude shale oil (CRM-2), and a petroleum crude oil (CRM-3). Aqueous and medium extracts were generated by slow stirring oil and aqueous milieux in a 1:8 ratio for 16 hours. Appropriate dilutions were made prior to short-term static testing for lethality and population growth impairment.

Mortality was determined by cell counting with the aid of dark-field microscopy. The concentration response in EPA "soft" four-salt test solution was examined by least-squares linear regression where Y is the number of viable cells per 10 ul following 24 hours of exposure and X is the concentration as percent extract. Similarly, concentration levels which alter population growth monitored spectrophotometrically at 540 nm were examined by linear regression where Y is the percent control 48-hr absorbance and X is the concentration as percent extract.

Data for the lethality are summarized in Table 2. The extract of the coal-derived liquid is the most toxic of the three CRMs assayed. Five percent extract causes 100% cell mortality within 3 hours exposure. Four percent causes 95+% cell death within 24 hours. On the other hand, 2% causes only a slight reduction in ciliary activity while 1% elicits no-observed effect. The extract of the crude shale oil is half as toxic as that from CRM-1 with 12% causing 100% cell death within 3 hours. Six percent CRM-2 extract only slightly decreases cell numbers. Whereas, 4% elicits no observable effect. The petroleum crude oil is the least toxic CRM tested. One hundred percent CRM-3 extract causes only a slight reduction in cell motility and no cell death.

Data for the 48-hr population growth impairment studies are presented in Table 3. Again only the CRM-1 and CRM-2 exhibit much adverse affect. The no-observed effects concentration is 0.5%, 1.0%, and 60.0% for the CRM-1, CRM-2, and CRM-3, respectively.

Table 2. Cytolethality of aqueous extracts of three crude oils.

Sample	Regression Eq.	Corr. Coeff.	24 hr LC_{50}(%)	24 hr LC_{100}(%)
CRM-1 (coal-derived liquid)	$Y=-11.000X + 45.30$	-0.975	2.62	5.0
CRM-2 (crude shale oil)	$Y=-2.725X + 37.88$	-0.952	7.85	12.0
CRM-3 (petroleum crude)			100% extract had no effect	

Table 3. Population growth impairment of medium-soluble extracts of three crude oils.

Sample	Regression Eq.	Corr. Coeff.	96hr LC_{50}(%)
CRM-1 (coal-derived liquid)	$Y=-30.701X + 103.9$	-0.967	1.76
CRM-2 (crude shale oil)	$Y=-8.147X + 102.7$	-0.963	6.47
CRM-3 (petroleum crude)	$Y=-0.999X + 163.8$	-0.903	113.88[a]

[a] 33% growth inhibition 97% extract

Embryotoxicity and teratogenicity of aqueous extracts of three crude oils.*

Studies are near completion in which the effects of aqueous extracts (AEs) of three comparative research materials (CRMs) on early embryos of *Xenopus laevis* are being tested. The three CRMs are a coal-derived liquid (CRM-1), a crude shale oil (CRM-2), and a petroleum crude oil (CRM-3). AEs are prepared by placing the individual CRMs and dechlorinated water in a flask in a ratio of 1:8. The flask contents are then slow-stirred for 16 hours prior to phase separation. For short-term static testing freshly prepared AEs are appropriately diluted with dechlorinated water to form a graded concentration series. Replicate groups of 50 embryos each are placed in 200 ml aliquots of test solution in 125 x 60 mm glass bowls which are covered to minimize evaporation. At representitive time points embryos are examined with the aid of a dissecting microscope and dead specimens removed. Following 96 hours of exposure information concerning mortality, number and type of abnormalities, stage of development, size, and relative motility and pigmentation are recorded. Mortality and abnormals/survivors were analysed by linear regression.

Mortality data are summarized in Table 4. The AE of CRM-1 is nine times more toxic than that of CRM-2 which, in turn, is four times more toxic than the one of CRM-3. Teratogenic data are summarized in Table 5. CRM-2 causes the largest proportion of nonlethal abnormalities as evidenced by the ratio of LC50 to EC50 for each CRM. The predominant abnormalities include: tail flexure, pericardial edma, and optic abnormalities. Embryo length, stage of development as well as motility and pigmentation also appear to be inversely related to extract concentration especially for CRM-1 and CRM-2.

---

*T. W. Schultz, L. J. Sutor, and J. N. Dumont, Biology Division, ORNL

Table 4. Embryolethality of aqueous extracts of three crude oils.

Sample	Toxicity Regression Eq.	Corr. Coeff.	96 hr LC_{50}(%)	96 hr LC_{100}(%)
CRM-1	$Y=58.46X - 42.28$	0.985	1.58	2.43
CRM-2	$Y=6.59X - 34.10$	0.993	12.77	20.36
CRM-3	$Y=2.35X - 60.16$	0.931	46.80	68.04

Table 5. Teratogenesis of aqueous extracts of three crude oils.

Sample	Teratogenesis Regression Eq.	Corr. Coeff.	96 hr EC_{50}(%)	96 hr EC_{0}(%)
CRM-1	$Y=51.78X + 1.46$	0.987	0.94	0.25
CRM-2	$Y=6.41X + 4.74$	0.975	7.06	2.00
CRM-3	$Y=2.32X - 46.26$	0.986	41.53	19.94

SUMMARY

1. H-coal samples from the PDU and shale oil samples contain mutagenic activity in bacterial testing (activation with mammalian extracts). See exception below.

2. Short-term bioassay such as the Ames *Salmonella* histidine reversion or yeast gene mutation assay can be effectively applied to the complex environmental samples.

3. Proper chemical extraction and fractionation methods should be coupled to the assay.

4. The shale- and coal-derived oils are relatively more mutagenic than is petroleum crude.

5. Mutagenic activity was mainly associated with the basic and neutral fractions. Subfractionation of the basic components leads to the identification of aromatic amines which are the major mutagenic component; subfraction of the neutrals leads to the identification of specific polynuclear aromatic hydrocarbons (PAHs). Comparative mutagenesis studies confirm the bacterial work.

6. Hydrotreatment reduces the mutagenic component and cytotoxic activity.

7. Refined oil samples from Paraho/Sohio refinery were not mutagenic.

REFERENCES

Cowser, K. E., ed. H-Coal pilot plant project quarterly progress report for the period ending Dec. 31, 1979, ORNL/TM-7270.

Cowser, K. E., ed. H-Coal pilot plant project quarterly progress report for the period ending March 31, 1980, ORNL/TM-7345.

Cowser, K. E., ed. H-Coal pilot plant status report for the period ending September 30, 1979, ORNL-TM-7274.

Cowser, K. E., ed. Proposed environmental and health program for H-coal pilot plant, Oak Ridge National Laboratory (June 11, 1979).

Epler, J. L., et al. "Analytical and biological analyses of test materials from the synthetic fuel technologies. I. Mutagenicity of crude oils determined by the Salmonella typhimurium/microsomal activation system," Mutat. Res. 57:264-76 (1978).

Epler, J. L., T. K. Rao, M. R. Guerin. Evaluation of feasibility of mutagenic testing of shale oil products and effluents. Environ. Health. Persp. 30:179-184, 1979.

Generoso, W. M., K. T. Cain, M. Krishna and S. W. Huff. Genetic lesions induced by chemicals in spermatozoa and spermatids of mice are repaired in the egg. Proc. Natl. Acad. Sci. USA 76:435-437, 1979.

Guerin, M. R., I. B. Rubin, T. K. Rao, B. R. Clark, J. L. Epler. Distribution of mutagenic activity in petroleum and petroleum substitutes. Fuel, in press, 1980.

Guerin, M. R., Ho, C-h., T. K. Rao, B. R. Clark, J. L. Epler. Polycyclic aromatic primary amines as determinant chemical mutagens in petroleum substitutes. Environ. Res. in press, 1980.

Ho, C.-h., M. R. Guerin, B. R. Clark, T. K. Rao, J. L. Epler. Preparative-scale isolation of alkaline mutagens from complex mixtures. Environ. Sci. and Technol., in press, 1980.

Rao, T. K., J. A. Young, A. A. Hardigree, W. Winton, J. L. Epler. Analytical and biological analyses of test materials from the synthetic fuel technologies. II. Mutagenicity of organic constituents from the fractionated synthetic fuels. Mutat. Res. 54:185-191, 1978.

Rao, T. K., B. E. Allen, D. W. Ramey, J. L. Epler, I. B. Rubin, M. R. Guerin, B. R. Clark, Analytical and biological analyses of test materials from the synthetic fuel technologies. III. Fractionation with LH-20 gel for the bioassay of crude synthetic fuels. Mutat. Res. in press, 1980.

Rao, T. K., J. L. Epler, J. J. Schmidt-Collerus, L. Leffler, M. R. Guerin. Biological monitoring of oil shale products and effluents using short-term genetic analyses, Presented at the Oil Shale Sampling, Analyses and Quality Assurance Symposium, Denver, Colorado, March 26-28, 1979.

Rao, T. K., J. L. Epler, M. R. Guerin, B. R. Clark and C. -h. Ho. Mutagenicity of nitrogen compounds from synthetic crude oils; collection, separation and biological testing. Proceedings of the Second Symposium on the Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures, Williamsburg, Va. March 4-7, 1980.

Rao, T. K., J. L. Epler, M. R. Guerin, B. R. Clark. Short-term microbial testing of shale oil materials. Proceedings of the Symposium on Health Effects Investigation of Oil Shale Development.

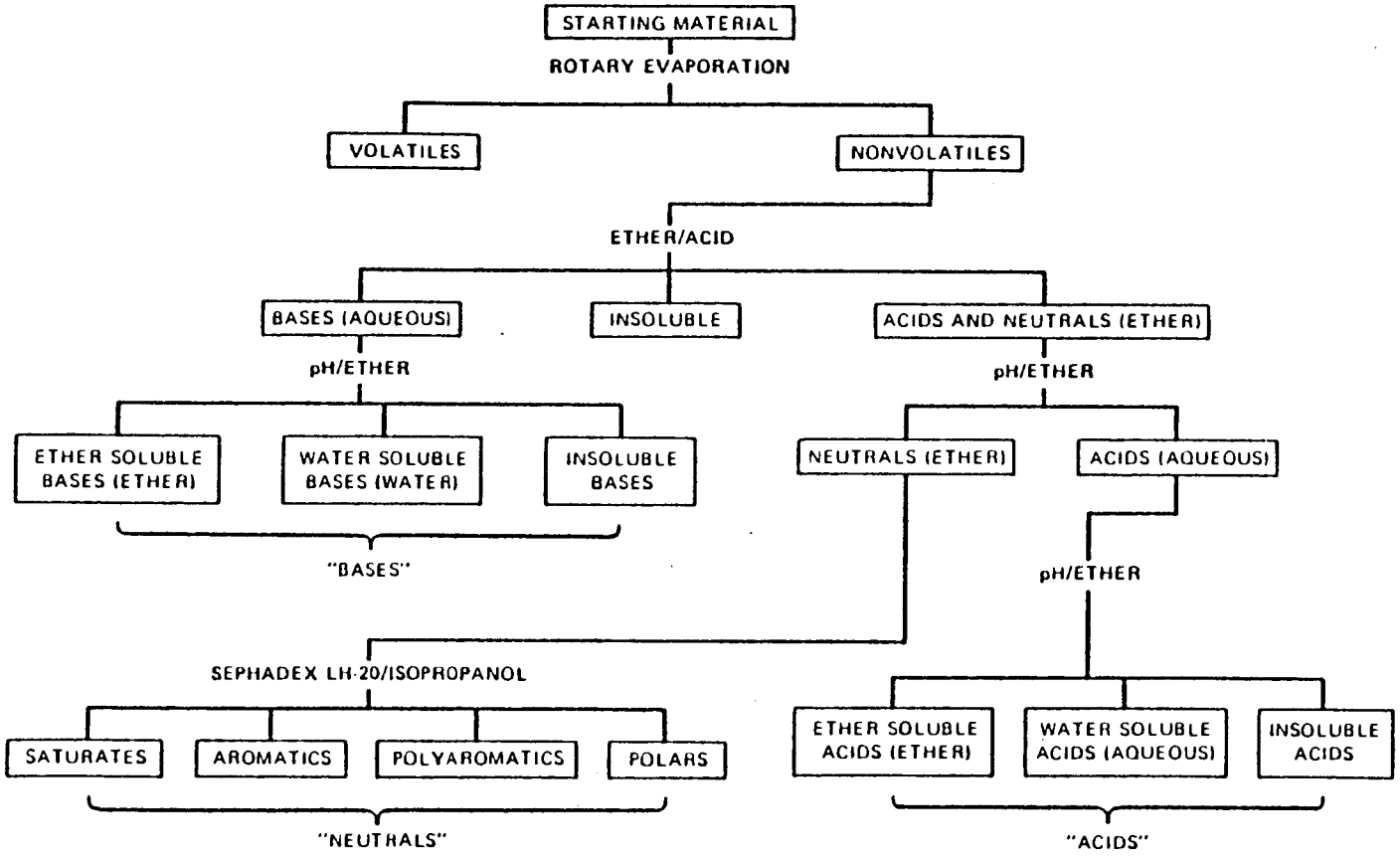
H-Coal liquefaction samples

Samples/Tests	Acute oral	Acute dermal	Skin irritation	Eye irritation	Skin sensitization	Carcinogenesis dermal[a]	Mutagenesis microbial	Mutagenesis In Vitro mammalian gene	Mutagenesis In Vitro cytogenetic mammalian	Analytical fractionation
H-Coal fuel oil										
HDT--low severity	X						X			X
HDT--medium severity	X						X			X
HDT--high severity	X						X			X
H-Coal distillate										
Raw	X	X	X	X	X	X	X	X		X
HDT--low severity	X	X	X	X	X	X	X	X	X	X
HDT--medium severity	X	X	X	X	X	X	X	X	X	X
HDT--high severity	X	X	X	X	X	X	X	X	X	X
Preproduct materials										
A. (PDU No. 5) Sept./Oct. 1977 Syncrude mode/Illinois No. 6										
Atmospheric separator bottoms	X						X			X
Vacuum separator overheads	X						X			X
Vacuum separator bottoms	X						X			X
B. (PDU No. 7) Sept. 1978 Fuel oil mode/Illinois No. 6										
Atmospheric separator overheads							X			X
Atmospheric separator bottoms							X			X
Vacuum separator overheads							X			X
Vacuum separator bottoms							X			X
Crude oil	X	X	X	X	X	X	X			X

DESCRIPTION OF SYNFUEL SAMPLES

Sample[a]	Source
Petroleum	
Wilmington crude oil (5301)	Bartlesville Energy Technology Center
Recluse crude oil (5305)	Bartlesville Energy Technology Center
Shale-derived oils	
Shale oil ('in situ') (4101)	Laramie Energy Technology Center
Paraho shale oil (4601)	US Navy/Standard Oil Company of Ohio
HDT-Paraho Shale oil (4602)	US Navy/Standard Oil Company of Ohio
Coal-derived oils	
SRC II-fuel oil (1701)	Pittsburgh and Midway Mining Company
H-Coal dist.-raw (1601)	Mobil Research/EPRI
H-Coal dist. HDT-low severity (1602)	Mobil Research/EPRI
H-Coal dist. HDT-medium severity (1603)	Mobil Research/EPRI
H-Coal dist. HDT-high severity (1604)	Mobil Research/EPRI
$ZnCl_2$ dist. (1801)	Conoco Coal Development Company

[a]Numbers in parentheses are repository numbers.



CHEMICAL CLASS FRACTIONATION FOR BIOTESTING
[MATERIAL], STEP, (PHASE)

Specific cellular bioassay tests — H-coal assessment

Cellular screens and validating tests	
Test	Applicable materials
Screening bioassays	
CS1. *Salmonella*	Solids
CS2. Yeast	— extracts, organic — leachates, aqueous
CS3. DNA repair	Liquids
CT1. Mammalian (CHO) cell cytotoxicity	Selected compounds
CT2. RAM	(Particulates only)
CT3. Invertebrate cytotoxicity	Selected aqueous samples and fractions
Screening/validating assays	
VT1. *Drosophila*	Selected compounds/fractions
VT2. Mammalian cell gene	Liquids
VT3a. Mammalian cell chromosomal (CHO)	
VT3b. Mammalian cell chromosomal (leukocyte)	
VT4. In vitro cell transformation	
Mammalian mutagenesis assays	
M1. Dominant lethals	
M2. Heritable translocations	Selected compounds
M3. Specific locus	
M4. "Spot test" for somatic mutation	Liquids (or solids processed in liquid form)
M5. Total reproductive capacity	

Specific cellular bioassay tests on H-coal samples

Sample type	Proposed study	Sample preparation	Type of bioassay	No. of samples	Sample size	Fractions per sample
Oils and tars						
6. Raw combined products	IB	Organic extract	CS1	6	10 g	
		Standard fractionation	CS1, CS2[a], VT2[a]	1	50 g	6
8. LP flask bottoms	IB	Organic extract	CS1	6	10 g	
9. Stripper bottoms	IB	Organic extract	CS1	6	10 g	
12. Light oil	IIB	Organic extract	CS1, M1, M4	3	10 g	
		Standard fractionation		1	50 g	6
13. Fuel oil mode/ syncrude oil mode	IIC	Organic extract	CS1	3/3	10 g	
		Standard fractionation	CS1, CS2[a], VT2[a]	1/1	50 g	6
17. Solvent	IB	Organic extract	CS1	1	10 g	
19. Fuel oil product	IIC	Organic extract	CS1, M1, M4	3	10 g	
		Standard fractionation	CS1, CS2[a], VT2[a]	1	50 g	6
27. Light slop oil	IB	Organic extract	CS1	1	10 g	
28. Heavy slop oil	IB	Organic extract	CS1	1	10 g	
37. Recycle oil	IB	Organic extract	CS1	3	10 g	
38. Waste oil	IB	Organic extract	CS1	3	10 g	

(cont.)

Sample type	Proposed study	Sample preparation	Type of bioassay	No. of samples	Sample size	Fractions per sample
Aqueous						
20. Sour water	IC	Organic extract/ concentrate	CS1, CT3, CT4	1	10 l	
21. Stripped water	IC	Organic extract/ concentrate	CS1, CT3, CT4	1	10 l	
27. Oily water	IC	Organic extract/ concentrate	CS1, CT3, CT4	1	10 l	
Solids						
7. Spent catalyst	II A,	Aqueous extract	CS1, CS2[a], CS3[a]	1	1 kg	
	IV	Organic extract	CS1	1	1 kg	
14. Solid flaked	II A,	Aqueous extract	CS1	1	1 kg	
	IV	Organic extract	CS1	1	1 kg	
15. Solid flaked	II A,	Aqueous extract	CS1	1	1 kg	
	IV	Organic extract	CS1	1	1 kg	
16. Flaked baghouse particulates	IIIA	Organic extract	CS1	1	250 g	
29. Sludge	IV	Aqueous extract	CS1, CS2[a], CS3[a]	1	1 kg	

[a]Only a selected number of samples or fractions will be assayed depending on bioassays, animal tests, and chemistry.

[b]Based on selected samples with three fuel sources and two operational modes.

MUTAGENICITY ASSAYS OF FRACTIONATED COAL CONVERSION PRODUCTS, SCREENING

J. L. Epler, Principal Investigator

Oak Ridge National Laboratory
Biology Division, Post Office Box Y
Oak Ridge, Tennessee 37830

EPA-IAG-D5-E681-AN
DOE-40-516-75

Project Contact: DOE/Biomedical Programs
EPA Coordination: EPA/ORD

Office of Research and Development
U.S.E.P.A.
Washington, D.C. 20460

ABSTRACT

The feasibility of using short-term mutagenicity assays to predict the potential biohazard of various crude and complex test materials from synthetic fuel technologies is being examined in an integrated chemical and biological approach. The principal focus of the research has been preliminary chemical characterization and preparation for bioassay, followed by testing in the Salmonella histidine reversion assay described by Ames and a battery of validating assays.

Using the rapid screening genetic assays, the group is attempting to identify mutagenic agents associated with coal and oil shale extraction, conversion or utilization.

Primary and subfractions of products and aqueous discharges are being assayed for the ability to inactivate and/or revert histidine requiring mutants of Salmonella. Potential mutagenic fractions are identified and attempts are made to ascertain the specific compounds(s) responsible for the mutagenic action.

Various fractionations have been carried out and the screening assays and validating assays (yeast, E. coli, Drosophila, and mammalian cells) are in progress. These investigations are the initial attempts to monitor environmentally important energy-related processes for genetic damage.

MAJOR RESEARCH AIMS AND ACCOMPLISHMENTS

Specific Aims: The specific aims of this project are (1) to ascertain the feasibility of utilizing short-term tests for mutagenicity coupled with chemical fractionation of energy-related materials as (a) a predictor of biological hazard and as (b) a means to search out the actual chemicals responsible for the activity; (2) to validate the response observed in short-term microbial assays with a battery of tests either with the same fractions or the actual chemicals identified; (3) to correlate changes in biological activity with changes in process condition or type; and (4) to determine the correspondence of the mutagenic potential with other profound long-range health effects such as teratogenicity, carcinogenicity, and other toxicological endpoints.

Accomplishments: In order to begin to approach these aims, this portion of the large and integrated interagency effort in energy and environment must accomplish a number of sequential developmental and/or experimental goals. As these goals are realized, the short-term mutagenicity tests will aid in identifying the specific hazardous compounds involved and in establishing priorities for further validative testing - testing in whole animals - and more definitive chemical analysis and monitoring. The overall application of mutagenicity testing to energy-related processes has been scheduled as follows:

(1) Development of a generally applicable extraction procedure(s) for (a) aqueous materials, (b) organic liquids and (c) solid and/or particulate materials;

(2) Development of a generalized class fractionation scheme;

(3) Appropriate application of short-term mutagenicity tests as coupled

to the above procedures with energy-related test materials;

(4) Development of a wide data base of generally encountered materials for comparison with the energy technologies;

(5) Extension of the energy-related data base to relevant samples (including evaluation of process changes), encompassing products, effluents (both aqueous and particulate), and combustion products;

(6) Identification of the actual components from energy-related processes;

(7) Further validation of the mutagenic hazard of the test materials and their components; and

(8) Intercomparison of synthetic fuel processes.

CONTENTS

Introduction

Technical Progress

- Fractionation
- Subfractionation of Neutral Components
- Subfractionation of Basic Components

References

Illustrations

Tables

Milestones

INTRODUCTION

Since the Oak Ridge National Laboratory functions as a key fundamental research arm of the Department of Energy and EPA the efforts of the Biology Division and the Life Sciences Divisions have focused, in general, on the potential biohazards of energy-related technologies - oil shale, coal liquefaction, and coal gasification.

The need for substitute fluid fuels and convenient energy sources had led to renewed efforts in the field of coal conversion. A number of coal liquefaction and coal gasification processes are being developed. For this approach to adequately and safely serve as an aid in solving energy problems, potential health hazards for workers and the general public should be considered. The overall environmental aspects of any energy technology include pollutant releases, physical disturbances, and socioeconomic disturbances along with health and safety impacts. This project will concentrate on health and safety aspects and utilize various rapid and inexpensive (short-term) biological assays. The realization that the number of specific compounds and complex effluents and streams in need of biotesting in any technology is overwhelming and would tax the scientific community's whole-animal testing capabilities has led to the development of this array of screening assays for toxicity, mutagenicity, teratogenicity, and carcinogenicity. The working hypothesis has been that the rapid tests can serve as predictors of biohazards early in the development of the technology. Thus, process changes might be initiated and/or definitive whole-animal testing considered. Conceivably, savings in both time and money on the technology and the biological testing would be gained.

The possibility of using short-term biological tests to isolate and identify the potential hazard of complex materials has been investigated with various coupled chemical and biological approaches. The work has usually involved chemical analyses and preparation for biological testing, e.g., cytotoxicity, mutagenicity, and carcinogenicity assays. As a prescreen for potential mutagenesis and/or carcinogenesis, the genetic test for Salmonella histidine reversion (Ames test) has been widely used. Subsequent fractionation procedures are carried out to aid in isolating and identifying the mutagens in the material, with the bioassay being used as a tool to trace the biological activity and guide the separations. The biological tests function as (1) predictors of long-range health effects such as mutagenesis, teratogenesis, and/or carcinogenesis; (2) predictors of toxicity for man and his environment; (3) a mechanism to rapidly isolate and identify a hazardous agent in a complex material; and (4) a key to relative biological activity through the correlation of control data with changes in environmental or process conditions. Since much of the exploratory work in the use of short-term assays has involved fractionated natural and synthetic oils and wastes, the health effects research progress reviewed here will emphasize the assays that have been applied to coal conversion processes.

To investigate the potential genetic (and carcinogenic) hazards associated with the developing synthetic fuel technologies, we initiated a coupled chemical and biological analysis of the products, process streams, and effluents of the existing or proposed energy-generating or -conversion systems. One phase of our investigation deals with known compounds expected to occur in the environment through energy production, conversion, or use; another phase deals with actual samples from existing or experimental processes. To approach the problems of coping with and testing large numbers of compounds, we set up a

battery of assays for mutagenicity testing. (This project has dealt largely with the development of fractionation procedures and the validation of the preliminary screening. Other EPA/DOE sponsored parallel research deals with site specific gasification and coal liquefaction pilot plants. Although much of the knowledge accumulated through this project is being applied to these sites, the approach has also been expanded to teratogenicity, cytotoxicity, and whole-animal toxicology, mutagenesis and carcinogenesis). For the study of applicability to complex mixtures, a battery of bacterial, fungal, insect, and mammalian cell assays was selected. Materials or samples chosen for testing were obtained on the basis of relevance to a generic comparison, not on the basis of examination of a specific site.

TECHNICAL PROGRESS

The feasibility of using short-term mutagenicity assays to predict the potential biohazard of various crude and complex test materials has been examined in a coupled chemical and biological approach. The principal focus of the research has been preliminary chemical characterization and preparation for bioassay, followed by testing in the Salmonella histidine reversion assay described by Ames and a battery of validative tests.

Fractionation

Biological effects, e.g., mutagenicity in a short-term test, of a crude material (or a crude extract or leachate) may be difficult to detect because of overall toxicity of the mixture or the very low concentrations of the active components, or both. Therefore, it is desirable to subdivide the materials into fractions that can be tested much more accurately and that lend themselves to chemical analyses for identification of the active compounds much more readily than does the starting test material. Possible disadvantages include the loss or modification of specific components or the loss of potential for synergistic effects. Various approaches to fractionation by chemical class are listed in the following sections. In the course of the project various procedures for general or specific purposes have evolved. In many cases, the results from bioassay guided the chemistry and led to the final procedure. A summary follows:

Extraction/Fractionation	Reference
1. Organic Extraction	Epler (1980)
2. Aqueous Extraction	Epler et al. (1980)
-Leachates	EPA-600/2-80-057

3. Class Fractionation	
-Liquid-Liquid	Epler et al. (1978)
-Thin-Layer Chromatography	Rao, Epler, Leffler and Guerin, 1979
-LH-20 Chromatography	Jones et al. (1977)
-Generalized	Rao et al. (1980)
4. Specific Classes	
-PAH's	Guerin et al. (1978)
-Basic Fractions	Epler et al. (1979)
-Alkylated PAH's	Griest et al. (1978)
-Neutral Nitrogen Compounds	Ho et al. (In Press)
-Polycyclic Aromatic Primary Amines	Guerin et al. (In Press)

The majority of the studies outlined utilized the rapid and inexpensive plate incorporation assay with the Salmonella histidine-reversion system. All strains (missense and frameshift) should be screened with unknown materials. However, in practice, most determinations can be made with strains TA100 and TA98 (containing the R plasmid pkM101). Crude materials or fractions for testing are usually dissolved in DMSO in the range of 5-10 mg of total solids/ml. Stock solutions are stored in the dark at 4OC. Fractions or subfractions are evaporated or lyophilized to dryness or to a concentrated "tar" before solubilization. In actual practice, many crude fractions are not completely soluble even in DMSO. The treatment can be carried out with the suspended material or the DMSO-soluble materials after suspension and centrifugation.

Subfractionation of Neutral Components

In the initial studies with neutral fractions of various petroleum crude and synthetic crude oils, gross toxicity of the fraction overcame or marked the mutagenic activity of the components. Fractionation with florisil column chromatography using successive elution with various organic solvents aided in identifying activity in the subfractions. The PAH's occurring in the neutral fractions appear to be major contributors to the mutagenicity. In natural petroleum crudes, the neutral (PAH) component accounts for most of the activity. Similarly, neutral material in coal tar and petroleum pitches contains

the bulk of the mutagenic activity. However, when shale oil or synthetic crude oils from coal liquefaction are considered, the contributions of both the basic and neutral materials must be taken into account. The definition of mutagenic components of these classes has become the key effort of research teams involved.

With the neutral components, various subfractionation techniques have been used, again, coupling the analytical chemistry to short-term mutagenicity assays. As previously described oils can be separated into lipophilic and hydrophilic fractions with the Sephadex LH-20 partition chromatography. The lipophilic fraction can then be further separated by use of neutral alumina and LH-20 with various solvents. The subfractions can be characterized as to ring size of the components. Assay of the fractions with the Salmonella histidine reversion system resolves the activity with a peak in the 4- and 5-ring subfraction. The observation is not surprising since known mutagens/carcinogens as benzo(a)pyrene, benzo(c)phenanthrene, and 3-methylcholanthrene would elute in this area.

The neutral or PAH isolates in all of these materials are highly complex. Much of this complexity can be attributed to multi-alkylated species. We reported the results of initial studies (sponsored by EPRI) characterizing the chemical and biological nature of multi-alkylated PAH's from a coal-derived crude oil. Similar complexity exists in PAH isolates of tobacco or marijuana smoke condensates; advances in techniques in tobacco smoke have provided methodology to separate parent and simple alkylated PAH's from multi-alkylated components.

With a coal-derived crude oil (Coal Liquid D), a gross PAH isolate was generated by Alumina-column chromatography of the neutral fraction from a Swain-Stedman-type procedure. A gel filtration method was used to separate

the isolate into multi-alkylated and parent plus simple alkylated PAH fractions. The gross isolate and its fractions were then assayed with the microbial mutagenesis system. Strain TA98 (frameshift) with an Aroclor-induced indicator of activity. The gross PAH isolate was too toxic to the bacterial system to assay in an appropriate concentration range; however, both the parent plus simple fraction and the multi-alkylated fraction showed activity. Removal of toxicity or inhibition by further fractionation often leads to increased resolution or "unmasking" of mutagenic activity. Although the multi-alkylated fraction shows less mutagenic potential (as measured by specific activity in rev/mg of a fraction), it contributes a greater weight proportion of the total sample. Therefore, the multi-alkylated components probably constitute a significant portion of the mutagenicity of PAH's in coal-derived oils.

Further separation of the multi-alkylated PAH fraction with Sephadex LH-20 column chromatography can lead to fractionation by ring size. Subfractions noted by their "cut-points" with PAH standards as "naphthalene", "phenanthrene", and "benz(a)anthracene" can be produced and correspond to fractions bearing two, three, and four or more rings, respectively. Assay of the fractions with the Salmonella system again seems to indicate an increase in mutagenic potential with increase in ring size; however, when weighted activities are considered, it appears that both the "phenanthrene" and "benz(a)anthracene" subfractions are significant contributors to the overall activity. Increase of cocarcinogenic activity of tobacco-smoke alkylated PAH fractions also follows increase in ring size.

Separation procedures coupled to short-term mutagenicity tests may also prove useful with a number of minor components of neutral fractions and their derived PAH isolates. For example, neutral N-heterocyclic aromatic compounds

(neutral N-PAHs) have considerable importance to environmental and health effects studies. For example, 7-H-dibenzo(c,g)carbazole is a potent carcinogen while 13 H-dibenzo(a,j)carbazole is a suspected carcinogen. Weak bases, such as the indoles and carbazole, are generally isolated along with the PAH fraction in acid-base extractions or chromatographic separations. Thus, if biological screening of these nitrogen containing compounds is desired with specific organic mixtures, a separation scheme had to be worked out. Basic nitrogen components have been reported as potent mutagens in synthetic crudes. Thus, separation of the neutral nitrogen aromatic components from PAH fraction becomes an important effort. Note also that in most isolates, this class is obscured by the major PAH compounds. Even greater importance for this neutral nitrogen class may be assumed in synthetic fuels. In these materials, basic nitrogen compounds may be controlled by acid-washing or some other control technology. However, neutral N-PAHs accompany neutral fractions and are not easily separated by conventional refining methods. The total nitrogen contents of crude synthetic fuels from coal or shale oil are higher than for conventional petroleum crudes.

We reported on a separation method for N-PAH compounds from crude oils and comparison studies of mutagenic activity in these materials. Coupled mutagenicity data on a coal-derived and a shale oil-derived liquid aided the development of the method.

Neutral fractions were produced by acid-base extractions. The neutral materials are then loaded onto a Sephadex LH-20 column and eluted sequentially with isopropanol and acetone. The acetone fraction was reduced in volume and the solvent removed. The residue was dissolved in benzene and transferred to a silicic acid column. Three subfractions are then eluted: Subfraction I (PAHs); Subfraction II (neutral N-PAHs); and Subfraction III (polar). The

silicic acid chromatography step was a modification of the work of Snook who developed the technique for tobacco smoke condensates.

Analytical data on Subfraction II indicates that the coal liquid contains mostly alkyl-substituted indoles, phenylpyrroles, phenylindoles, alkyl-substituted carbazoles and alkyl-substituted benzocarbazoles while the shale oil contains mostly alkyl-substituted indoles, carbazoles, and benzocarbazoles.

The mutagenic activities of the neutral N-PAH fractions were determined in a coal-derived oil and a shale oil. The separation of the aliphatic and aromatics materials shows the mutagenic activity separating as expected with the aromatics. Subfractionation shows a higher total mutagenicity for the coal-derived oil with no mutagenic activity in the neutral N-PAH fraction of the shale oil. However, the PAH fraction of the shale oil shows little overall activity. Considerable difference in composition in the oils has also been observed.

On the basis of the known composition of the neutral N-PAH fraction and the availability of compounds, several typical compounds have been tested for mutagenicity in microbial mutagenesis. One-, two-, and three-ring neutral N-PAHs as pyrrole, indole, 2-methylindole and carbazole showed no mutagenicity. However, the highly carcinogenic 7H-dibenzo(c,g)carbazole (5-rings) showed positive results in the Salmonella system. A number of other predicted constituents, for example, benzocarbazoles, have been tested and shown to be mutagenic. The correlation between the increase in ring size and mutagenic activity is probably general and agrees with the observation with PAHs and with basic N-PAHs. With respect to the neutral N-PAHs, the mutagenic activity may originate from small quantities of the higher molecular weight compounds composed of 4-5 rings.

Subfractionation of Basic Components

Although neutral materials compose a great proportion of the mutagenic activity derived from various synthetic fuels and natural petroleums, the

suspected and identified constituents of the separated fraction were expected to be mutagens. What was not entirely expected was the high mutagenic activity observed in basic fractions. Considering the results with class fractionation procedures as the Swain-Stedman or the LH-20 column chromatographic steps, the activity of the basic fractionation demanded considerable alterations. We developed a combined extraction and chromatographic procedure for the polar, basic fraction(s). An elution sequence using alumina and Sephadex LH-20 with various solvents isolates approximately 90% of the mutagenic activity from basic fractions into 0.5 wt. % fraction of both synfuels and shale oil.

A class fractionation procedure was used initially to separate acidic and basic fractions through liquid/liquid (acid-base) extraction procedure. An Ether Soluble Bases (ESB) fraction was generated. The general observation has been that petroleum (natural) crudes differ from synfuels by a relative lack of mutagenic activity in the basic fractions. ESB fractions of a number of materials from energy-related processes and other environmentally encountered complex organic mixtures have been examined with the same procedure and isolation of the basic materials in this manner appears to have general applicability.

With acid-base extraction, the ESB fraction contains the bases which are back-extracted out of the initial acid extract following pH adjustment to pH 11. In a series of studies using ESB fractions from shale oil and a syncrude oil, a fractionation method was developed specifically to isolate the mutagenic constituents. Thus, chemical knowledge and an empirical combination of separations and mutagenicity testing led to the procedure. The ESB is separated on basic alumina using benzene and ethanol followed by Sephadex LH-20 column separation using first isopropanol and then acetone. The acetone subfraction contains the bulk of the mutagenic activity as detected by the

Salmonella histidine reversion assay and also by mammalian cell mutagenesis.

Specifically with a crude shale oil sample, the acetone subfraction accounts for 90% of the mutagenic activity (as measured by Salmonella histidine reversion) is 9% of the original ESB. No activity is found in the benzene fraction and little in the isopropanol. With a coal-derived syncrude, the enrichment is much the same. However, in the mixed petroleum crude, although activity is seen in the acetone subfraction, the absolute mutagenicity is much less. The assumption is made that we are dealing with the same types of constituents in all materials with differences in the relative quantities.

Analyses of the various subfractions can be correlated with the mutagenic data. The benzene fraction (inactive) contains a large variety of pyridines and some quinolines with alkyl substitution. The isopropanol (inactive or slightly active) appears to contain partially hydrogenated 1-2 ring azaarenes. The acetone subfraction (active) contains a variety of azaarenes and aromatic amines. Mutagenicity testing of compounds that are suspected to occur points to little activity in 1-4 ring azaarenes but significant activity in the 5-ring class.

Tests were also carried out with an array of quinolines and alkyl pyridines. Although activity is not observable in the subfractions, we have seen detected mutagenic activity in various quinolines.

Testing with a group of 5-ring azaarenes reveals considerable mutagenic activity. The nitrogen analogue of benzo(a)pyrene, azabenzopyrene, shows considerable activity. Methyl substitution appears to reduce the activity: although azaarenes are implicated as potential mutagens, the activity in the ESB is not necessarily confined to this class. Further studies suggest that aromatic amines are also involved and in fact may be the major hazardous constituents.

Initial evidence from Infra Red Spectra from analytical studies guided by the mutagenicity work pointed to the presence of aromatic amines in the acetone subfraction. Gas chromatographic and mass spectral analyses of the subfraction agreed with the presence of aromatic amines. Control separations with known compounds confirmed the behavior of the primary amines and azaarenes in the separation procedure. Mass spectral data suggest the presence of amino-naphthalenes, -biphenyls, and -anthracenes along with parent and alkyl substituted anilines. The mutagenic potential of the amino substituted constituents is much higher than the parent hydrocarbons and the corresponding azaarenes. For example, aminopyrene is much more active than is benzo(a)-pyrene as measured by the Ames plate tests. Very high activities of primary amines may explain the high activities observed in the crude fractions.

The studies have also been extended to the refinement of conditions for separating the primary amines from the azaarenes. The further separation of the primary amines from the azaarenes has shown that the bulk of the acetone subfraction is made up of primary amines and, furthermore, that almost all of the mutagenic activity is due to the primary amines rather than azaarenes. The fact that these nitrogenous materials appear to differentiate mutagenic activity in syncrudes from activities on petroleum crudes may be significant. Technically, the removal of the basic components may be approachable in the upgrading process with synthetic fuels.

REFERENCES

1. Epler, J. L., W. Winton, A. A. Hardigree, and F. W. Larimer, "The appropriate use of genetic toxicology in industry," In: Developments in Toxicology and Environmental Science, Vol. 6, The Scientific Basis of Toxicity Assessment (H. R. Witschi, ed.), Elsevier/North Holland Biomedical Press, Amsterdam, 1980, pp. 45-59.

2. Epler, J. L., "The use of short-term tests in the isolation and identification of chemical mutagens in complex mixtures," In: Chemical Mutagens: Principles and Methods for Their Detection (F. J. deSerres and A. Hollaender, eds.), Vol. 6, Plenum Press, New York and London, 1980, pp. 239-270.

3. Hsie, A. W., P. A. Brimer, J. P. O'Neill, J. L. Epler, M. H. Hsie, and M. R. Guerin, "Mutagenicity of alkaline constituents of a coal-liquefied crude oil in mammalian cells," Mutat. Res. 78: 79-84 (1980).

4. Larimer, F. W., A. A. Hardigree, W. Lijinsky, and J. L. Epler, "Mutagenicity of N-nitrosopiperazine derivatives in Saccharomyces cerevisiae. Mutat. Res. 77: 143-148 (1980).

5. Rao, T. K., J. L. Epler, M. R. Guerin, B. R. Clark, and C-h. Ho, "Mutagenicity of nitrogen compounds from synthetic crude oils: Collection, separation, and biological testing," In: Proc. Second Symposium on the Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures, Williamsburg, Virginia, March 4-7, 1980.

6. Tomkins, B. A., H. Kubota, W. H. Griest, J. E. Caton, B. R. Clark, and M. R. Guerin, "Unambiguous determination of BaP in petroleum substitutes," Anal. Chem. 52: 1331-1334 (1980).

7. Epler, J. L., F. W. Larimer, T. K. Rao, C. E. Nix, and T. Ho, "Energy-related pollutants in the environment: Use of short-term tests for mutagenicity in the isolation and identification of biohazards," Environ. Health Perspect. 27: 11-20 (1979).

8. Epler, J. L., T. K. Rao, and M. R. Guerin, "Evaluation of feasibility of mutagenic testing of shale oil products and effluents," Environ. Health Perspec. 30: 179-184 (1979).

9. Epler, J. L., B. R. Clark, C.-h. Ho, M. R. Guerin, and T. K. Rao, "Short-term bioassay of complex organic mixtures: Part II. Mutagenicity testing," In: Environmental Science Research, Vol. 5, Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures (M. D. Waters, S. Nesnow, J. L. Huisingh, S. S. Sandhu, and L. Claxton, eds.), Plenum Press, New York and London, 1979, pp. 269-289.

10. Epler, J. L., C. E. Nix, T. Ho, F. W. Larimer, T. K. Rao, and A. W. Hsie, "Short-term mutagenicity testing," In: Proc. Symp. Potential Health and Environmental Effects of Synthetic Fuel Technologies, Gatlinburg, Tennessee, September 25-28, 1978, Oak Ridge National Laboratory, CONF-780903, 1979, pp. 129-136.

11. Guerin, M. R., B. R. Clark, C.-h. Ho, J. L. Epler, and T. K. Rao, "Short-term bioassay of complex organic mixtures: Part I. Chemistry," In: Environmental Science Research, Vol. 15, Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures (M. D. Waters, S. Nesnow, J. L. Huisingh, S. S. Sandhu, and L. Claxton, eds.), Plenum Press, New York and London, 1979, pp. 247-268.

12. Griest, W. H., B. A. Tomkins, J. L. Epler, and T. K. Rao, "Characterization of multialkylated polycyclic aromatic hydrocarbons in energy-related materials," In: Polynuclear Aromatic Hydrocarbons, Third International Symposium on Chemistry and Biology - Carcinogenesis and Mutagenesis (P. W. Jones and P. Leber, eds.), Ann Arbor Science Publishers, Inc., Ann Arbor, 1979, pp. 395-409.

13. Ho, C.-h., B. R. Clark, M. R. Guerin, C. Y. Ma, and T. K. Rao, "Aromatic nitrogen compounds in fossil fuels -- a potential hazard?" In: American Chemical Society, Division of Fuel Chemistry, Vol. 24, No. 1, pp. 281-301, Preprints of Papers Presented at Honolulu, HI, April 2-6, 1979.

14. Nix, C. E., B. Brewen, R. Wilkerson, W. Lijinsky, and J. L. Epler, "Effects of N-nitrosopiperidine substitutions on mutagenicity in Drosophila melanogaster," Mutat. Res. 67: 27-38 (1979).

15. Nix, C. E., and B. Brewen, "The role of Drosophila in chemical mutagenesis testing," In: Environmental Science Research, Vol. 15, Application of Short-term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures (M. D. Waters, S. Nesnow, J. L. Huisingh, S. S. Sandhu, and L. Claxton, eds.), Plenum Press, New York and London, 1979, pp. 111-123.

16. Rao, T. K., J. L. Epler, J. J. Schmidt-Collerus, L. Leffler, and M. R. Guerin, "Biological monitoring of oil shale products and effluents using short-term genetic analyses," In: Proc. Symposium on Oil Shale Sampling, Analyses and Quality Assurance, Denver, Colorado, March 26-28, 1979.

17. Rao, T. K., J. A. Young, C. E. Weeks, T. J. Slaga, and J. L. Epler, "Effect of the co-carcinogen benzo(e)pyrene on microsome-mediated chemical mutagenesis in Salmonella typhimurium," Environ. Mutagen. 1: 105-112 (1979).

18. Rao, T. K., J. A. Young, W. Lijinsky, and J. L. Epler, "Mutagenicity of aliphatic nitrosamines in Salmonella typhimurium," Mutat. Res. 66: 1-7 (1979).

19. Rao, T. K., D. W. Ramey, W. Lijinsky, and J. L. Epler, "Mutagenicity of cyclic nitrosamines in Salmonella typhimurium: Effect of ring size," Mutat. Res. 67: 21-26 (1979).

20. Rao, T. K., F. W. Larimer, C. E. Nix, and J. L. Epler, "Mutagenicity testing of aqueous materials from alternate fuel production," In: Proc. Symposium on Analysis of Water Associated with Alternate Fuel Production, Pittsburgh, Pennsylvania, June 4-8, 1979.

21. Weeks, C. E., T. K. Rao, J. A. Young, T. J. Slaga, and J. L. Epler, "Effect of weak, non- and co-carcinogenic chemicals on 2-acetylamino-fluorene-induced mutation in Salmonella typhimurium," Toxicology 14: 255-262 (1979).

22. Epler, J. L., J. A. Young, A. A. Hardigree, T. K. Rao, M. R. Guerin, I. B. Rubin, C.-h. Ho, and B. R. Clark, "Analytical and biological analyses of test materials from the synthetic fuel technologies. I. Mutagenicity of crude oils determined by the Salmonella typhimurium/ microsomal activation system," Mutat. Res. 57: 265 (1978).

23. Epler, J. L., A. A. Hardigree, J. A. Young, and T. K. Rao, "Feasibility of application of mutagenicity testing to aqueous environmental effluents," Mutat. Res. 53: 88 (1978) (abstract).

24. Epler, J. L., and M. D. Waters, "Status of bioscreening of emissions and effluents from energy technologies," In: EPA Decision Series, Energy/ Environment III, U.S. Environmental Protection Agency, EPA-600/0-78-022, pp. 29-50 (1978).

25. Guerin, M. R., "Energy sources of polycyclic aromatic hydrocarbons," In: Polycyclic Hydrocarbons and Cancer, Vol. 1, Academic Press, New York, 1978. pp. 3-42.

26. Guerin, M. R., J. L. Epler, W. H. Griest, B. R. Clark, and T. K. Rao, "Polycyclic aromatic hydrocarbons from fossil fuel conversion processes, In: Carcinogenesis - A Comprehensive Survey, Vol. 3, Polynuclear Aromatic Hydrocarbons (P. W. Jones and R. I. Freudenthal, eds.), Raven Press, New York, 1978, pp. 21-33.

27. Griest, W. H., M. R. Guerin, B. R. Clark, C.-h. Ho, I. B. Rubin, and A. R. Jones, "Relative chemical composition of selected synthetic crudes," In: Proc. Symposium on Assessing the Industrial Hygiene Monitoring Needs for the Coal Conversion and Oil Shale Industries (O. White, ed.), Brookhaven National Laboratory, Upton, New York, November 6, 1978 (March 1979), pp. 61-78.

28. Rao, T. K., J. A. Young, A. A. Hardigree, W. Winton, and J. L. Epler, "Analytical and biological analyses of test material from the synthetic fuel technologies. II. Extended genetic and biological studies with mutagenic fractions," Mutat. Res. 54: 185-191 (1978).

29. Rao, T. K., A. A. Hardigree, J. A. Young, and J. L. Epler, "Correlation of mutagenic activity of energy-related effluents with organic constituents," Mutat. Res. 53: 88-89 (1978) (abstract).

30. Ho, C.-h., C. Y. Ma, B. R. Clark, M. R. Guerin, T. K. Rao, and J. L. Epler, "Separation of neutral nitrogen compounds from synthetic crude oils for biological testing," Environ. Res., in press.

31. Nix, C. E., B. Brewen, C. McKinley, and J. L. Epler, "A comparison of the induction of X-linked recessive lethals by chemical mutagens in wild-type and excision-repair-deficient, mei-9^a, Drosophila melanogaster males," Mutat. Res., in press.

32. Nix, C. E., B. Brewen, W. Lijinsky, and J. L. Epler, "Effects of methylation and ring size on mutagenicity of cyclic nitrosamines in Drosophila melanogaster," Mutat. Res., in press.

33. Nix, C. E., B. Brewen, D. Moore, W. Lijinsky, and J. L. Epler, "Effects of structural differences in aliphatic nitrosamines on mutation induction in Drosophila melanogaster," Mutat. Res., in press.

34. Nix, C. E., B. Brewen, and J. L. Epler, "Microsomal activation of selected polycyclic aromatic hydrocarbons and aromatic amines in Drosophila melanogaster," Mutat. Res., in press.

35. Rao, T. K., B. E. Allen, D. W. Ramey, J. L. Epler, M. R. Guerin, I. B. Rubin, and B. R. Clark, "Analytical and biological analyses of test materials from the synthetic fuel technologies. III. Use of Sephadex LH-20 gel chromatography technique for the bioassay of crude synthetic fuels," Mutat. Res., in press.

ILLUSTRATIONS

Fig. 1 Subfraction of Basic Materials

2 Isolation of Neutral N-PAH Fraction

3 Generalized Fraction Scheme

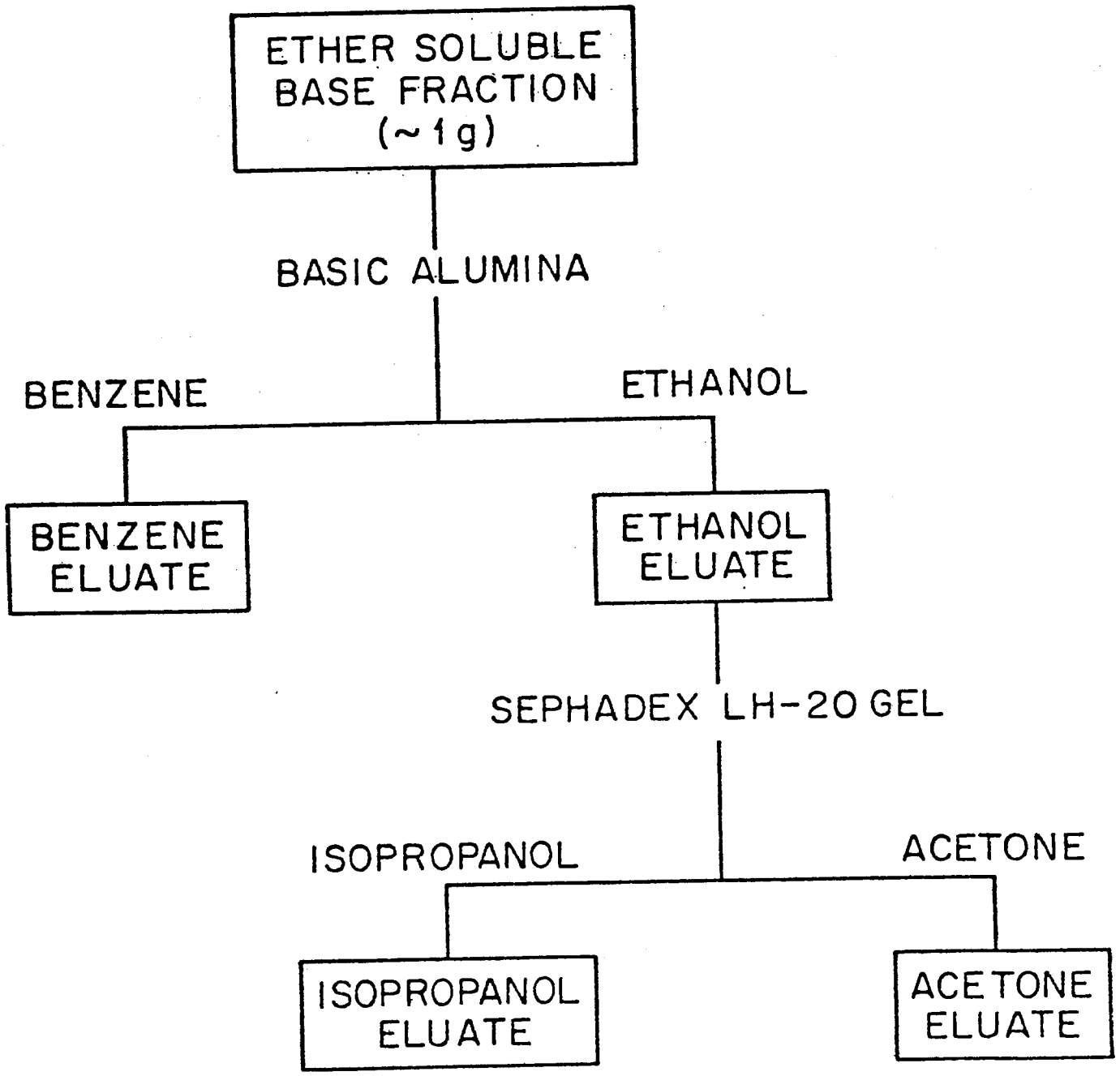


FIGURE 1. Subfraction of basic materials.

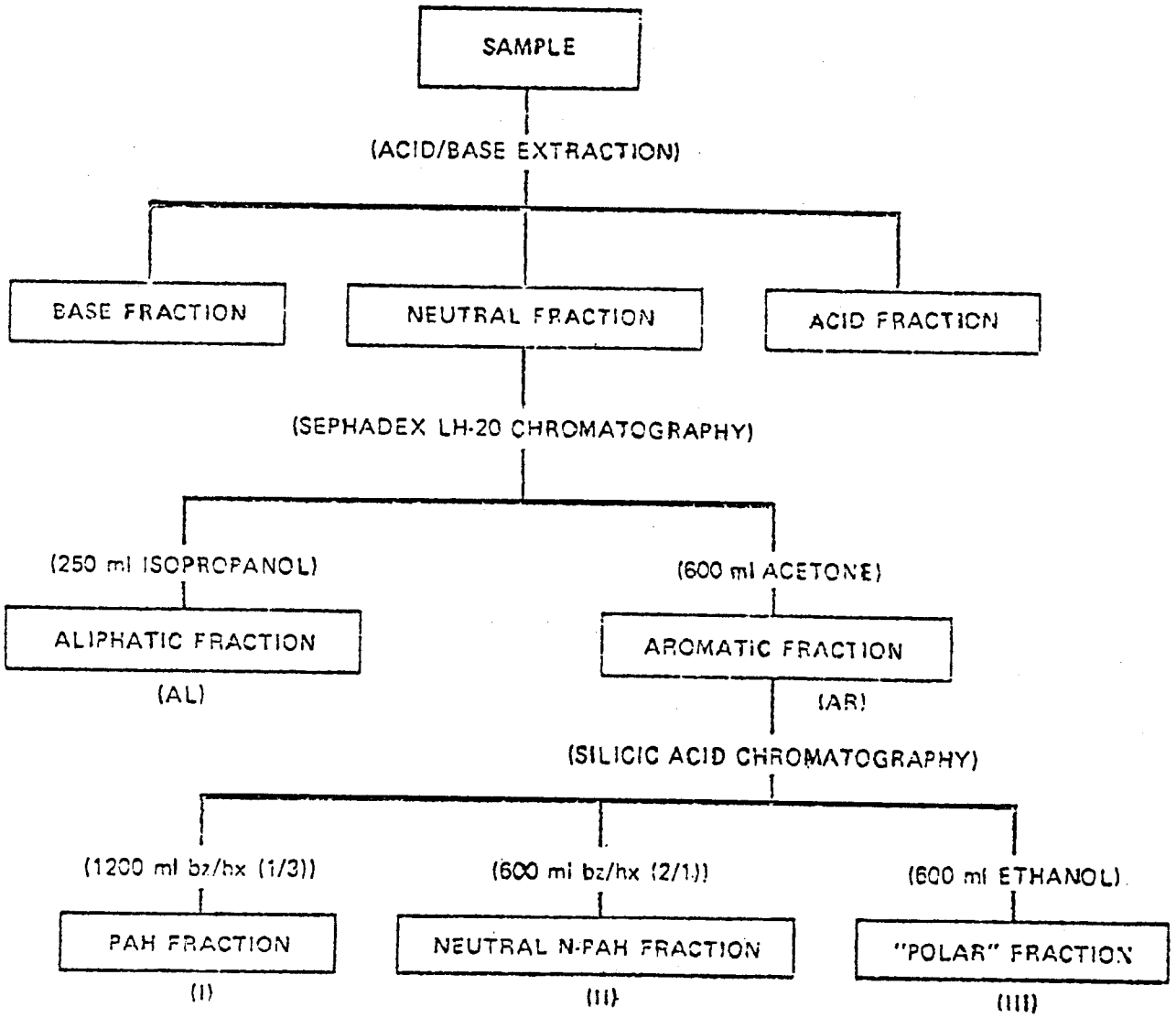
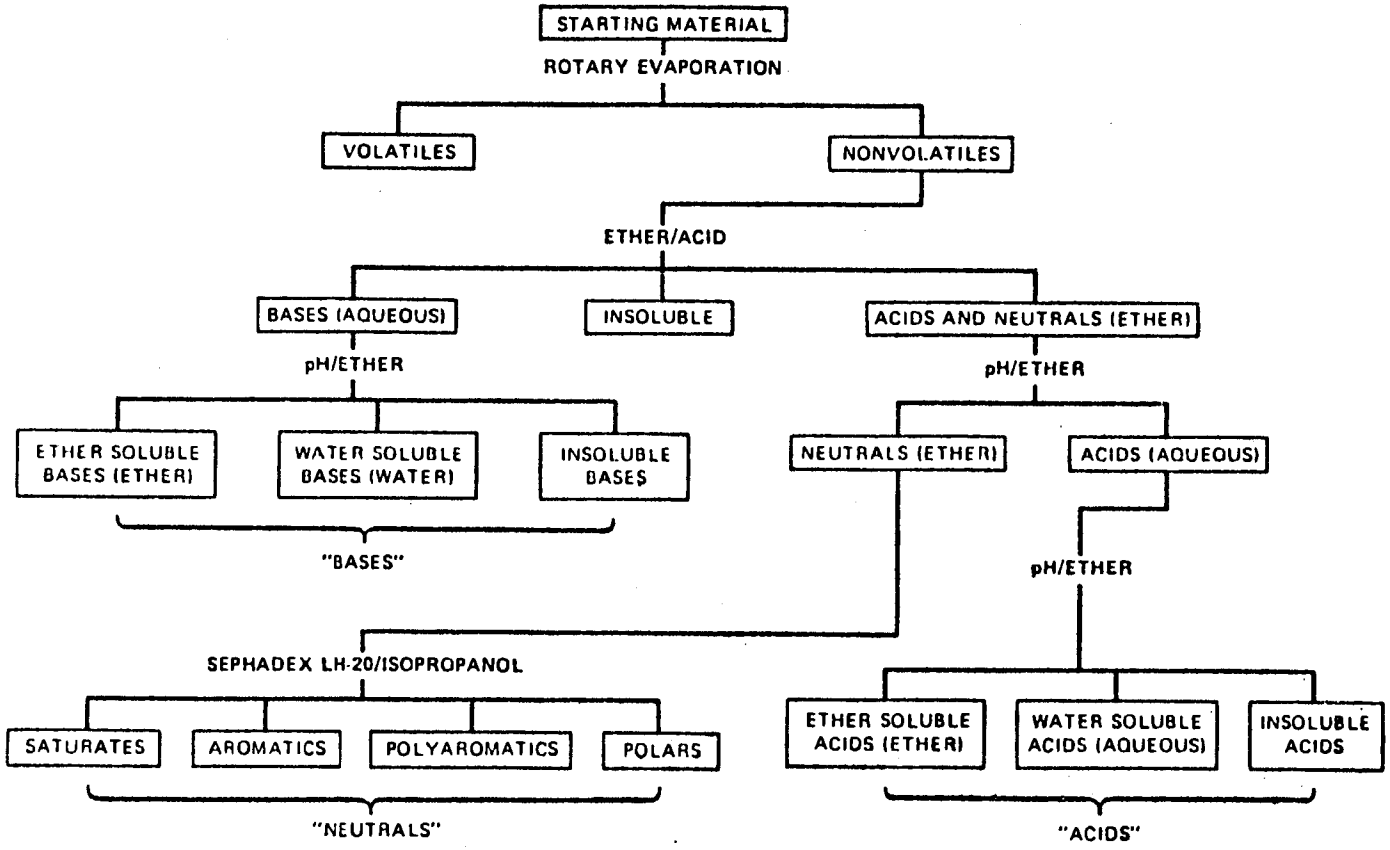


FIGURE 2. Isolation of neutral N-PAH fraction.



CHEMICAL CLASS FRACTIONATION FOR BIOTESTING
MATERIAL, STEP, (PHASE)

FIGURE 3. Generalized fraction scheme.

TABLES

Table 1 Detailed weight distribution of biotested samples in weight percent

2 Mutagenicity of class fractionated samples - Specific mutagenicity in revertants per milligram of fraction

3 Mutagenicity of class fractionated samples - weighted mutagenicity in revertants per milligram of original sample

4 Summary - approximate mutagenicity and weight distributions by chemical class

Table 1. Detailed Weight Distribution of Biotested Samples in Weight Percent

	Volatiles V	Ether Insolubles I	Ether Soluble Acids ESA	Insoluble Acids IA	Ether Soluble Bases ESB	Insoluble Bases IB	Neutral-Saturates N-S	Neutral-Aromatics N-Ar	Neutral-Poly-aromatics N-Ply	Neutral-Polar N-Pol	Total[a] T	Experimental N_e	Summation N_s	Recovery %
Petroleum Crude Oils														
Wilmington No.[b] 5301	7.2	-	0.3	2.2	0.2	0.6	55.7	16.5	9.8	6.2	98.8	90.2	88.2	97.7
Recluse No. 5305	19.6	-	0.2	<0.1	0.6	-	51.0	7.4	3.9	1.1	83.9	-	63.4	90.3
Shale-Derived Substitute														
Paraho No. 4601	0.3	-	0.4	0.3	3.3	0.5	55.6	27.4	7.0	2.2	96.9	95.4	92.1	96.5
Coal-Derived Substitute														
SRC II FO Bld No. 1710	3.0	-	3.8	0.1	5.6	0.1	5.0	59.2	7.3	0.2	84.2	78.5	71.5	91.1
ZnCl Dist No. 1801	0.8	-	0.5	0.4	4.9	0.1	11.8	64.0	21.2	0.6	104.2	96.8	97.6	100.8
H-Coal ASB (Syn) No. 1309	0.6	-	0.4	-	2.0	0.8	20.2	62.6	10.3	<0.1	96.9	97.1	93.1	95.9
H-Coal VSOH (Syn) No. 1310	0.2	-	0.6	-	2.5	0.4	10.2	63.7	22.8	<0.1	100.4	97.6	96.7	99.1
H-Coal VSB (Syn) No. 1311	-	65.1	2.0	0.6	2.1	3.6	0.8	5.8	20.0	0.5	98.4	28.9	27.1	94.0
H-Coal ASOH (FO) No. 1312	34.7	-	0.9	-	1.1	0.5	11.9	24.6	0.5	<0.1	74.2	54.9	37.0	67.4
H-Coal ASB (FO) No. 1313	0.8	-	0.6	0.1	2.1	-	22.9	55.8	6.9	<0.1	89.2	94.1	85.6	91.0
H-Coal VSOH (FO) No. 1314	0.3	-	1.2	0.3	2.6	-	13.9	58.5	19.7	<0.1	96.5	95.1	92.1	96.8
H-Coal VSB (FO) No. 1315	-	59.5	0.2	0.8	1.8	9.6	1.3	8.0	18.4	0.3	99.9	27.7	28.0	101.1
H-Coal Dist No. 1601	4.2	-	1.4	0.1	1.5	0.1	12.7	48.9	6.3	0.2	75.5	83.9	68.1	81.1
H-Coal Dist HDT/L No. 1602	9.3	-	0.2	1.0	1.2	1.0	13.9	47.6	3.0	-	77.1	84.0	64.5	76.8
H-Coal Dist HDT/M No. 1603	9.7	-	<0.1	1.0	0.8	0.9	15.8	47.9	1.9	-	77.8	82.1	65.6	79.9
H-Coal Dist HDT/H No. 1604	12.8	-	<0.1	1.1	0.3	1.0	18.5	42.3	1.4	-	77.3	79.0	62.2	78.7

[a]Using summation of neutral subfractions.

[b]USEPA/USDOE Synfuels Research Materials designation.

Table 2. Mutagenicity of Class Fractionated Samples - Specific Mutagenicity[a] in Revertants per Milligram of Fraction

	V[b]	I	ESA	IA	ESB	IB	N-S	N-Ar	N-Ply	N-Pol
Petroleum Crude Oils										
Wilmington No. 5301[c]	0	-	0	0	0	0	0	20	15	0
Recluse No. 5305										
Shale-Derived Substitute										
Paraho No. 4601	0	-	0	0	8000	141	20	0	1600	0
Coal-Derived Substitute										
SRC II FO Bld No. 1710	0	-	-	0	6000	0	104	0	10000	800
ZnCl Dist No. 1801	0	-	-	0	0	0	0	0	2500	0
H-Coal ASB (Syn) No. 1309	0	-	1200	0	22500	643	12	0	5000	3000
H-Coal VSOH (Syn) No. 1310	0	-	700	200	40000	600	40	0	6000	1800
H-Coal VSB (Syn) No. 1311	-	500	350	0	50000	8333	0	0	1700	300
H-Coal ASOH (FO) No. 1312	0	-	0	0	0	0	0	0	0	0
H-Coal ASB (FO) No. 1313	0	-	0	0	7000	1750	0	0	0	0
H-Coal VSOH (FO) No. 1314	0	-	0	0	40000	110	32	0	16000	0
H-Coal VSB (FO) No. 1315	0	200	3000	0	300000	3000	30	140	3000	700
H-Coal Dist No. 1601	0	-	-	0	9000	0	0	0	3500	0
H-Coal Dist HDT/L No. 1602	0	-	-	0	0	0	0	0	18000	0
H-Coal Dist HDT/M No. 1603	0	-	-	0	0	0	0	0	11000	0
H-Coal Dist HDT/H No. 1604	0	-	-	0	0	0	0	0	0	0

[a]All tests using strain TA-98 with Arochlor 1254-induced S9 activation.

[b]See Table 2 for names of fractions.

[c]USEPA/USDOE Synfuels Research Materials designation.

[d]Dash, "-", indicates "not tested" or "unable to test due to toxicity or interference".

Table 3. Mutagenicity of Class Fractionated Samples - Weighted Mutagenicity[a] in Revertants per Milligram of Original Sample

	V[b]	I	ESA	IA	ESB	IB	N-S	N-Ar	N-Ply	N-Pol	T
Petroleum Crude Oils											
Wilmington No.[c] 5301	0	-[d]	0	0	0	0	0	4	2	0	6
Recluse No. 5305											
Shale-Derived Substitute											
Paraho No. 4601	0	-	0	0	265	1	11	0	111	0	388
Coal-Derived Substitute											
SRC II FO Bld No. 1710	0	-	0	0	349	0	5	0	748	1	1070
ZnCl Dist No. 1801	0	-	0	0	0	0	0	0	530	0	530
H-Coal ASB (Syn) No. 1309	0	-	4	0	445	5	2	0	513	1	970
H-Coal VSOH (Syn) No. 1310	0	-	4	1	988	3	4	0	1367	1	2368
H-Coal VSB (Syn) No. 1311	-	325	7	0	1040	298	0	0	340	2	2012
H-Coal ASOH (FO) No. 1312	0	-	0	0	0	0	0	0	0	0	0
H-Coal ASB (FO) No. 1313	0	-	0	0	141	<2	0	0	0	0	143
H-Coal VSOH (FO) No. 1314	0	-	0	0	1048	<1	4	0	3155	2	4210
H-Coal VSB (FO) No. 1315	-	119	5	0	5340	288	<1	11	551	2	6317
H-Coal Dist No. 1601	0	-	-	0	129	0	0	0	217	0	346
H-Coal Dist HDT/L No. 1602	0	-	-	0	0	0	0	0	544	0	544
H-Coal Dist HDT/M No. 1603	0	-	-	0	0	0	0	0	212	0	212
H-Coal Dist HDT/H No. 1604	0	-	-	0	0	0	0	0	0	0	0

[a]All tests using strain TA-98 with Arochlor 1254-induced S9 activation.

[b]See Table 2 for names of fractions.

[c]USEPA/USDOE Synfuels Research Materials designation.

[d]Dash, "-", indicates "not tested" or "unable to test due to toxicity or interference".

Table 4. Summary - Approximate Mutagenicity and Weight Distributions By Chemical Class

Sample	Mutagenic Activity					Weight Distribution Percentage of Total				
	Total Rov/mg	Percentage Contribution Acids %	Bases %	Neutrals %	Other %	Acids %	Bases %	Neutrals %	Other %	Total Recovered %
Petroleum Crude Oils										
Composite No. 5107	150	2	3	95	0	1	2	85	1	89
Wilmington No. 5301	5	0	0	100	0	2	1	90	7	99
Recluse No. 5305	5	0	0	100	-	<1	<1	65	20	85
LMS No. 5101	75	0	0	100	0	2	1	80	3	86
Shale-Derived										
LETC No. 4101	180	2	42	54	2	2	7	85	1	95
Paraho No. 4601	390	0	69	31	0	1	4	95	0	100
Coal-Derived										
Syntholl No. 1202	4200	3	80	10	8	8	10	65	20	103
COED HDT No. 1106	530	0	11	89	0	4	7	85	1	97
SRC II FO Bld No. 1701	1100	0	35	65	0	4	5	80	3	92
ZnCl Dist No. 1801	530	0	0	100	0	1	1	95	1	98
H-Coal ASB (Syn) No. 1309	970	1	46	53	0	<1	3	95	<1	99
H-Coal VSOL (Syn) No. 1310	2400	0	42	58	-	1	3	95	1	100
H-Coal VSB (Syn) No. 1311	2000	0	66	17	16	2	5	30	65	102
H-Coal ASOH (FO) No. 1312	0	0	0	0	-	1	2	55	35	93
H-Coal ASB (FO) No. 1313	140	0	100	0	-	1	2	95	1	99
H-Coal VSOH (FO) No. 1314	4200	0	25	75	-	1	2	95	1	99
H-Coal VSB (FO) No. 1315	6300	0	89	9	2	1	10	30	60	101
H-Coal "Dist" No. 1601	350	0	37	63	0	1	1	85	4	91
H-Coal "Dist" HDT-L No. 1602	540	0	0	100	0	1	2	85	10	98
H-Coal "Dist" HDT-M No. 1603	210	0	0	0	0	1	2	80	10	93
H-Coal "Dist" HDT-H No. 1604	0	0	0	0	0	1	1	80	13	97

MILESTONES

The most important role that short-term biological assays may serve is providing the rationale for prioritizing research activities, both in chemical measurements and further biological testing. Specific milestones are listed below. Year 1 - current period.

Year	Milestone
1 & 2	(1) Development of a generally applicable extraction procedure(s) for (a) aqueous materials, (b) organic liquids and (c) solid and/or particulate materials;
1 & 2	(2) Development of a generalized class fractionation scheme;
1 & 2	(3) Appropriate application of short-term mutagenicity tests as coupled to the above procedures with energy-related test materials;
1,2,3,4 & 5	(4) Development of a wide data base of generally encountered materials for comparison with the energy technologies;
1,2,3,4 & 5	(5) Extension of the energy-related data base to relevant samples (including evaluation of process changes), encompassing products, effluents (both aqueous and particulate), and combustion products;
1,2,3,4 & 5	(6) Identification of the actual components from energy-related processes;
1,2,3,4 & 5	(7) Further validation of the mutagenic hazard of the test materials and their components; and
3,4 & 5	(8) Intercomparison of synthetic fuel processes.

MUTAGENICITY AND CARCINOGENICITY OF ENERGY RELATED POLLUTANTS IN CULTURED MAMMALIAN CELL SYSTEMS

A. W. Hsie, Principal Investigator

Oak Ridge National Laboratory
Biology Division, Post Office Box Y
Oak Ridge, Tennessee 37830

EPA-IAG-D5-E681-AN
DOE-40-516-75

Project Contact: DOE/Biomedical Programs
EPA Coordination: EPA/ORD

Office of Research and Development
U.S.E.P.A.
Washington, D.C. 20460

A. W. Hsie

Mutagenicity and carcinogenicity of energy-related pollutants in cultured mammalian cell systems: mutagenic and cytotoxic effects of dimethyl and monomethyl sulfates.

It has recently been shown that coal fly ash collected from coal fired plants, contains dimethyl sulfate (DMS) and monomethyl sulfate (MS) at concentrations as high as 830 parts per million. DMS was both mutagenic and cytotoxic but MS, even at a concentration as high as 100 mM did not exhibit either activity. DMS is readily hydrolysed to MS and thus methylation by the highly reactive methyl group of DMS appears to account for both of these activities. On a molar basis, DMS is twice as mutagenic as methyl methanesulfonate under standard assay conditions (1480 cf 690 x 10^{-6} mutants/clonable cell/mM for a 5 h treatment time). Maximum mutation induction and cytotoxicity were obtained after approximately 1 h of treatment. The mutagenic potency of DMS was more stable in aqueous solutions at 4° than at an ambient temperature of 22°, and was least stable in dimethyl sulfoxide solutions at 22°. Although aqueous solutions of DMS rapidly lost its mutagenic potency within 2-3 h at 22°, this compound cannot be overlooked as posing a risk to workers who are closely involved in coal combustion technology.

IN VIVO SCREENING FOR GENE MUTATIONS IN MOUSE GERM CELLS AND SOMATIC CELLS

L. B. Russell, Principal Investigator

Oak Ridge National Laboratory
Biology Division, Post Office Box Y
Oak Ridge, Tennessee 37830

EPA-IAG-D5-E681-AN
DOE-40-516-75

Project Contact: DOE/Biomedical Programs
EPA Coordination: EPA/ORD

Office of Research and Development
U.S.E.P.A.
Washington, D.C. 20460

Subagreement: Screening

Task: *In vivo* screening for gene mutations in mouse germ cells and somatic cells

Principal Investigator: L. B. Russell

During this 3-month period, we completed two sets of experiments using the *in vivo* somatic mutation test ("mouse spot test"): (1) Tests on Coal Liquid No. 2 with two doses at each of 2 stages, and (2) Expansion of work to develop an efficient positive control. The mouse spot test can detect gene mutations, deficiencies of various sizes, and certain other types of genetic events involving marked chromosomal regions.

(1) Tests With Coal Liquid No. 2

Coal Liquid No. 2 is derived from the experimental SRC process No. 2 (supported by DOE and Gulf Oil). We had determined previously that 400 mg/kg, a dose about one-third that fully tolerated by adult mice, would permit intrauterine as well as postnatal survival following exposure of the embryos.

We tested two doses, 400 mg/kg and 500 mg/kg, at each of two stages, days 9 1/4 and 10 1/4 post-conception. Coal Liquid No. 2, (SRC2) was dissolved in Mazola corn oil and injected intraperitoneally into C57BL females, 9 or 10 days following the finding of a vaginal plug from matings with T-stock (multiple-recessive) males. The volume was adjusted by mouse weight, so that a 25 mg mouse received 0.2 ml of one of the two SRC2 solutions, or of corn oil only (control). The exposed and control embryos were checked at birth for external features, then classified at 12-14 days and again at about 4 weeks for spots of various types.

The results are summarized in Table 1. In addition, to the corn-oil group in this experiment, a historical (corn oil) control of 537 may be used for comparison with the experimental groups.

There was no clear evidence that SRC2, either at 400 or 500 mg/kg, affected prenatal survival. Postnatal survival was somewhat reduced. The incidence of external anomalies at birth was 3.3% in the group treated on day 10 1/4 with 500 mg/kg of SRC2. Although one abnormality was also observed in the contemporary control (0.6%), no abnormalities had been observed in our historical corn-oil control of 539. We conclude that SRC2 may have a slight teratogenic effect.

The incidence of midventral white spots (WMVS), which are probably the result of cell (melanocyte) killing, is clearly increased following SRC2 treatment on day 10 1/4. On the other hand, there is no clear evidence for a mutational effect. The frequency of RS (recessive spots, due to gene mutation, deficiency, etc) is 0.5% and 1.0% following, respectively, SRC2 and control injections on day 10 1/4; the corresponding frequencies are 1.3% and 2.2% for day 9 1/4. The historical corn-oil control frequency of RS is 0.8%.

We conclude that SRC2 has some survival, teratogenic, cell-killing effects, but is not mutagenic in the spot test.

(2) Expansion of Work to Develop an Efficient Positive Control

Since scoring in the spot test is somewhat subjective, being dependent on visual acuity, a positive control is desirable for laboratories that have no prior experience with this test.

We have recently tabulated, for all chemicals so far tested in the mouse spot test, the ratio of experimental RS frequency to RS frequency in the appropriate vehicle control. These ratios range from 4 to 19. For ethyl nitrosourea (ENU), however, our ratio from preliminary experiments was as high as 57. This compound thus appears to be a highly favorable positive control.

During the previous reporting period, we checked 263 animals that had been exposed to ENU on day 10 1/4. In the present report period, we increased the number to 651 by expanding, both, stages and doses tested. At day 10 1/4 p.c., we have nowtried 100, 75, 50 and 25 mg/kg; and on day 9 1/4 p.c., 75, 50 and 25 mg/kg.

It is clear that embryos treated on day 9 1/4 p.c. are more vulnerable to lethal effects of ENU than those treated on day 10 1/4 p.c. Most of the deaths occur postnatally. A dose of 75 mg/kg on day 9 1/4 killed every one of 18 animals postnatally, whereas the same dose administered day 10 1/4 allowed 54% postnatal survival among 232 animals. At 50 mg/kg, the results for postnatal survival are in the same direction, although the test is less complete: only 3 animals were born to 5 pregnant females injected day 9 1/4 and none survived to day 12. Following 50 mg/kg injection on day 10 1/4, survival is virtually normal (89%).

Therefore, it is only at the lowest dose tried, 25 mg/kg, that comparisons of mutation rate (RS frequency) and melanocyte killing (WMVS frequency) can be made between days 9 1/4 and 10 1/4. With that dose, there does not appear to be a very clear difference between these two stages for either parameter; RS frequencies are 9.7% and 8.5% for days 9 1/4 and 10 1/4, respectively; WMVS frequencies are 18.4 and 15.9%, respectively.

For injections on day 10 1/4 p.c., where different doses of ENU can be compared, there is a dose-related increase of the various frequencies that can be measured. For doses of 25, 50 and 75 mg/kg (there was no survival at 100 mg/kg), respective frequencies are as follows: RS, 8.5, 17.7 and 19.2%; WMVS, 15.9, 29.7 and 42.4%, and morphological anomalies, 8.4, 22.1, and 33.6%.

It may therefore be recommended that ENU injections on day 10 1/4 p.c. be used as a positive control wherever the use of such a control appears indicated in the mouse spot test.

TABLE 1. Results of spot test with Coal Liquid SRC No. 2

Treatment	Exposure (mg/kg i.p.)	Stage (day p.c.)	No. born	Av. litter size	Survival to day 12 (%)	Abn. morphology	WMVS*	RS** %
SRC2 in cornoil	400	10¼	182	5.1	74.7	0.6	22.1	0.5
SRC2 in cornoil	500	10¼	96	3.8	75.0	3.3	29.2	
SRC2 in cornoil	400	9¼	92	6.1	78.3	1.1	5.6	1.3
SRC2 in cornoil	500	9¼	110	5.5	80.0	0	22.7	
Cornoil contemporary	-	10¼ or 9¼	167	4.9	85.6	0.6	9.8	1.4
Cornoil historical	-	10¼ or 9¼	539	6.4	90.5	0.0	9.2	0.8

*White midventral spots, indicative of cell killing

**"Recessive spots," indicative of genetic lesion

USE OF MOUSE SPECIFIC-LOCUS METHOD TO QUANTIFY THE GENE MUTATION HAZARD FROM MUTAGENS ASSOCIATED WITH NON-NUCLEAR ENERGY TECHNOLOGIES

W. L. Russell and L. B. Russell
Principal Investigators

Oak Ridge National Laboratory
Biology Division, Post Office Box Y
Oak Ridge, Tennessee 37830

EPA-IAG-D5-E681-AQ
DOE-40-516-75

Project Contact: DOE/Biomedical Programs
EPA Coordination: EPA/ORD

Office of Research and Development
U.S.E.P.A.
Washington, D.C. 20460

Subagreement: Mutagenicity and Teratogenicity

Task: Use of the Mouse Specific-Locus Method to Quantify the Gene Mutation Hazard from Mutagens Associated with Non-Nuclear Energy Technologies

Principal Investigators: William L. Russell and Liane B. Russell

For scope and objectives, and for background and uniqueness of approach, see 3-year summary dated June 1978.

Coal Liquid No. 2

Since the latest report, a fifty percent increase has been obtained in data from the specific-locus experiment with Coal Liquid No. 2 (from SRC process No. 2 supported by DOE and Gulf Oil). Male mice were injected intraperitoneally with 1300 mg/kg of crude liquid dissolved in corn oil. The volume injected was adjusted to the weight of each mouse, but never exceeded 0.5 ml. The males were mated more than 7 weeks later with the test stock females. This ensures that all offspring come from germ cells treated in spermatogonial stage. A total of 12,675 offspring has been scored for mutations at the seven loci, and none has been found. The observed zero mutation frequency in this many offspring excludes with 95% confidence the possibility that the true induced mutation frequency from this heavy an injection of Coal Liquid 2 could be as high as 3.5 times the control, spontaneous mutation rate. This result would be accepted by the GENE-TOX panel on the mouse specific-locus test as adequate negative evidence for mutagenicity of Coal Liquid 2.

Concurrent with this experiment, a positive control with the same test system has been producing specific-locus mutations at the rate of about 1 mutation per 200 offspring.

The zero mutagenic effect in this *in vivo* test on transmitted gene mutations in a mammal is in agreement with the lack of mutagenic effect observed in our related programs on dominant lethality and somatic mutations (spot test) in mice.

ANIMAL TOXICOLOGY STUDIES OF EFFLUENTS FROM LOW BTU GASIFIERS

R. J. M. Fry, J. M. Holland, H. R. Witschi
Principal Investigators

Oak Ridge National Laboratory
Biology Division, Post Office Box Y
Oak Ridge, Tennessee 37830

EPA-IAG-D5-E681-AQ
DOE-40-516-75

Project Contact: DOE/Biomedical Programs
EPA Coordination: EPA/ORD

Office of Research and Development
U.S.E.P.A.
Washington, D.C. 20460

Animal Toxicology Studies of Effluents from Low BTU Gasifiers

R. J. M. Fry, J. M. Holland and H. P. Witschi

Acute toxicity studies of UMD gasifier samples

This report presents the data obtained in acute toxicity studies with 3 samples from the UMD gasifier, Duluth, Minnesota. At present, work with 2 samples is still ongoing (i.e. UMD19 and UMD83).

Summary

The acute $LD_{50's}$ using oral administration for the samples tested were between 5.0-6.4 g/kg and 1.1-3.2 g/kg for intraperitoneal administration, and therefore, the samples are considered toxic. No local nor lethal toxicity occurred with topical applications. The weight gain of the rats did vary with the samples and the least weight gain was 14.0 g $\pm$ 1.79 per animal over a two week period.

All samples caused some irritation under the conditions of the test. The reactions included edema and injection, and corneal damage. In rabbits some skin irritation was detected,and no delayed hypersensitivity was found in guinea pigs. The degree of eye irritation was unexpected and should be noted. The carcinogenesis tests on mouse lung and skin are still in progress and will be reported later.

1. Objective

The objective of this study was to determine the acute toxicity of two samples, collected *in situ* at the experimental gasifier, Univ. of Minnesota, Duluth.

2. Experimental design

A total of 5 acute toxicity tests was performed: determination of the acute toxicity following oral and ip administration of the test materials to mice; acute dermal toxicity in rats; primary skin irritation; eye irritation in rabbits; and delayed contact sensitivity

in guinea pigs. All experimental protocols followed standard procedures as adopted in our laboratory.

3. Materials and Methods

a) Materials

Materials were obtained from ORNL depository, Analytical Chemistry Division (Dr. M. R. Guerin). Two samples were collected during the run of 5/31/1979 and were sampled at ports No. 13 and No. 19; they are identified by these numbers. Sample No. 83, an electrostatic precipitator tar, was collected from post No. 15 during the run of 2/6-7/1980.[1]

All three samples were tars, practically solid at room temperature and below. This viscosity produced some handling problems. After several preliminary experiments emulsions of samples were prepared by heating them and diluting 1:1 with propylene glycol. This dilution was used as starting material for all experiments.

b) Young adult BALB/c mice, 6-8 weeks old and weighing 20-25g, bred in our facilities, were fasted for 4 hours prior to the test. Appropriate dilutions of the test agent were administered either by stomach tube or by intraperitoneal injection.

Since liquefaction of the samples was only possible by diluting them 1:1 with propylene glycol, the highest dose administered was a 1:1 mixture of this solution, corresponding to 500 mg/ml of tar and 500 mg/ml of propylene glycol (assumed density of both agents = 1).

Lower dose levels were achieved by diluting this stock solution serially with corn oil. In control animals, the toxicity of propylene glycol was tested, the tar being replaced with corn oil.

All solutions were administered at a dose of 0.1 ml/10g body weight, except when a dose of 10 g/kg was given; for this dose solution No. 1 was given at a dose of 0.2 ml/10g body weight. The animals were observed for 2 weeks after dosing. The LD_{50} was calculated according to Weil.[2]

c) Acute skin toxicity

Female CD/CR rats (Charles River), 2-1/2 months old and weighing 200-400g were used. The animals were lightly anesthetized with ether and the hair was removed from the back with clippers. Tar dilutions corresponding to a dose of 2 g/kg were evenly spread over an area of 25-50 cm^2 and the animals were then housed individually for 4 hours. At the end of this period, excessive amounts of tar were wiped off the skin with gauze and the animals were periodically observed for 2 weeks. They were reweighed at the end of the test period.

d) Eye irritation

White male New Zealand rabbits, 2-3 kg (Dutchland) were used. The lower lid of the left eye was gently pulled away and 0.1 ml of the test agent was instilled into the lower conjunctival sac. The eyes were examined 24, 48 and 72 hrs after instillation. Following

visual inspection, two drops of an 0.05% sodium fluorescein solution (in 0.9% NaCl) was placed onto the cornea. Two minutes later the eyes were gently rinsed with 5 ml of 0.9% NaCl and inspected under a UV lamp.

e) Skin irritation

White male New Zealand rabbits, 2-3 kg (Dutchland) were used. Both flanks of the animals were clipped and 0.5 ml of the test agent was spread evenly over an area of 2.5 cm^2. The site was covered with a gauze patch and the trunk of the animals was wrapped with elastic bandage. After 4 hours, the bandage was removed and the test site was inspected and scored at this time as well as 24 and 72 hours later.

f) Delayed type hypersensitivity

Male and female guinea pigs, Hartley strain, 2-3 months old (Charles River) were used. The skin between the shoulder blades was clipped and 0.1 ml of test agent (0.1% of sample in propylene glycol, v/v) was injected intradermally within an area of 3-4 cm^2 on 3 consecutive days a week for 3 weeks. The site of injection was inspected and scored 24 and 48 hours after each injection. After the last sensitizing injection, the animals were left untreated until 2 weeks later, when a challenging dose (0.05 ml of the 0.1% solution) was injected intradermally into a test site distant from the original field of injection. The reaction was scored 24 and 48 hrs later.

g) Scoring

Lesions for skin irritation, eye irritation and delayed type hypersensitivity were scored by at least two observers according to the Draize procedure as spelled out in reference 3.

4. Results

a) Acute LD_{50}

Groups of 6 mice per dose level were used. The following LD_{50}'s were calculated:[2]

Sample No. 13

Oral administration:	5 g/kg
	(confidence limits: 3.2--7.9 g/kg)
Intraperitoneal administration:	2.2 g/kg
	(confidence limits: 1.7--3.0 g/kg)

Sample No. 19

Oral administration	5 g/kg
	(confidence limits: 3.4--7.2 g/kg)
Intraperitoneal administration	1.1 g/kg
	(confidence limits: 0.8--1.5 g/kg)

Sample No. 83

Oral administration	6.4 g/kg
	(confidence limits 5.0--8.0 g/kg)
Intraperitoneal administration	3.2 g/kg
	(confidence limits 2.5--4.0 g/kg

Solvent (propylene glycol)

Oral administration:	greater than 10 g/kg
Intraperitoneal administration:	4 g/kg
	(confidence limits: 2.6--6.0 g/kg)

b) Acute skin toxicity

Five female rats per group were exposed to 2 g/kg of samples No. 13 and 19 applied to the skin; for sample No. 81, male rats were used. No animal died within the observation period and no abnormal skin lesions developed at the application site.

The following average weight gains within 2 weeks were noted:

Sample No. 13:	14.0 ± 1.7 g per animal
Sample No. 19:	16.2 ± 3.8 g per animal
Sample No. 83:	28.7 ± 2.9 g per animal

c) Eye irritation

In the first set of experiments, 4 animals per compound were tested. A positive individual reaction was noted if there was damage of the cornea or of the iris, or inflammation or edema of the conjunctiva. This scoring procedure is a simplified version of the scoring procedure as outlined in reference 3, page 123. The test was considered to be positive if there were 2 or more positive animals per group, ambiguous if there was one positive animal per group, and negative if no lesions were seen. The following results were obtained:

Compound	Time	Positive animals/animals tested	Test results
13	24 h	3/4	positive
	48 h	3/4	positive
	72 h	2/4	positive
	7 d	1/4	ambiguous
19	24 h	4/4	positive
	48 h	4/4	positive
	72 h	4/4	positive
	7 d	1/4	ambiguous
83	24 h	4/4	positive
	48 h	4/4	positive
	72 h	4/4	positive
	7 d	1/4	ambiguous

The positive reactions were mainly conjunctival (edema and injection). The lids were much less affected (injection, little exudation), except with sample 83 (edema and exudate during first 3 days). The iris was not evaluated, since no reliable observations could be made due to clouding of the cornea.

Corneal damage was evident with agent No. 13, 2 animals out of 4 showed moderate opacity at 24 and 48 hrs, and the entire cornea stained diffusely with fluorescein; the two other animals were negative. With compound No. 19, all animals developed corneal opacity and in 3 out of 4 eyes the entire cornea was fluorescent, in the fourth animal half of the cornea stained. With sample No. 83, the entire cornea of all 4 animals stained diffusely with fluorescein 24 hours after instillation

of the substance, and staining persisted in two animals on day three. After 7 days the corneal lesions essentially had disappeared in all animals, and no fluorescein staining was visible. A low grade of conjunctival irritation was found in one animal treated with No. 83.

The positive response to the eye test was not anticipated and the possibility was considered that heating of the test agents to bring them into solution and to keep them in a liquid state might have been responsible for eye irritation. The test was therefore repeated using 3 animals per compound and the temperature of the test agents was carefully kept at not more than 40°C.

Compound No. 13 produced again a positive response in 2 out of 3 animals, and compound No. 19 a positive response in all three test animals. Diffuse staining with fluorescein was seen in two animals treated with No. 13 and one animal treated with No. 19. In all test animals no signs of eye irritation were seen one week after application of the test agent. In two animals treated with a 1:1 mixture of corn oil and propylene glycol, no ocular lesions were detectable.

d) Skin irritation

Compound No. 13 and 19 were applied to the clipped skin of 6 rabbits and the test sites were inspected for visible lesions 4, 24, and 72 hours later. No erythema or necrosis was produced in any animal with either compound. Compound No. 13 produced slight edema in 4 out of 6 animals and compound No. 19 in two out of 6 animals 72 hours after application only. At this time, edema was detected by palpitation only. No palpitation was done at 4 and 24 hours. With

sample No. 83 no necrosis was observed in any of the animals and only one rabbit exhibited slight edema at 24 hours. All six rabbits, however, showed a low grade but definite erythema 24 and 72 hours after application. In addition, two animals showed slight dry desquamation on day 7.

e) Delayed type hypersensitivity

Each compound was tested in 4 male and 4 female Hartley guinea pigs. The site of each sensitizing injection was inspected at 24 and 48 hours. The lesions were identical with each compound in each sex, slight edema and erythema in an area with a diameter of 4 to 11 mm. No vesiculation or necrosis of the skin was seen.

Two weeks after the last injection the challenge dose was injected into a site distant from the original injection field. Again the lesions, read 24 and 48 hours later, were identical in each animal with either compound: slight erythema and edema, no necrosis, the diameter of the reddened skin area varying between 5 and 7 mm. No tests for delayed type hypersensitivity were done with sample No. 83.

Comments

The 3 samples, collected at the UMD gasifier facility, have an overall toxicity of 5 g/kg or more when given orally to mice. They are considered to be slightly toxic. The vehicle alone, propylene glycol, did not cause any death if given at 5 g/kg orally and its LD_{50} was greater than 10 g/kg; the data on oral toxicity probably reflect only the toxicity of the test agents.

Following intraperitoneal injection toxicity was increased two to five fold. However, the test probably overestimates the true toxicity of the compounds, since intraperitoneal injection of propylene glycol produced an LD_{50} of 4 g/kg. Even so, all three compounds can be labeled as moderately toxic if given by the intraperitoneal route.

A comparison with other data on the acute toxicity of coal conversion products in mice shows that the UMD samples have approximately the same acute toxic potential as have hydrotreated coal distillate, HDT hydroprocessed coal fuel oil or SR1 fuel oil blend, and are more toxic compared to, for example, mixed petroleum crudes, Wilmington crude, crude shale oil (Paraho) and its derivatives.[4,5]

The test compounds did not produce any toxicity when applied to the skin of rats at a dose of 2 g/kg and are therefore, for all practical purposes, not acutely toxic if skin exposure occurs. This agrees with earlier observations on other coal conversion products. Also, the compounds do not produce acute skin irritation or corrosion.

The test for delayed type hypersensitivity was negative. The findings are of interest if compared to a previous study in which we tested crude shale oil (Paraho) and two of its derivatives, where skin sensitization occurred.[6]

An unexpected result was the eye irritation produced by both compounds. The conjunctiva of all test animals became moderately to severely erythematous and edematous, and corneal opacity and fluorescein-staining of the cornea were evident in the majority of the animals. Repetition of the test with the temperature of the test solution carefully controlled confirmed the original observation, although

the lesions appeared slightly less severe. Seven days after the test all lesions were greatly reduced or had disappeared in the first experiment and in the second experiment (controlled temperature of test agent) had disappeared completely.

The test materials thus caused minor, superficial and transient changes of the cornea and conjunctiva as determined by external examination and fluorescein staining. The changes cleared within 7 days. The eye irritation produced by compound No. 13 and 19 must therefore be labeled as moderate and reversible.

b) Conclusions

Samples No. 13 and 19 from the UMD are slightly toxic if ingested, practically non toxic if in contact with the skin and do not cause skin damage, or skin sensitization. However, caution should be exercised with eye exposure, since they are moderately irritating.

7. Bibliography

1. Toxicology Group, Biology Division ORNL, File Report Tox. 80-1.
2. Weil, C. Tables for convenient calculation of median-effect dose (LD_{50} or ED_{50}). Biometrics 8, 249-263, 1952.
3. Principles and Procedures for Evaluating the Toxicity of Household Substances. National Academy of Sciences, Washington, D.C.,1977.
4. Toxicology Group, Biology Division, ORNL, File Report Tox 79-1.
5. Toxicology Group, Biology Division, ORNL, File Report Tox 79-5.

ANIMAL TOXICOLOGY STUDIES OF SHALE OIL AND H-COAL LIQUEFACTION OPERATIONS

R. J. M. Fry, J. M. Holland, H. R. Witschi
Principal Investigators

Oak Ridge National Laboratory
Biology Division, Post Office Box Y
Oak Ridge, Tennessee 37830

EPA-IAG-D5-E681-AQ
DOE-40-516-75

Project Contact: DOE/Biomedical Programs
EPA Coordination: EPA/ORD

Office of Research and Development
U.S.E.P.A.
Washington, D.C. 20460

Animal Toxicology Studies of Shale Oil and H-Coal Liquefaction Operations

R. J. M. Fry, J. M. Holland and H. P. Witschi

Summary

Crude shale oil was found to be slightly toxic by the oral, intraperitoneal and topical routes although no appreciable eye or skin irritation was noted. The evidence suggests that acute exposures to fuels produced from shale oil do not present any more hazard than the petroleum fuels in current use, and are less toxic than the coal conversion materials as judged by $LD_{50's}$. One area of concern arises from the skin sensitization bioassays.

The provisional data from the mouse lung tumor assay suggest that crude shale oil is tumorigenic.

The results for the skin carcinogenicity and toxicity resulting from protracted dermal exposures are included. Skin tumors have resulted from protracted exposures to shale oil crude, and both the hydrotreated crude and hydrotreated residue. The effects of the distillates are proving more complicated due to the demonstrated dermal toxic effects, such as inflammation and hyperplasia, etc. Furthermore, water intake was increased after about 8 months exposure and the mechanism of this change and its significance requires further study.

ACUTE TOXICITY OF SELECTED CRUDE AND REFINED SHALE OIL- AND PETROLEUM-DERIVED SUBSTANCES

INTRODUCTION

To help evaluate and predict hazards of substances to which humans may be exposed in the workplace, several kinds of tests with laboratory animals can be used. These tests are usually designed to provide information on overall toxicity of a given compound and to detect its potential to cause untoward effects.

It was the objective of the present study to obtain general information on the toxicity of selected samples of crude Paraho shale oil and some of its derivatives, some crude petroleums, and three refined petroleum products. Five tests were used to determine the acute toxicity of these substances: acute lethality in mice following oral or intraperitoneal administration of a single dose; acute dermal toxicity of a single dose in rats; delayed-type allergic contact hypersensitivity in guinea pigs; primary eye irritation and primary skin irritation of a single dose in rabbits. In addition, we have examined histopathologic changes induced in mice following intraperitoneal (ip) injection of a single large dose of crude shale oil and two of its hydrotreated derivatives. Studies also have been initiated to examine the tumor inducing potential of selected samples in the mouse lung adenoma bioassay. The present report describes our findings and shows that all compounds tested have very low or no acute toxic effects in laboratory animals.

MATERIALS AND METHODS

Substances Evaluated

Bulk samples of the materials were received from the ORNL repository. Viscous samples were thoroughly mixed by shaking after being warmed in a water bath at 70–80°F. Where feasible, samples were applied undiluted.

For the experiments in which it was necessary to administer graded doses, measured portions were diluted with corn oil (laboratory grade, Fisher Scientific) to provide the desired final concentrations; the density of all substances was assumed to be 1.0.

The following substances were studied:

ORNL repository no.	Name
Shale oil and its derivatives	
4601	Crude shale oil
4602	Hydrotreated shale oil
4607	Hydrotreated shale oil residue
4608	JP-5 product (jet fuel)
4609	JP-8 product (jet fuel)
4610	Diesel fuel marine product
Petroleum and its derivatives	
5107	Mixed petroleum crudes
5301	Wilmington crude oil
5305	Recluse crude oil
4614	JP-5 product (jet fuel)
4615	JP-8 product (jet fuel)
4616	Diesel fuel marine product

Acute LD_{50} in Mice

The objective of this test was to determine acute toxicity following a single oral or ip administration of a substance. The animals used were

BALB/c male or female mice that weighed 20 — 25 g.

Mice were randomly grouped six per cage and were fasted for 18 hr before administration of a substance. For oral administration a ball-tipped needle (20 gauge) fitted to a 1-ml syringe was used. The administration volume was 10 μl/g body weight except when a dose of 16 g/kg was delivered; in this case 20 μl/g body weight of a solution containing 0.8 ml/ml of the test agent was administered. The doses were 2, 4, 8, and 16 g/kg. For ip injections, a 1-ml syringe fitted with a 22-gauge needle was used. The injection volume was 10 μl/g body weight, and doses were 1, 2, 4, and 8 g/kg. Additional doses were tested when necessary. Corn oil was the vehicle used for both routes of administration.

Animals were returned to their cages with free access to food and water and were observed for signs of toxicity. The number of survivors was recorded daily. Two weeks after administration of the substance, the surviving mice were killed. The LD_{50} was calculated from the number of 14-day survivors according to the procedures of Weil.[1]

Acute Dermal Toxicity in Rats*

The objective was to determine the acute toxicity of a substance following a single application to the skin. The animals used for this test were albino male (300—400 g) and female (200—250 g) rats. Each substance was tested on five or six animals of each sex.

The animals were lightly anesthetized with ether, and the hair was clipped from the sides and back. The substance was applied to the center of the back and spread over an area of 25 — 50 cm^2. An attempt was made to cover the same area on each animal and to apply the substance uniformly. A microliter pipetting device was used to apply 2 ml/kg body weight of

undiluted sample. The rats were then placed in individual cages where they remained for 4 hr. During this time they were observed for signs of systemic toxicity. At the end of the 4 hr period any residual substance was removed and signs of skin injury were noted. The animals were returned to maintenance cages and observed over a period of 14 days. At the end of this period, body weights were obtained and necropsies were performed.

Delayed-Type Allergic Contact Hypersensitivity in Guinea Pigs*

The objective was to determine the delayed-type allergic response resulting from cutaneous contact with a substance. The animals used for this test were Hartley albino male and female guinea pigs about 3 months old. Each substance was tested on four different animals of each sex.

Hair was clipped from a 3- to 4-cm^2 area on the back between the shoulder blades. Clipping was done on the first day of injection and when necessary during the course of further injections. A 0.1-ml portion of a 0.1% solution of the substance in 20% DMSO—80% saline or in propylene glycol was injected intradermally three times a week for 3 weeks. The injections were within a 3- to 4-cm^2 field at different sites within the field. Twelve days after the ninth injection, a challenge dose of 0.05 ml of the 0.1% solution of the substance was injected intradermally into a fresh site of the clipped area. Readings for skin reactions were made 24 and 48 hr after each of the nine injections and after the challenge dose. The response was graded and evaluated according to the method of Draize *et al.*[2] which scores sensitization in terms of redness, edema, blistering, and necrosis.

Primary Eye Irritation in Rabbits*

The objective was to determine surface irritation to ocular tissues by

a substance following a single application. The animals used were New Zealand albino male rabbits that weighed about 2 kg. Each substance was tested on one eye of four animals; the other eye served as a control. The lower lid was gently pulled away from the eye, and 0.1 ml of the undiluted agent was instilled into the conjunctival sac by use of a pipetting device. The eyes were examined for signs of irritation 1, 2, and 3 days after instillation. The response was graded and evaluated according to a modified Draize method[2] which scores irritation in terms of redness of the lid and the conjunctiva, edema of the lid and conjunctiva, and opacity of the eye surface.

Primary Skin Irritation in Rabbits*

The objective was to determine skin irritation by a substance following a single application. The animals used for this test were New Zealand albino male rabbits that weighed about 2 kg. Each substance was tested on six different animals.

Hair was clipped from the sides of the rabbits, and 0.5 ml of the undiluted substance was distributed evenly over a 6- to 7-cm^2 area of flank skin by use of a pipetting device. The area was covered with a gauze pad secured with strips of adhesive tape, and the flank was wrapped with an elastic bandage. Four hours later, the bandage and pad were removed and any residual substance was wiped off with dry gauze. At this time the first reading for a skin response was made, followed by further readings at 24 and 72 hr after application. If a definite response was observed at 72 hr, another reading was made 7 days after application. The response was graded and evaluated according to the method of Draize _et al._[2] which scores irritation in terms of redness and edema of the skin.

Histopathology in Mice

Male BALB/c mice 6 — 8 weeks old, 10 per group, were given a single ip injection of the test agent. The mice were killed 2 or 4 days later, and gross necropsy was performed. Tissues were removed and fixed in 10% buffered formalin. They were embedded in paraffin, sectioned at 4–6 μm, and stained with hematoxylin and eosin. The following organs were examined histologically: brain, heart, liver, kidney, esophagus, stomach, intestine, colon, pancreas, spleen, gall bladder, adrenal, lung, trachea, lymph node, and thymus.

Lung Adenoma Assay in Strain A Mice

The objective was to determine if multiple ip injections of a substance can induce lung adenomas in strain A/Jax male mice 6 — 8 weeks old. In preliminary toxicity studies, the maximum tolerated dose (MTD) was determined. The MTD is defined as the highest dose producing no mortality when given in a total of six injections over a 2-week period. After 6 weeks, survivors were killed and a gross necropsies were performed. In the adenoma test proper, each substance was injected into 30 mice three times a week (Monday, Wednesday, Friday) for 8 weeks. The following doses were used: the MTD, one-half the MTD, and one-fifth the MTD. Control groups were a) 30 mice injected with the same vehicle (corn oil) used to dissolve the test substance, b) 50 mice given a single injection of urethan at 1 g/kg body weight (positive control), and c) 50 mice which received nothing.

Body weights were recorded every 2 weeks, and the mice were killed 20 weeks after the first injection. The lungs were fixed in Tellyesniczky's solution and the number of tumors on the lung surface was

counted. Selected tissues were fixed in 10% buffered formalin and Bouin's fixatives for histopathology.

RESULTS

Acute Toxicity in Mice and Rats

Data for acute toxicity of shale oil and its derivatives are given in Table I. Crude shale oil was the only one that produced acute deaths in both male (at doses of 8 and 10 g/kg) and female (at doses of 4, 8, and 16 g/kg) mice when given orally. Following ip injection, deaths also occurred at doses of 2 g/kg. Because the mortality data were similar for both sexes, substances 4608–4610 were tested in female mice only. The LD_{50} was between 6 and 8 g/kg for substances 4608 and 4609 and greater than 16 g/kg for substance 4610.

Results for petroleums and derivatives are shown in Table II. Acute toxicity data were for all practical purposes similar to the ones observed with shale oil and its derivatives.

In both male and female rats, there were no visible toxic responses arising from the dermal application of 2 g/kg of compounds 4601, 4602 and 4607. Neither visible skin lesions nor manifestations of central nervous system pertubations were observed. Deaths did not occur during the 2 weeks following application of the substances, and all rats showed a progressive gain in body weight (Table III). Similar observations were made when rats were treated with 2 g/kg of shale oil- or petroleum-derived JP-5, JP-8, or diesel fuel marine product: no animals died, no skin lesions developed, and weight gains over a 2-week period were normal.

Delayed-Type Contact Hypersensitivity

Guinea pigs were sensitized with crude shale oil, hydrotreated shale

oil, hydrotreated shale oil residue, or shale oil- and petroleum-derived jet and diesel fuels. The data are summarized in Table IV. During the sensitization procedure, only slight redness and focal scab formation was noted in some of the animals for all nine substances; edema and necrosis were not seen. After the challenge injection, all animals sensitized with shale oil, hydrotreated shale oil, and residue developed a degree of redness and edema greater than that observed after the sensitizing injections. The challenge injection of these three substances also produced a slight necrosis in most guinea pigs, and for two of the substances, a marked lesion with a necrotic center developed. No difference in reaction between the sensitizing and challenge injection was observed in animals exposed to the shale oil- or petroleum-derived jet and diesel fuels.

Eye and Skin Irritation in Rabbits

The data in Table V show that only the shale oil and to a lesser extent its hydrotreated derivative produced a visible redness of the rabbit eye. However, irritation was slight and transient and is considered insignificant in evaluating substance toxicity. None of the other substances produced visible irritation at the three observation intervals. There were no indications that the shale oil, its five derivatives, or the petroleum products were irritating to rabbit skin to the extent of producing visible redness or edema (Table VI).

Histopathology

Mice killed 2 or 4 days following ip injection of 4601 at 4 g/kg, or 4602 or 4607 at 16 g/kg showed superficial pyogranulomatous serositis of the peritoneal cavity resulting from local reaction to the substances.

Lymphoid depletion of the thymic cortex was present in all animals at 2 and 4 days, but was most marked in mice injected with 4602. Serositis and thymic cortical depletion were the only histopathologic alterations observed for these substances. No lesions were present in animals injected with corn oil alone.

Lung Adenoma Assay

This assay was done essentially as described by Shimkin and Stoner[3] In preliminary toxicity studies, the MTD for 4601 was determined to be 2.5 g/kg per injection and for 4602, 16 g/kg. However, during the course of the adenoma assay it was found that the MTD, as determined, was too high. Only in one group was the survival rate higher than 50%. Cumulative mortality rates are shown in Figure 1A and B, and the average weight again of survivors is shown in Figure 2. It is interesting to note that with both crude shale oil and hydrotreated shale oil animals continued to die even after cessation of exposure to the test substances. The reasons for this delayed toxicity remains to be established. Treated animals also generally failed to gain weight at rates comparable to that of controls.

As shown in data of Table VII, tumor incidence was 30 — 60% in treated animals and not significantly different from animals injected with vehicle (corn oil) alone. On the other hand, tumor multiplicity was significantly higher in animals injected with 1.25 g/kg of crude shale oil. In all other groups, the differences from the control value were statistically not significant. However, few animals survived when injected with 2.5 g/kg of crude shale oil or with 16, 8, or 3.2 g/kg of hydrotreated shale oil. In view of this general toxicity, the adenoma assay in these groups is probably not representative and needs to be repeated at lower dose levels. Detailed results on histopathology will be reported later.

DISCUSSION

Our data show that, when ingested, crude shale oil may be considered slightly toxic and all other samples are practically nontoxic; no sex difference was found where both male and female mice were tested. Following ip injection the substances were slightly toxic. Acute skin toxicity was invariably greater than 2 g/kg, and no evidence was obtained to document appreciable eye and skin irritation. Histopathologic findings following acute administration of large amounts of test material were not remarkable. Lymphoid reduction in the thymic cortex was interpreted as a stress reaction rather than a substance-specific effect.

Compared with petroleum and some of its derivatives, shale oil and its derivatives appear to have practically the same overall low toxicity. Fuels from both sources have about the same LD_{50}'s and are essentially nonirritating to the skin and eye. These observations corroborate our earlier studies which showed that three other petroleum-based substances did not elicit skin or eye toxicity (Table VIII). We would not expect, therefore, that acute exposure to these jet and diesel fuels produced from shale oil would be any more hazardous than exposure to their petroleum-derived equivalents currently in use.

From the present data, we conclude that a single exposure of humans to any one of the six Paraho/SOHIO substances tested would result in little if any systemic toxicity or skin or eye injury. One possible exception arises from the findings of the skin-sensitizations bioassay. Our data suggest that repeated topical exposures to the crude shale oil or the hydrotreated products 4602 or 4607 could possibly elicit an immunologically mediated reaction in humans. This type of reaction which could take weeks or months

to develop may become manifest as itching, reddening, eruption, and edema of the skin. These reactions may disappear if there is no further contact with the sensitizing substance, but the state of sensitization may be permanent.

The data obtained in the lung adenoma bioassay suggest that crude shale oil has tumorigenic potential. This appears to agree with preliminary data obtained in skin painting experiments.[6] Before any more definitive conclusions can be drawn, particularly as to the carcinogenic potency of shale oil, additional studies with the lung adenoma bioassay will have to be completed.

It is interesting to note that the oral LD_{50}'s of shale oil, petroleum, and their derivatives are consistently higher than the oral LD_{50} found with materials derived from various coal conversion processes. Table IX lists some data for comparative purposes.

In conclusion, acute toxicity of Paraho shale oil and of five derivatives does not appear to be a problem of immediate concern. The substances produced slight acute effects similar to those of petroleum-derived materials. Our data essentially agree with those in a report by Weaver and Gibson,[4] except that they failed to obtain a positive response in the assay for skin sensitization. This discrepancy might need to be resolved in further studies.

REFERENCES

1. Weil, C. S., "Tables for convenient calculation of median-effective dose (LD_{50} or ED_{50}) and instructions in their use," Biometrics, 8, 249-263 (1952).

2. Draize, J. H., G. Woodard, and H. O. Calvery, "Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes," J. Pharmacol. Exp. Ther., 82, 377-390 (1944).

3. Shimkin, M. and B., G. D. Stoner, "Lung tumors in mice: Application to carcinogenesis bioassay," Adv. Cancer Res., 21, 1-58 (1975).

4. Weaver, N. K., and R. L. Gibson, "The US oil shale industry: A health perspective," Am. Ind. Hyg. Assoc. J., 40, 460-467 (1979).

5. Cowser, K. E., et al. (ed.), "Briefing on Oak Ridge National Laboratory Life Sciences Synthetic Fuels Program," September 27, 1979, pp. 86-94.

6. Holland, J. M., These proceedings.

FOOTNOTE

*The test methods used are based on those described in Principles and Procedures for Evaluating the Toxicity of Household Substances, National Academy of Sciences, Washington, D.C., 1977. Acute toxicity, pp. 10–17; dermal toxicity, pp. 23–26; delayed-type sensitivity, pp. 36-39; eye irritation, pp. 123-124; skin irritation, pp. 28-31.

Table I. Acute Lethality Test in Mice: Shale Oil and Its Derivatives

Substance	LD_{50} (g/kg)			
	Oral		Intraperitoneal	
	Male	Female	Male	Female
4601	11.3	11.3	6.1 (4.5–8.2)*	4.3 (2.0–6.1)
4602	>16.0	>16.0	>16.0	>16.0
4607	>16.0	>16.0	>16.0	>16.0
4608	ND†	>16.0	ND	8.0 (5.4–12.8)
4609	ND	>16.0	ND	6.4 (5.0–8.0)
4610	ND	>16.0	ND	>16.0

*95% confidence limits.

†ND = not done.

Table II. Acute Lethality Test in Mice: Petroleums and Their Derivatives

	LD_{50} (g/kg)			
	Oral		Intraperitoneal	
Substance	Male	Female	Male	Female
5107	>10.0	ND[†]	ND	ND
5301	>16.0	ND	ND	ND
5305	>16.0	ND	ND	ND
4614	ND	>16.0	ND	11.2 (8.13-15.5)[‡]
4615	ND	>16.0	ND	8.0 (5.5-12.8)
4616	ND	>16.0	ND	>16.0

[†]ND = not done.

[‡]95% confidence limits.

Table III. Weight gain in male and female rats following dermal application of 2 g/kg of shale oil, hydrotreated shale oil, or residue*

	Mean body weight (g) ± SE			
	Male		Female	
Substance	Day 0	Day 14	Day 0	Day 14
4601	358 ± 8	415 ± 7	209 ± 5	241 ± 2
4602	352 ± 9	422 ± 9	209 ± 3	238 ± 4
4607	370 ± 4	435 ± 7	225 ± 5	256 ± 6

*Six animals per group except for substance 4602 females, for which there were 5 rats.

Table IV. Delayed-Type Allergic Contact Hypersensitivity Test in Guinea Pigs

Substance	Average response* 24 and 48 hr after each of nine sensitizing injections			Average response* 24 and 48 hr after the challenge injection		
	Redness	Edema	Necrosis	Redness	Edema	Necrosis
Shale oil and its derivatives						
4601	±	0	0	1–2	2	±
4602	0	0	0	2	1	±
4607	0	0	0	2	1	±
4608	0–1	0	0	0	0	0
4609	0–1	0	0	0	0	0
4610	0–1	0	0	0	0	0
Petroleum derivatives						
4614	0–1	0	0	0	0	0
4615	0–1	0	0	0	0	0
4616	0–1	0	0	0	0	0
Propylene glycol†	0–1	0	0	0	0	0
Dinitrochlorobenzene‡	1–2	0–1	1–2	1–2	1–2	2–3

*Scale: 0, ±, 1–4.

†Vehicle.

‡Positive control.

Table V. Primary Eye Irritation Test in Rabbits

Substance	Response at indicated times after instillation*		
	24 hr	48 hr	72 hr
Shale oil and its derivatives			
4601	6 of 6 showed grade 1 redness†	0	0
4602	2 of 6 showed grade 1 redness†	0	0
4607	0	0	0
4608	0	0	0
4609	0	0	0
4610	0	0	0
Petroleum derivatives			
4614	0	0	0
4515	0	0	0
4616	0	0	0

*Four or six eyes per substance.

†On the basis of a 0 – 4 response of lid, conjunctiva, and cornea.

Table VI. Primary Skin Irritation Test in Rabbits

Substance	No. of rabbits responding at indicated times after application*					
	4 hr		24 hr		72 hr	
	Redness	Edema	Redness	Edema	Redness	Edema
Shale oil and its derivatives						
4601	0	0	0	0	0	0
4602	0	0	0	0	0	0
4607	0	0	0	0	0	0
4608	0	0	0	0	0	0
4609	0	0	0	0	0	0
4610	0	0	0	0	0	0
Petroleum derivatives						
4614	0	0	0	0	0	0
4615	0	0	0	0	0	0
4616	0	0	0	0	0	0

*Six animals per substance.

Table VII. Mouse Lung Adenoma Assay: Shale Oil and Hydrotreated Shale Oil

Substance	Dose per injection* (g/kg)	No. of mice initially	No. of survivors	No. of mice with tumors	No. of tumors/mouse†
4601	2.5	30	5	3	0.6 ± 0.3
	1.25	30	12	6	1.3 ± 0.5‡
	0.5	30	16	7	0.6 ± 0.2
4602	16	30	0	—	—
	8	30	1	0	0
	3	30	6	3	1.0 ± 0.6
Corn oil	10	20	19	6	0.4 ± 0.1
Untreated	—	20	20	3	0.2 ± 0.1
Urethan	1.0§	20	20	20	23.2 ± 1.7‡

*Given three times a week for 8 weeks.

†Total number of tumors divided by total number of surviving animals; mean ± SE.

‡$P < 0.05$ compared with corn oil control.

§One ip injection of 1 mg/g body weight.

Table VIII. Skin and Eye Toxicity of Petroleum-Based Substances

Substance	ORNL repository No.	Acute LD_{50}, skin (g/kg)	Eye irritation	Skin irritation
Syncrude	1106	>2	0	0
Mixed petroleum crude	5107	>2	0	0
Synthoil crude	1201	>2	0	0

Table IX. Acute Oral Lethality Test of Selected Coal Conversion Products in BALB/c Male Mice [from ref. 5]

Substance	ORNL repository no.	LD_{50} (g/kg)
H-coal		
H-coal distillate (raw)	1601	3.6 (2.8–4.5)
HDT distillate (low severity)	1602	4.0 (3.4–4.7)
HDT distillate (high severity)	1604	5.5 (3.8–7.2)
HDT fuel oil (low severity)	1617	6.2 (3.7–8.7)
HDT fuel oil (high severity)	1619	6.2 (5.1–7.3)
Atmospheric bottoms (ASB)	1313	2.3 (1.9–2.6)
Vacuum overhead (VSOH)	1314	2.6 (2.2–3.2)
Atmospheric bottoms (ASB)	1309	3.6 (2.4–5.2)
Vacuum overhead (VSOH)	1310	2.5 (1.7–3.1)
Coal SRC I		
HDT light organic liquid (low severity)	1606	3.6 (3.1–4.1)
HDT light organic liquid (high severity)	1608	10.0
HDT recycle solvent (low severity)	1614	2.6 (1.9–3.2)
HDT recycle solvent (high severity)	1616	5.3 (4.4–6.3)
Coal SRC II		
Fuel oil blend	1701	3.1 (2.7-3.5)
Zinc halide hydrocracking process		
Product distillate	1801	3.4 (0.5-2.3)

*95% confidence limits

FIGURE LEGENDS

Fig. 1. Survival of mice given (A) 24 injections of crude shale oil 4601 or (B) hydrotreated shale oil 4602. (A): ●, 2.5 g/kg; ■, 1.25 g/kg; ▲, 0.5 g/kg; ○, corn oil. (B): ●, 16 g/kg; ■, 8 g/kg; ▲, 3.2 g/kg; ○, corn oil. Brackets indicate SE's.

Fig. 2. Body weight of mice given 24 injections of crude shale oil 4601 or hydrotreated shale oil 4602. 4601: ●, 2.5 g/kg; ■, 1.25 g/kg; ▲, 0.5 g/kg. 4602: □, 8 g/kg; △, 3.2 g/kg; ○, corn oil. Number of mice shown in parentheses. Brackets indicate, SE's.

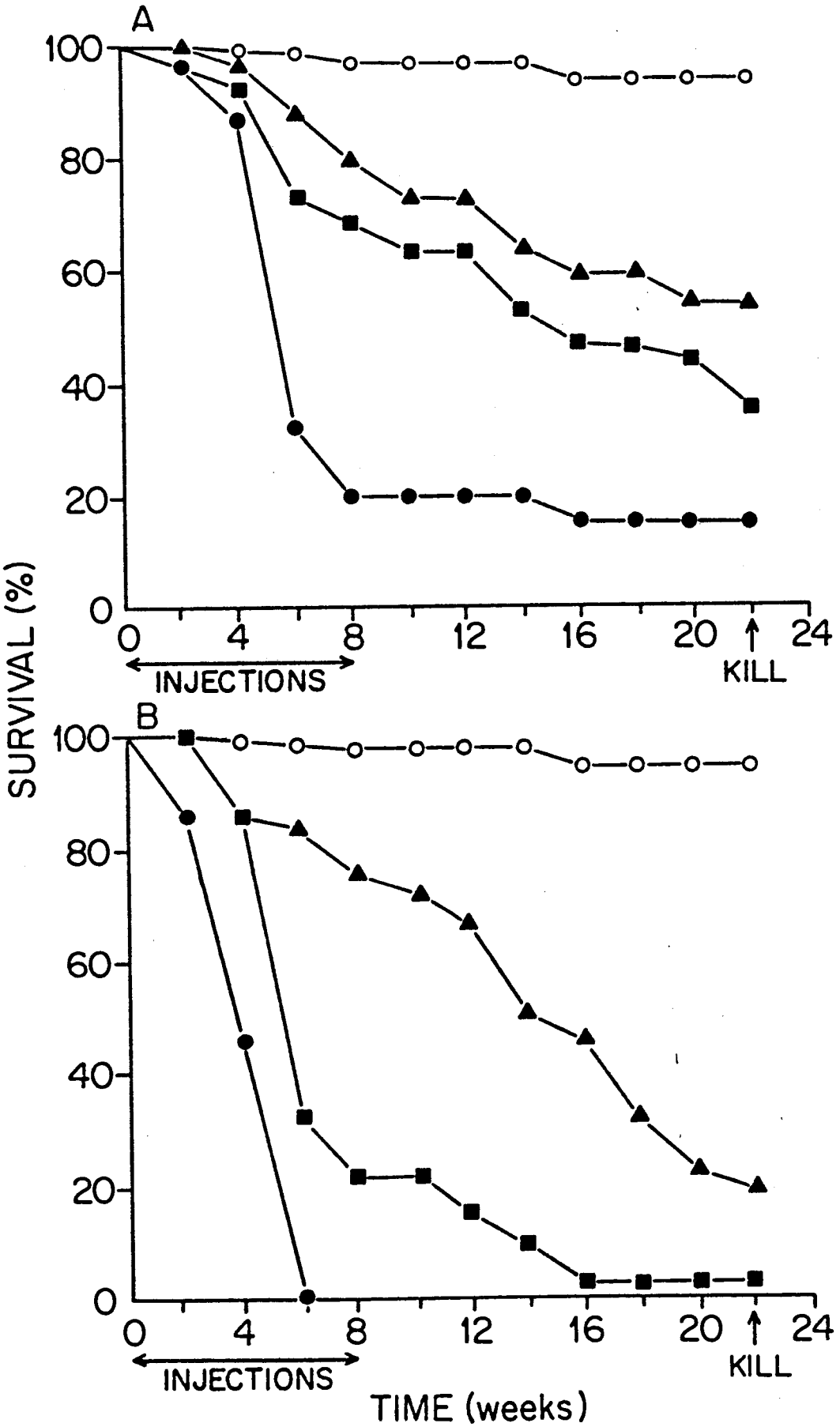

A
B
SURVIVAL (%)
100
80
60
40
20
0
0
4
8
12
16
20
24
INJECTIONS
KILL
TIME (weeks)

Fig. 1

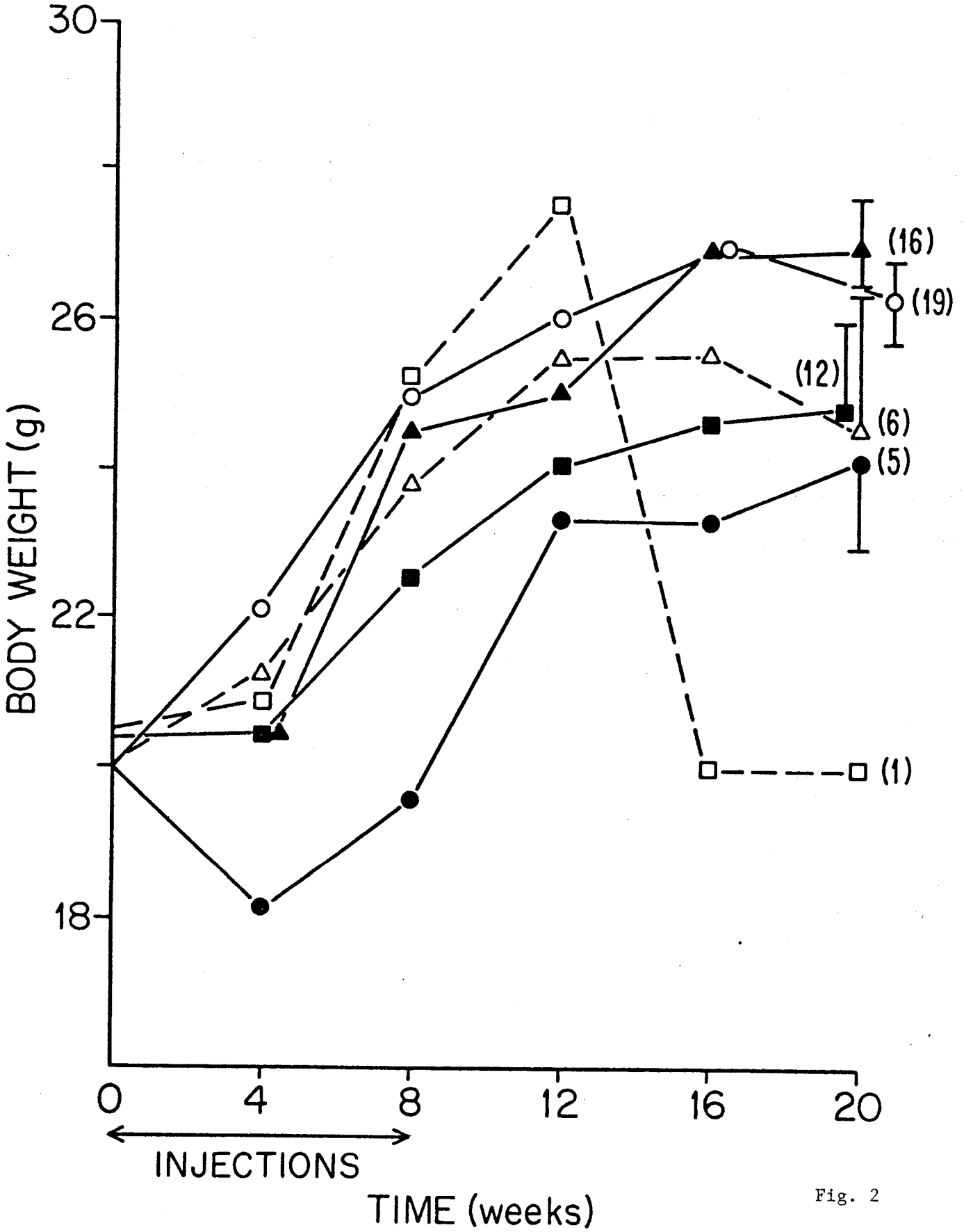

30
26
22
18
BODY WEIGHT (g)
0
4
8
12
16
20
INJECTIONS
TIME (weeks)
(16)
(19)
(12)
(6)
(5)
(1)

Fig. 2

CHRONIC DERMAL TOXICITY OF PARAHO SHALE OIL AND DISTILLATES*

J. M. Holland, L. C. Gipson, M. J. Whitaker, T. J. Stephens, and G. M. Clemmer, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

D. A. Wolf, Computer Sciences Division, Union Carbide Corporation, Oak Ridge, Tennessee 37830

INTRODUCTION

The purpose of this experiment was to estimate the relative skin oncogenicity of a Paraho process shale oil and related distillates. A secondary objective was to determine whether materials applied to the skin were absorbed, ingested or possibly inhaled in quantities sufficient to induce systemic toxicity.

The results obtained reflect continuous exposure of mice for more than 12 months. Exposures will continue for another 12 months and for this reason the current results should be treated as preliminary pending terminal sacrifice and examination of surviving animals.

MATERIALS AND METHODS

Animals

Equal numbers of male and female C3Hf/Bd inbred mice were randomly distributed to treatment groups at 10 weeks of age. Mice were housed 5 per cage on sterilized hardwood chip bedding and given free access to sterilized Purina 5010C diet and hyperchlorinated-acidified water.

---

*Research supported by the Office of Health and Environmental Research, U. S. Department of Energy, under contract W-7405eng-26 with the Union Carbide Corporation.

Treatment groups, concentrations and the number of animals in each group are given in Table I. To determine whether the interval between successive doses (dose rate) made any difference in toxicity the materials were applied on both a three times and a two times weekly schedule. For materials capable of evoking skin tumors on the 3 times weekly schedule we plan to stop treatment while continuing twice weekly exposures until the same number of dosages have been applied on both schedules. By contrasting the cummulative hazard rates as a function of dose rate, material which show a lesser degree of dose rate dependence can be identified. It is these mixtures which represent the greatest potential occupational hazard because even infrequent or widely spaced exposure could represent a finite risk due to either to long skin residence times or irreversible tissue injury with each exposure.

Application of Test Materials

An appropriate amount of each test material was weighed into a number of glass vials. The vials, containing a known weight of material, were kept at 4°C in an explosion proof refrigerator. Each week a vial was removed and a volume of spectrograde cyclohexane added to yield the highest concentration required for the study. Lower concentrations of the same material were made from this sample. Benzo(a)pyrene solutions were also made fresh weekly.

Hair was removed from the upper back with electric clippers. Materials were applied using a 50 μL micropipette. Concentration of each test material was determined as the maximum permitted either by physical characteristics or acute local or systemic toxicity. Whenever possible the materials

were applied without dilution in order to demonstrate toxicity without possible influences by the solvent.

Parameters of Acute and Chronic Dermal Toxicity

The area directly exposed to the test materials is monitored for nonspecific inflammatory, hyperplastic and neoplastic changes and these lesions are recorded for each animal at the time of treatment or when the mouse is reshaved. In addition to local effects on skin texture, thickness, pigment and hair growth, there are potential systemic toxic effects due to absorption of components through the skin, ingestion or a combination of both. Also, for the more volatile components of the mixture, there is a distinct possibility that inhalation exposure occurs. Therefore a dermal "carcinogenesis" bioassay results in a multipath, natural route, exposure of the animal to the test material.

Given the protracted nature of the exposure there is ample time for cummulative toxicity to become manifest. This toxicity can be reflected as decreased motor activity, neurological signs, or more readily quantifiable effects on body weight, organ weight or in the extreme, an increased mortality rate. An additional physiologic parameter is the average daily water intake required to maintain a favorable water balance. And finally tissues in animals that die or that survive to the end of the study are examined systematically and any lesions or abnormalities observed are taken for histologic examination.

Water intake for individual cage groups was determined by placing a known quantity of drinking water into the standard water bottle and measuring the remainder at the end of one week. Bottles were placed on the cages and removed at the same time for all treatment groups and vehicle controls.

No attempt was made to account for spillage because it was assumed that this would be comparable between treated and vehicle control groups. Twenty-four-hour water intake, urine output and feces weight was also determined for individual mice from representative treated and vehicle control groups by placing them into metabolism cages.

Statistical Analysis of Skin Tumor Responses

The temporal distributions of skin tumor latencies, for all groups in which two or more animals had developed grossly typical skin tumors at the site of application, were fit using both the non-parametric Kaplan-Meier procedure and the parametric Weibull model relating dose in mg per week to the expectation of survival without a treatment related skin tumor. Interim relative potency estimates were obtained by comparing the dose response obtained with benzo(a)pyrene to that observed for the unknown through the current point in the experiment. Details concerning the approach used and other examples can be found elsewhere.[1-4] As additional animals develop tumors in the various treatment groups we do not anticipate that the estimated relative potencies will change substantially, however, the confidence limits will narrow.

For continuous variables, such as body weight and water consumption, group means were compared using student's tstatistic with significance at the 0.05 level.

RESULTS AND DISCUSSION

Skin Tumor Induction

Both neoplastic and non-neoplastic changes have been induced to different degrees by the various treatments. Grossly typical neoplasms have been

observed in animals exposed to the Shale Oil Crude, the Hydrotreated Crude and the Hydrotreated Residue on both the three times and the two two times weekly dosage schedule (Table II). At the two highest benzo(a)pyrene dose levels all the animals at risk have developed a skin neoplasm and thus exposure was discontinued. Neither tumors or non-specific inflammatory lesions have been observed in mice exposed to the cyclohexane vehicle.

Interim calculation of relative skin carcinogenicities was accomplished by comparison of the dose responses obtained for the benzo(a)pyrene reference carcinogen to that obtained for each of the unknowns, with dose rate expressed on a mg per week basis. The observed proportion of mice surviving without a skin tumor in cohorts exposed to tumorigenic materials is shown in Figures 1-4 for the benzo(a)pyrene, Shale Oil Crude, Hydrotreated Shale Oil and Hydrotreated Residue, respectively. The solid, step curves represent the Kaplan-Meier estimates and the superimposed dashed lines the fitted Weibull with the shape and location parameters of this model constant for all groups. Wherever the solid line stops represents the cut-off for the interim relative potency estimates for that dose-treatment group.

By comparison of the fitted Weibull curves, relative potencies are obtained for each of the positive unknowns in terms of benzo(a)pyrene dose equivalents. Table III lists these relative potencies together with upper 95% confidence limits. These values can be interpreted as the relative carcinogenic efficiency of the test material to an equivalent amount of BaP when both are applied continuously. In order of decreasing potency are the Shale Oil Crude (1/998), the Hydrotreated Residue (1/1622) and the Hydrotreated Shale Oil (1/2780) applied on the same 3 times weekly schedule as were the BP positive controls. Since there were no BP twice weekly exposure

groups, the twice weekly and three times weekly relative potency estimates are not entirely comparable. However, the potency estimates are based upon dosage per week and thus difference in the schedule of application would not affect the calculation. It is noteworthy that reducing the dose rate by a factor of 1/3 reduced the tumorigenicity of the Shale Oil Crude by a factor of 1.7, the Hydrotreated Residue 2.8, and the Hydrotreated Shale Oil, 3.2. The implication of this is that the probability of tumor occurrence decreases multiplicatively as the dose rate decreases arithmetically.

Non-Neoplastic Effects

To date, none of the distillates, whether derived from shale oil or natural petroleum, have induced skin neoplasms. However the materials have induced local inflammatory, degenerative and hyperplastic skin changes. The skin exposed to the test materials demonstrates loss of hair proportionate to the dosage level. At the highest exposure levels the surface hair loss is complete. The exposed skin is thickened, coarsely folded and deeply pigmented (Figure 5). Microscopically, the surface epidermis is both more cellular and covered by a thick layer of amorphous keratin (Figure 6). Small and large defects in the epidermis (ulcers) are present with attendant acute inflammation in the exposed dermis and the wound margins.

Hair follicles are always prominent features in chemically damaged mouse skin. In this particular instance hair follicles are exhibiting abortive hyperplasia with production of incompletely keratinized hair. Many branched and otherwise malformed follicles are observed deep within a generally disorganized and edematous dermis. The dermis is notable in that there are increased numbers of pigment cells clustered at the dermal-subcutaneous junction. These focal aggregates represent both phagocytized

melanin arising from degenerating hair follicles as well as melanocyte proliferation. The upper portion of the follicle is filled with keratinaceous debris, hair fragments and occasional inflammatory cells. The epithelium of the external root sheath has become continuous with the surface epidermis and in most areas the keratinization pattern noted on the surface is of the trichohyaline type. This indicates that epidermal keratinocytes have been overgrown by hyperstimulated follicular keratinocytes with attendant alteration in the type of keratin present on the surface.[5]

Marked ectasia of the superficial blood vessels is also noted in the dermis (Figure 7). In addition to the pigment aggregates mentioned previously, the dermis is infiltrated by variable numbers of mononuclear and neutrophil leukocytes. In summary, the skin of mice exposed to the undiluted distillates manifests a partially compensated chemical dermatitis with focal ulcerative and diffuse hyperplastic changes.

In addition to local irritant effects the distillate materials have demonstrated systemic toxicity reflected both by a reduction in average body weight in groups exposed to the highest concentration of shale oil derived JP8 and DFM (Table IV), and an increase in average daily water intake observed in all groups exposed to the distillates but <u>not</u> the crudes (Table V).

The rather remarkable treatment and dose related increase in average daily water intake was noted commencing at about the eighth month of exposure and has continued through the present time without marked change. That the water consumption effect was treatment related was reinforced by the observation that when animals were not treated over the Memorial Day holiday water intake returned to control levels. When treatment was reinstituted

after the holiday the water intake again increased to levels similar to those noted before the interruption in treatment schedule.

Increased water intake can occur as a consequence of pituitary/hypothalmic imbalances, kidney damage, changes in plasma osmolality such as accompany diabetes mellitus, and fluid losses that accompany severe burns, diarrhea, vomiting or external hemorrhage. Of these the most likely possibility was kidney damage arising from exposure to percutaneously absorbed materials or reactive metabolites generated by the hyperplastic skin. To evaluate this possibility individual mice were placed in a metabolism cage in which water intake, urine production and feces production can be accurately monitored. The availability of only one cage has limited the amount of data we were able to collect for this presentation but the limited data we have obtained (Table VI) reveal an interesting phenomenon. We have three independent measurements of mice treated with cyclohexane (solvent) three measurements of mice treated with shale oil derived DFM (DFMS), and two measurements in mice treated with natural petroleum derived DFM. These data confirmed the higher average daily intake in mice exposed to distillates relative to vehicle controls but this greater water intake cannot be explained as a consequence of loss of renal concentrating ability because urine production rates were only slightly above control. The only other possible sources of water loss was through feces, percutaneous diffusion and the respiratory tract. Fecal weights did not differ between treated and control. By a process of elimination this leaves the respiratory tract and precutaneous diffusion as the remaining alternatives. Of these, water loss through damaged skin is the more likely possibility. This hypothesis is ammenable to experimental test by treating the damaged skin in a way to

reduce its permeability to water molecules, while continuously monitoring the water intake and output profiles.

The possibility that a fraction of the increased water requirement was a consequence of kidney damage is suggested by histologic finding in two animals dying acutely in the group exposed to 100% natural petroleum derived JP5. The tissues from these animals were subjected to histologic evaluation. The changes in the skin were described previously. The liver exhibited lobular atrophy and focal coagulation necrosis. There was arrest of follicular maturation in the ovary with persistence of corpora lutea and concomitant cystic hyperplasia of the uterus and mammary tissue; the mice appeared pseudopregnant. The adrenals exhibited mild cortical atrophy and the thyroid and pituitary were unremarkable.

The kidneys were grossly normal. Microscopic lesions were observed at the corticomedulary junction. The lesions were restricted to the terminal straight segment of the proximal tubule and the thick ascending limb and distal tubule. The glomerulus, convoluted proximal tubule and collecting ducts were not involved. The lesions consisted of patchy necrosis of tubular epithelial cells with segmental loss of epithelium (Figure 8). Affected tubules were filled with pink amorphous material probably representing cytoplasmic debris from the disintegrating epithelium. Pycnotic nuclei were common in the affected tubules. The lesions, while easily recognized were not considered to be either sufficiently extensive or long standing to explain increased water consumption. Both mice died per-acutely, literally "in their tracks," (see Fig. 5). This fact, coupled with other anatomic indications of a shock syndrome (contracted spleen, acute ischemic myocardial necrosis) make it likely that the mice died as a consequence of water

deprivation. Dehydration can produce focal tubular necrosis similar in character, degree and distribution to the lesions described. Sequential sacrifice and perfusion fixation of mice from the twice weekly exposure group will clarify the frequency and extent of this lesion.

In spite of the possible contribution of skin damage to water loss and the potential role of dehydration in the pathogenesis of renal lesions noted in this study, similar renal lesions were noted by Carpenter et al.[6] in rats exposed by inhalation to certain petroleum solvents. Human contact exposures to diesel fuel have also been associated with acute renal failure on at least two occasions.[7,8] These observations, combined with the present experience, increase the likelihood that "middle distillates" are, at least potentially, nephrotoxic. The basis for nephrotoxicity may vary depending upon the route of exposure, dose rate and chronicity. If exposures are massive, direct effects are more likely. Alternatively, chronic dermal exposures may sufficiently alter skin permeability both to body water as well as toxic materials. In an animal as small as the mouse, a small percentage change in skin permeability could have a large effect upon water requirements given the proportionately high surface to volume ratios involved.

Several reports have appeared that describe the acute toxicity of petroleum and shale oil derived military fuels. Smith et al. (this volume) report a low level of systemic toxicity. Cowan et al.[9] reported LD_{50}'s for petroleum and shale oil derived JP5 and DFM. These authors reported that shale oil products were slightly more toxic than petroleum derived materials. JP5 inhalation for 120 days caused no unusual hematologic or histopathologic changes in rats and guinea pigs. Neurobehavioral effects

have also been identified in a case-control study of workers exposed to petroleum base jet fuel.[10]

In a study of the chronic toxicity of JP4 jet fuel, Kinkead et al.[11] reported no marked toxic effect in mice, rats, dogs and monkeys exposed repeatedly to 2.5 or 5.0 mg JP-4 vapor/L for 6 hours/day, 5 days/week. However, a slight increase in tumor incidence in mice, activity depression in dogs and monkeys, enhanced erythrocyte fragility in dogs and increased organ weights in the rat led the authors to recommend an occupational exposure limit not to exceed 2.5 mg vapor/L.

In summary, it would appear that the principal toxiologic concern associated with chronic dermal exposure to Paraho Shale Oil Crude, the Hydrotreated Crude and the Hydrotreated Residue is skin tumor induction. For distillates in the boiling range of those in the current study the probability of skin tumor induction is very low; however, the potential for local irritation and systemic toxicity is greater than with the crude materials.

REFERENCES

1. M. A. Pike, "A Suggested Method of Analysis of a Certain Class of Experiments in Carcinogenesis." Biometrics 22, 142-161 (1966).

2. R. Peto and P. N. Lee, "Weibull Distributions for Continuous-Carcinogenesis Experiment." Biometrics 29, 457-470 (1973).

3. R. Peto, P. N. Lee, and W. S. Paige, "Statistical Analysis of the Bioassay of Continuous Carcinogens," Br. J. Cancer 26, 258-261 (1972).

4. J. M. Holland, D. G. Gosslee and N. J. Williams, "Epidermal Carcinogenicity of Bis(2,3-epoxycyclopentyl)ether, 2,2-Bis(p-glycidyloxyphenyl)-propane, and mPhenylenediamine in Male and Female C3H and C57BL/6 Mice," Cancer Res. 39, 1718-1725 (1978).

5. H. Pinkus, "Keratosis Senilis: A Biologic Concept of Its Pathogenesis and Diagnosis Based on the Study of Normal Epidermis and 1730 Seborrheic and Senile Keratoses," Am. J. Clin. Path. 29, 193-207 (1958).

6. C. P. Carpenter, E. R. Kinkead, D. L. Geary, L. J. Sullivan and J. M. King, "Petroleum Hydrocarbon Toxicity Studies. VI. Animal and Human Responses to Vapors of '60 Solvent'," Toxicol. and Appl. Pharmacol. 34, 374-394 (1975).

7. A. M. T. Barrientos. T. Ortuno, J. M. Morales, F. Martinez-Tello and J. L. Rodicio, "Acute Renal Failure After Use of Diesel Fuel as Shampoo," Arch. Intern. Med. 137, 1217 (1977).

8. A. J. Crisp, A. K. Bhalla and B. I. Hoffbrand, "Acute Tubular Necrosis After Exposure to Diesel Oil," British Med. J. 2, 177 (1979).

9. M. J. Cowan, L. J. Jenkins, Jr., L. E. Doptis and R. W. Young, "Evaluation of Comparative Toxicity and Shipboard Hazards of Selected Petroleum and Oil-Shale-Derived Fuels," Oil Shale Symposium Proceedings Vol. II, 1978, pp. 21-31.

10. B. Knave, B. A. Olson, S. Elofsson, F. Gamberale, A. Isaksson, P. Mindus, H. E. Persson, G. Struwe, A. Weinberg and P. Westerholm, "Long Term Exposure to Jet Fuel. II. A Cross-Sectional Epidiologic Investigation on Occupationally Exposed Industrial Workers with Special Reference to the Nervous System," Scand. J. Work Environ. Hlth. 4, 19-45 (1978).

11. E. R. Kinkead, L. C. DiPasquale, E. H. Vernot and J. D. MacEwen, "Chronic Toxicity of JP-4 Jet Fuel," Proceedings of 5th Annual Conf. Environ. Toxicol., ISS AMRL-TR74-125, 1974, pp. 145-154.

Table I. Experimental Design

Material (Repository No.)	Concentrations (w/v%)		No. of Mice/Dose		Total No. of Mice at Risk
	3/wk	2/wk	3/wk	2/wk	
Crude shale oil (4601)	50	50,25,12	30	20	90
Hydrotreated shale oil (4602)	50	50,25,12	30	20	90
Hydrotreated residue (4607)	50	50,25,12	30	20	90
Shale oil JP5 (4608)	100,50	100,50,25,12	30	20	140
Shale oil JP8 (4609)	100,50	100,50,25,12	30	20	140
Shale oil DFM (4610)	100,50	100,50,25,12	30	20	140
Petroleum JP5 (4614)	100,50	100,50,25,12	30	20	140
Petroleum JP8 (4615)	100,50	100,50,25,12	30	20	140
Petroleum DFM (4616)	100,50	100,50,25,12	30	20	140
Benzo(a)pyrene	0.1,0.05,0.025	-	30	-	90
Cyclohexane	100	-	30	-	60

Table II. Skin Tumor Frequency in Mice Exposed Either 3 Times or 2 Times Weekly to Graded Doses of Shale Oil Crude, Hydrotreated Shale Oil or Hydrotreated Residue for 10 Months

Material	Concentration (w/v%)	Percent with Tumor 3/wk	Percent with Tumor 2/wk	Average Time to 1st Tumor (days+S.E.) 3/wk	Average Time to 1st Tumor (days+S.E.) 2/wk
Shale oil crude	50	93	75	206 (6)	261 (11)
	25	nt[a]	35	-	271 (18)
	12.5	nt	10	-	278 (52)
Hydrotreated shale oil	50	46	5	228 (12)	280
	25	nt	0	-	-
	12.5	nt	0	-	-
Hydrotreated residue	50	70	20	192 (11)	270 (26)
	25	nt	0	-	-
	12.5	nt	0	-	-
Benzo(a)pyrene	0.1	100	nt	169(6)	-
	0.05	100	nt	215 (4)	-
	0.025	67	nt	254 (4)	-

[a]Not tested.

Table III. Relative Skin Carcinogenic Potencies of Shale Oil Crude, Hydrotreated Crude and Hydrotreated Residue on the Basis of 10 Months Exposure

Material	Dose Rate	Relative Potency	Upper 95% Confidence Limit
Shale oil crude	3/wk	1/998	1/425
Shale oil crude	2/wk	1/1698	1/713
HDT-Shale oil	3/wk	1/2780	1/1086
HDT-Shale oil	2/wk	1/8974	1/2851
HDT-Residue	3/wk	1/1622	1/664
HDT-Residue	2/wk	1/4634	1/1722

Table IV. Average Body Weight of Mice Exposed to Shale Oils and Distillates 3 Times Weekly for a Minimum of 30 Weeks

Material	Concentration (w/v %)	Average Body Wt. (gms. ± S.E.)
Shale oil crude	50	31.6 ± 0.9
HDT Shale oil	50	31.8 ± 0.9
HDT Residue	50	31.9 ± 1.3
JP5 Product	100	30.6 ± 0.5
JP8 Product	100	28.7 ± 1.0[a]
DFM Product	100	29.6 ± 0.9
Petroleum JP5	100	30.3 ± 0.6
Petroleum JP8	100	30.3 ± 0.7
Petroleum DFM	100	30.4 ± 0.7
Cyclohexane	100	31.5 ± 0.8

[a]Significant at the 0.05 level.

Table V. Average Daily Water Consumption in Mice Exposed to Shale Oils and Distillates for a Minimum of 30 Weeks

Material	3/week Exposures Concentrations: 100	50% (w/v)	2/week Exposures Concentrations: 100	50	25	12% (w/v)
Shale oil crude	nt[a]	nt	-	5.1(0.3)	4.9(0.1)	5.1(0.1)
HDT Shale oil	nt	nt	-	5.0(0.1)	4.7(0.2)	4.7(0.2)
HDT Residue	nt	nt	-	4.8(0.3)	4.0(0.1)	4.8(0.3)
JP5 Product	9.9(0.3)[b,c]	7.3(0.3)	8.3(0.3)	6.6(0.1)	5.7(0.3)	4.7(0.1)
JP8 Product	9.4(0.4)[c]	7.3(0.3)	8.2(0.2)	6.7(0.3)	5.7(0.3)	5.1(0.2)
DFM Product	10.9(0.3)[c]	7.8(0.2)	9.2(0.4)	6.9(0.2)	6.1(0.3)	5.0(0.1)
Petroleum JP5	10.4(0.4)[c]	7.3(0.2)	8.1(0.1)	6.7(0.3)	5.1(0.1)	4.4(0.1)
Petroleum JP8	10.2(0.3)[c]	7.5(0.1)	8.2(0.1)	6.8(0.1)	5.4(0.3)	4.9(0.2)
Petroleum DFM	10.2(0.3)[c]	7.7(0.2)	9.3(0.2)	6.8(0.2)	5.4(0.1)	5.3
Cyclohexane	5.1(0.1)	-	-	-	-	-

[a]Not tested. [b]Average daily water intake in ml/mouse/day (± S.E.).
[c]Significant at the 0.001 level.

Table VI. Water Balance in Mice Exposed Chronically to Petroleum and Shale Oil Derived Middle Distillates

Treatment	Determinations	Average Water Intake (ml)	Average Urine Output (ml)	Average Fecal Weight (gm)
Cyclohexane	3	3.7	1.3	1.1
DFM Product	3	7.7	1.6	1.3
Petroleum DFM	2	9.0	2.4	1.5

FIGURE CAPTIONS

Figure 1. Proportion of mice surviving without a skin tumor following three times weekly application of benzo(a)pyrene at 0.15 (□), 0.075 (Δ) and 0.035 (X) mg per week. Solid lines refer to the Kaplan-Meier estimates and the dashed lines the fitted Weibull.

Figure 2. Proportions of mice surviving without skin tumor following application of shale oil crude on a three times weekly schedule at 75 (□) mg per week and 50 (Δ), 25 (X) and 12 (∇) mg per week, twice weekly.

Figure 3. Proportion of mice surviving without skin tumor following application of the hydrotreated shale oil on a three times weekly schedule at 75 (□) mg per week and 50 mg (Δ) per week, twice weekly.

Figure 4. Proportions of mice surviving without skin tumor following application of the hydrotreated shale oil residue on a three times weekly schedule at 75 (□) mg per week and 50 mg (Δ) per week, twice weekly.

Figure 5. This mouse was one of two found dead in an upright and normal position in a group that had been exposed chronically to natural petroleum derived JP-5 (100% three times weekly). The exposed skin over the dorsal thorax is thickened, scaling and deeply pigmented.

Figure 6. A photomicrograph of the skin of this mouse reveals an excess of amorphous keratin covering a hyperplastic epidermis. Hair follicles are also hyperplastic and sebaceous glands absent. The follicle lumina are filled with keratin, hair fragments and, occasionally, collections of neutrophil leukocytes. The dermis contains scattered aggregates of melanin which also is observed at the dermal-subcutaneous boundary.

Figure 7. Dilated and congested capillaries are noted in the superficial dermis (arrows). Intraepithelial foci of inflammatory cells are also apparent.

Figure 8. Renal corticomedullary junction. Tubular epithelium was undergoing necrosis as reflected by an increased staining intensity and disintegration. Nuclei in affected tubule segments were infrequent and often pyknotic. Degenerating tubules were also dilated and occluded by cellular debris (arrows).

Fig.1

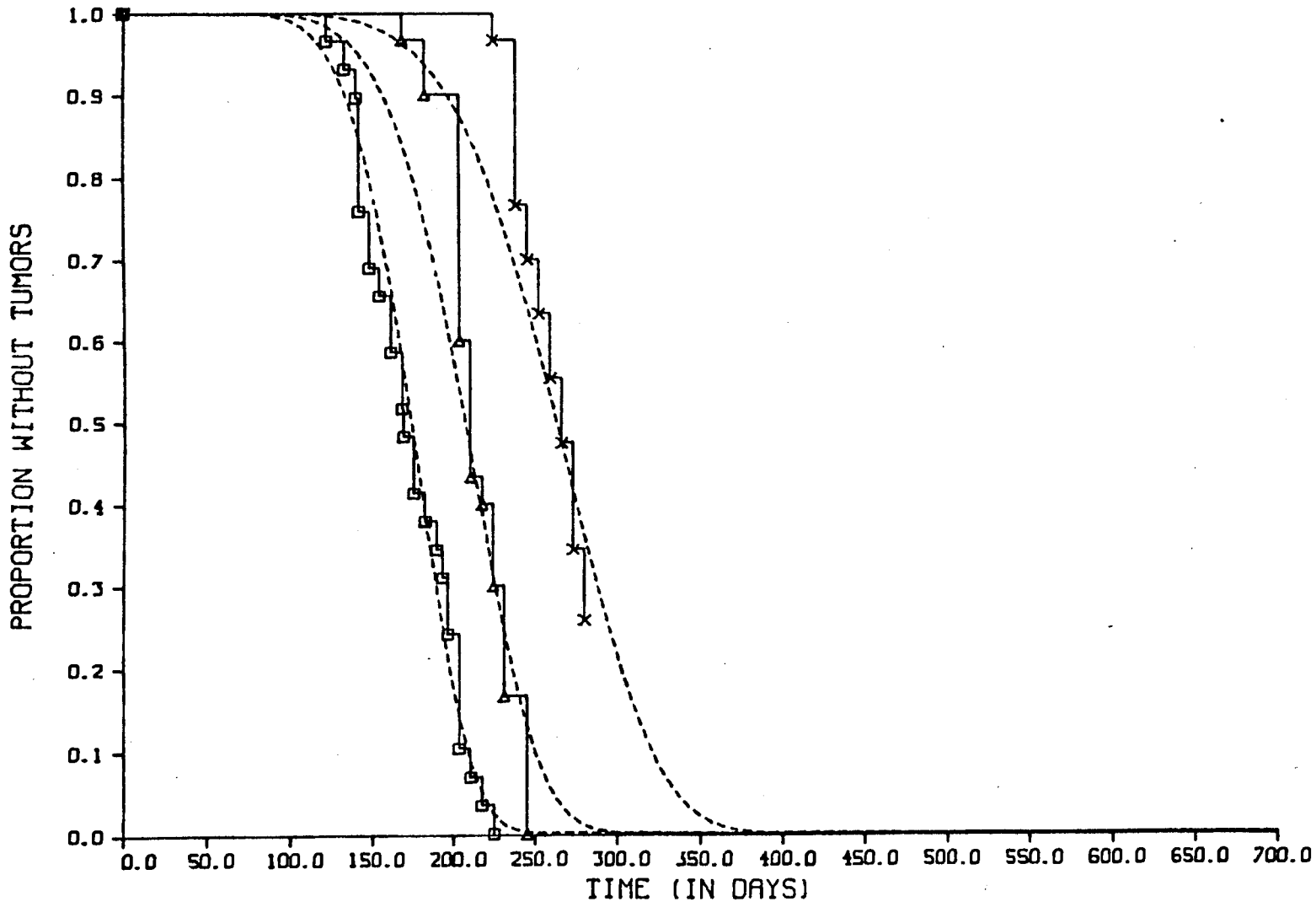

PROPORTION WITHOUT TUMORS
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0
450.0
500.0
550.0
600.0
650.0
700.0
TIME (IN DAYS)

Fig.2

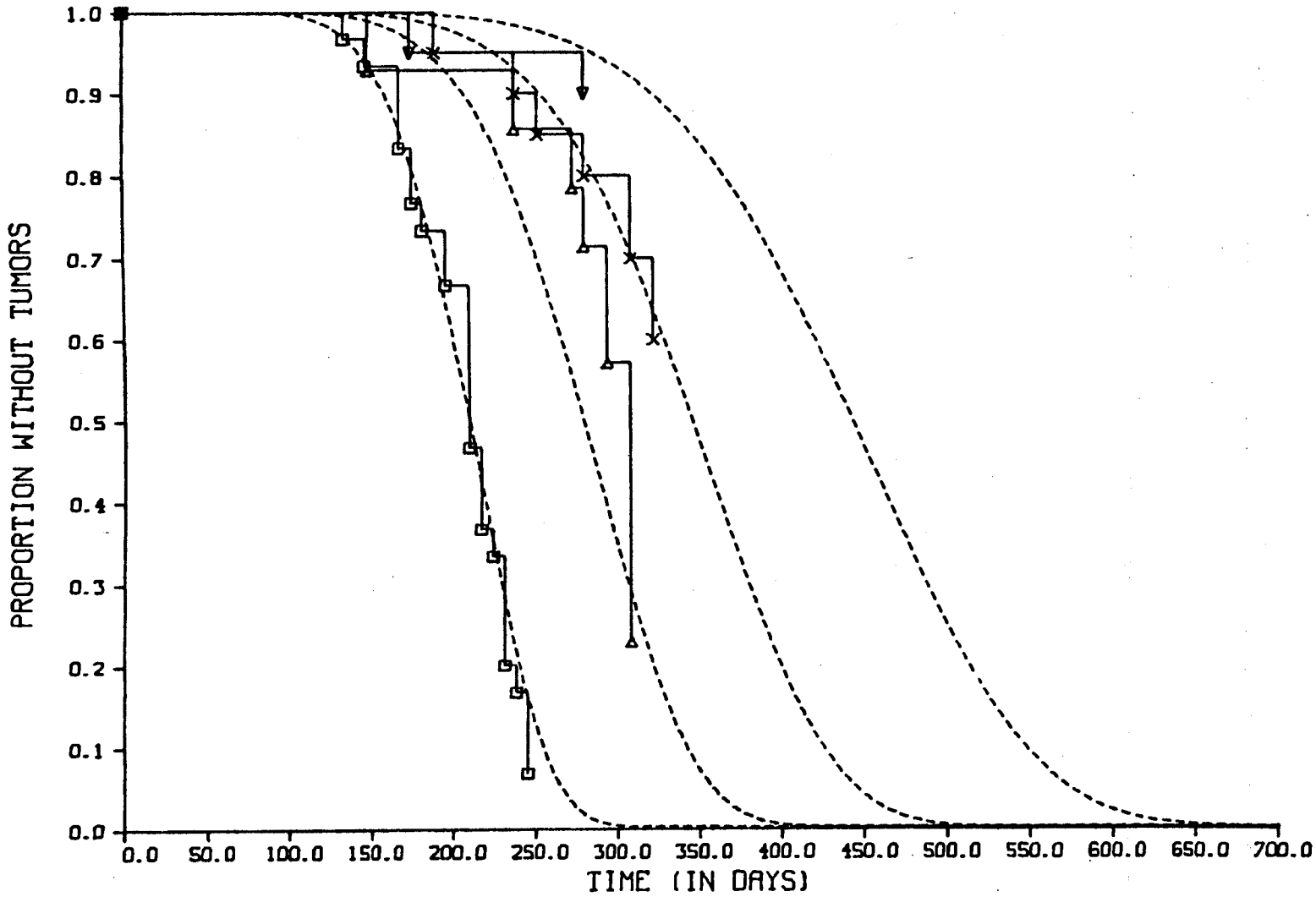

PROPORTION WITHOUT TUMORS
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0
450.0
500.0
550.0
600.0
650.0
700.0
TIME (IN DAYS)

Fig.3

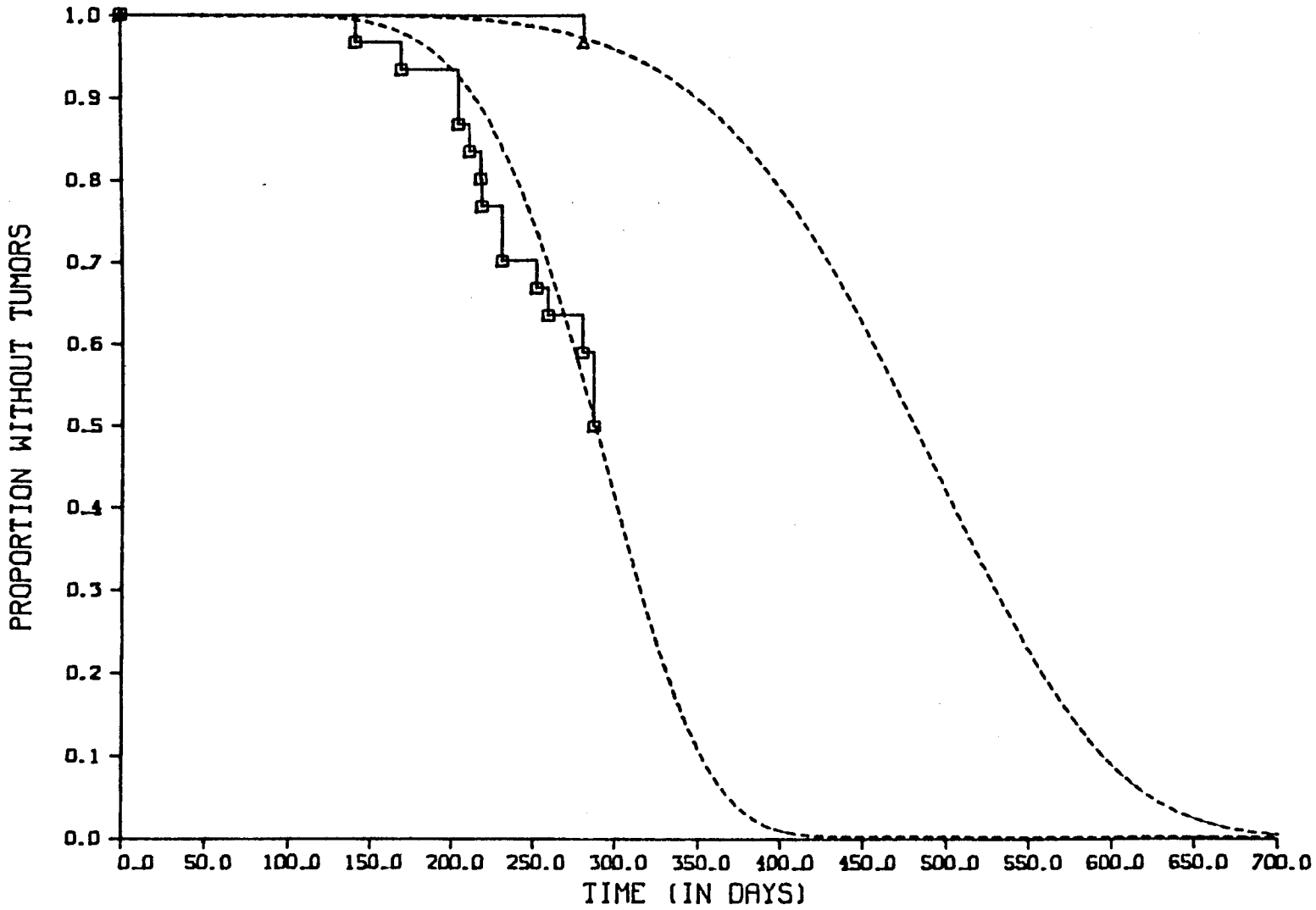
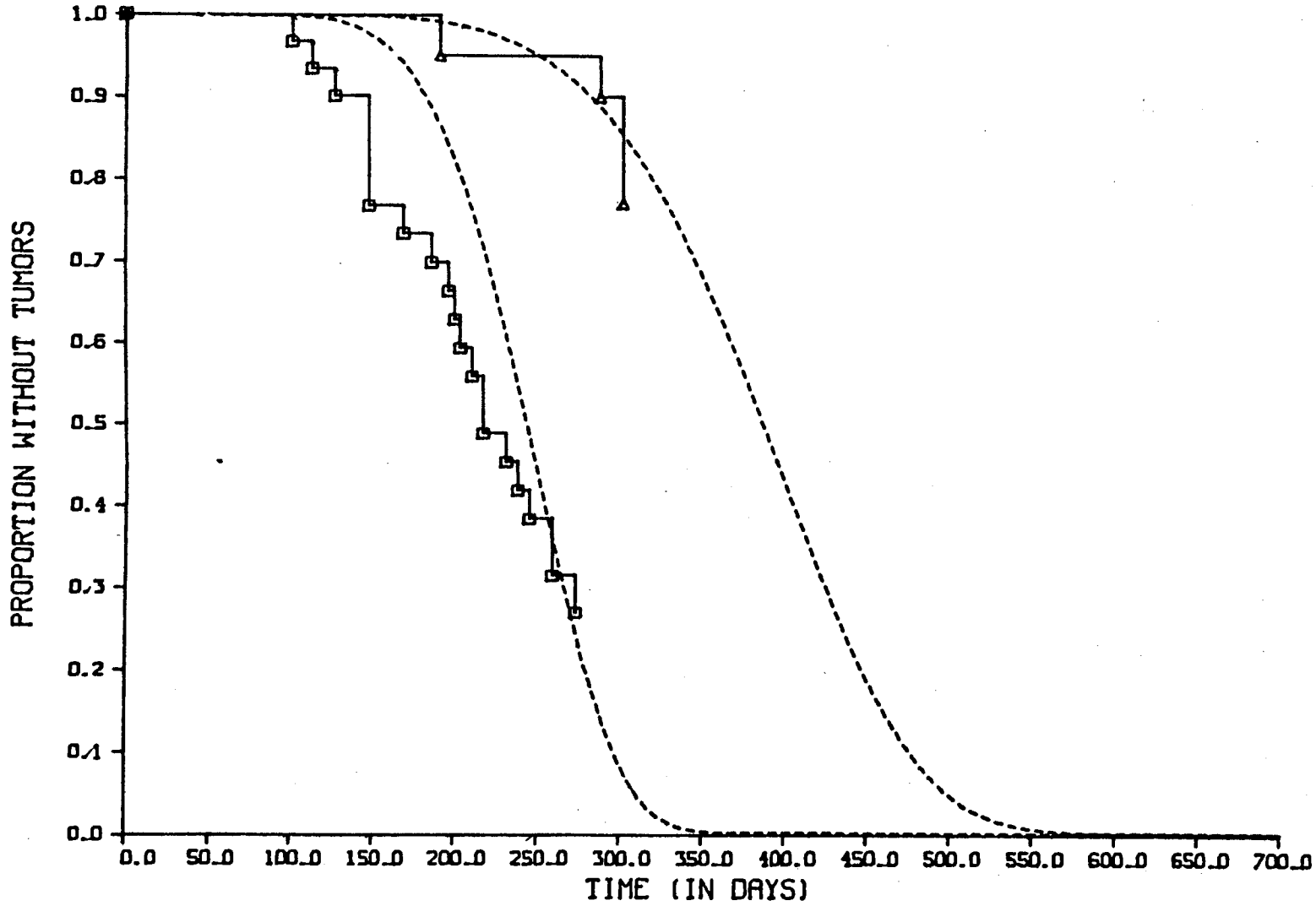
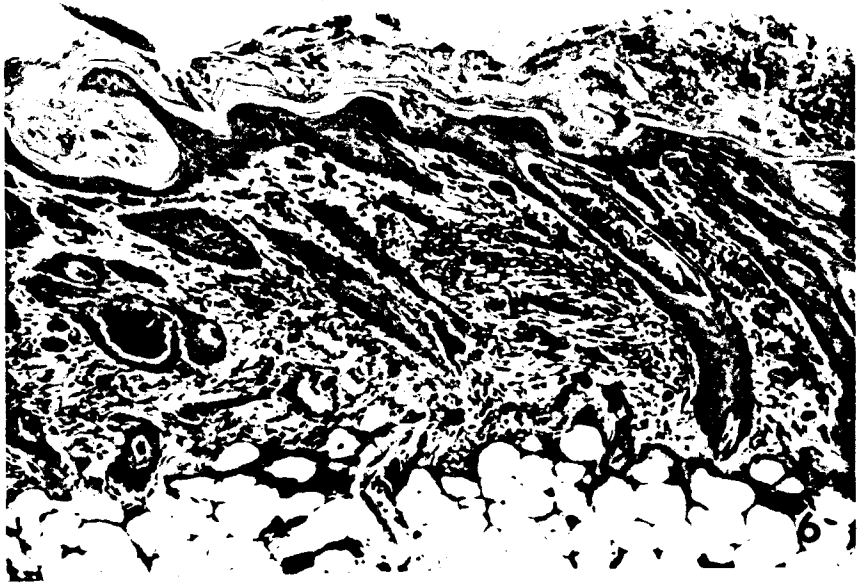

PROPORTION WITHOUT TUMORS
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0
450.0
500.0
550.0
600.0
650.0
700.0
TIME (IN DAYS)

Fig.4


PROPORTION WITHOUT TUMORS
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.0
50.0
100.0
150.0
200.0
250.0
300.0
350.0
400.0
450.0
500.0
550.0
600.0
650.0
700.0
TIME (IN DAYS)

5



6

6

In Press:

Environ. Health Perspect.

Relative Potency Estimation for Synthetic Petroleum Skin Carcinogens

by J. M. Holland,* D. A. Wolf,† and B. R. Clark‡

By acceptance of this article, the publisher or recipient acknowledges the right of the U.S. Government to retain a nonexclusive, royalty-free license in and to any copyright covering the article.

---

*Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.

†Computer Sciences Division, Union Carbide Corporation, Oak Ridge, Tennessee 37830.

‡Analytical Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.

Research supported by the Office of Health and Environmental Research, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

Send proof to: Dr. J. M. Holland
Biology Division
Oak Ridge National Laboratory
Post Office Box Y
Oak Ridge, Tennessee 37830

KEY WORDS - skin cancer potency, petroleum hydrocarbons

The skin carcinogenic potencies of three synthetic petroleums and one composite natural petroleum were established relative to benzo(a)pyrene by repeated dermal application of the materials for 24 months. The question of what chemical class is most responsible for skin carcinogenicity was addressed in two ways: following the chemical class fractionation of each crude, the weight percent polynuclear aromatic (PNA) material was determined gravimetrically. Each crude was then diluted with a solvent so that the dose rate of PNA material was normalized for all materials. The second method involved application of graded dosages of the PNA fraction alone, after its separation from the parent mixtures.

Based upon comparison of dose-response relationships, all three of the synthetic fossil liquids were tumorigenic in mouse skin, with relative potencies ranging from 1/500 to 1/1400 the activity of an equivalent dosage of benzo(a)pyrene. Under the dose rate and exposure conditions used in this study, a composite sample of five natural petroleums failed to elicit skin tumors.

That PNA-related materials or compounds recovered in this fraction were responsible for most, if not all, the activity represented in the parent material was suggested by the fact that the potency for the isolate

was greater than or equal to that of its respective crude. Failure of the normalization of PNA concentration to equalize skin tumorigenicity of the crudes is most readily explained on the basis of quantitative and qualitative differences in the chemical composition of this fraction.

Introduction

The number of chemical and physical agents that are capable of evoking skin neoplasms is, for all practical purposes, limitless. Since many of these known or potential oncogens are valuable commodity chemicals, it is essential that methods be developed to rank them in order of carcinogenic potency. While we are in no position to judge the level of potency at which risk to human beings becomes unacceptable, we feel that an accurate means for estimating relative potency is an essential first step in this process.

This reportillustrates the approach we are using to obtain relative potency estimates for various synthetic and natural petroleum products applied to the skin. Groups of genetically homogeneous inbred mice are exposed repetitively to serial dilutions of the test material. In the simplest case, observation establishes time to appearance of a clinically typical epidermal neoplasm or to death of the animal before it develops a tumor. A Weibull distribution is fit to the time to tumor appearance for each material—dose combination. Tumorigenic efficiency of a combination is then measured by the logarithm of the estimated Weibull scale parameter. The Weibull model is preferred over other alternatives because it has been shown to fit a wide variety of time to tumor data (1—4).

To relate skin tumorigenic potencies for different materials and mixtures tested at the same or different times, we include a reference carcinogen standard with each series of unknowns; this choice is arbitrary. For our purposes we have selected benzo(a)pyrene (BP), which is

commercially available in high purity, chemically stable, and a highly efficient mouse skin carcinogen.

For estimation of relative potencies, the Weibull scale parameters are assumed to be a linear function of dose for each material on a log-log scale; dose responses for materials in the same strain are also assumed to be parallel. These assumptions appear to be reasonable in this and in previous experiments (2, 5). The potency of an unknown material relative to the reference carcinogen is estimated by the ratio of dosages required to elicit comparable oncogenic effects.

Materials and Methods

Animal Exposure

Groups consisting of 25 male and 25 female C3Hf/Bd mice were exposed to graded dosages of synthetic petroleums, their respective polynuclear aromatic (PNA) fractions, reference standard BP, and a vehicle control consisting of 70% acetone and 30% cyclohexane by volume. Exposures were to the shaved dorsal skin in a volume of 50 µl commencing at 10 weeks of age and continuing for 24 months or until death. The materials used and absolute amounts applied are given in Table 1. Details concerning the source and history of the crude materials are given in Table 2. The natural petroleum sample consisted of six separate components, whose volume percents are given in Table 2.

Mice were treated three times weekly, and at each exposure were examined for evidence of skin neoplasia arising in the area directly exposed to the test material. When grossly typical skin neoplasms were observed, the exact date of initial observation was recorded. Only animals

in which the initial tumor grew progressively were included in the analysis. When an animal died or was killed at the end of an experiment, the skin tumors were excised and the animal was subjected to gross necropsy for detection of internal lesions and evidence of regional metastasis of skin neoplasms.

The observations, consisting of an animal identification number, birth date, date of initial treatment, and observation of initial skin neoplasm (if any), and death or sacrifice date, were coded and verified. Two computer programs provided by the National Cancer Institute were used to estimate the statistical distribution of time to tumor observation for each material—dose combination (6). One program fits the Weibull distribution (D. G. Thomas, personal communication), and the other obtains the Kaplan-Meier nonparametric estimate of the distribution. Each program utilizes the information on animals that die or are killed before a treatment-related tumor is observed.

Dosage selection

These experiments were designed to evaluate the relative contributions of PNA components of a fossil liquid as determining factors in skin tumor induction. If additivity of the effect of active components is assumed, it should be possible to correlate skin carcinogenicity with the amount and composition of the PNA fraction.

To accomplish this we determined the weight percent of the crude contributed by the PNA fraction. Details concerning the methods used and results for the remaining chemical class fractions of these materials can be found elsewhere (7–9). Gravimetrically determined percent "PNA

materials" were 5.1, 2.0, 6.0, and 2.6% for materials A, B, C, and D, respectively. Under the assumption that the PNA fraction of each crude was equivalent in potency to BP, we set the weekly exposure for each whole crude at a level equivalent to 0.15, 0.03, and 0.006 mg of BP. Since each crude differed in the amount of material recovered as the PNA fraction, the doses differed accordingly. To compare the oncogenic potency of the whole material with that of its respective PNA isolate and to compare isolates with each other, we treated separate groups with equal quantities of each isolate.

Results and Discussion

Skin Tumor Dose Responses

The skin tumor data were analyzed separately for each sex; however, significant ($p < 0.05$) differences were not observed, thus estimations and comparisons were made after the data were pooled. Skin tumors were induced in groups exposed to A, B, C, AN, BN, and BP (Table 3). At the very low rates used in this study, skin tumors were not induced in mice exposed to natural petroleum or its PNA fraction. Neither were skin tumors observed in mice exposed to the PNA isolate derived from the COED process hydrotreated coal liquid. On this basis we conclude that the PNA compositions of these materials differ considerably, a conclusion amply illustrated by chemical analysis (7–9). Mean time to tumor is also given in Table 3, but very little can be concluded from this summary, primarily because so few responses were obtained in many groups.

Mortality

Over the 24-month duration of the experiment, differences in mortality rate between treated and vehicle control mice were also noted in mice exposed to whole crudes. A chi-square and exact P value (Cox's test) were used for the comparison of treated with vehicle control mice. The results are given in Table 4. Mortality significantly greater than in controls ($p \leq 0.05$) occurred in groups A and B at the highest dosage and in group C at next to the lowest dosage. Mortality in BP-treated mice is reported at the lowest dosage only, since the two higher dosage groups were killed at 250 and 360 days of exposure, respectively. With the exception of the 0.5-mg dose of C, mortality significantly greater than in controls occurred in groups with a high incidence of skin tumor. The most likely interpretation of this observation is that skin tumors were lethal, as a result of either metastasis, toxemia, or both. There was no clear pattern of systemic toxicity induced by chronic dermal application of these liquid hydrocarbons.

Estimation of Time-to-Tumor Distributions

The time-to-tumor distributions provide a clear picture of the tumorigenic effects of the various treatments by illustrating the patterns of tumor occurrences. Furthermore, if a particular parametric form for the distribution is assumed, relative potency estimates can be calculated. The distribution of the time to tumor is estimated parametrically and nonparametrically by methods that fully utilize the information in both the uncensored and censored observations. ("Censored observations" represent

mice that die from natural or accidental causes or are killed at the end of the study before a treatment-related skin tumor is induced.)

The nonparametric estimates are calculated by the Kaplan-Meier method (10). With unique observation times and no censoring, the Kaplan-Meier estimate is a step function starting at 1 at the first exposure time and decreasing at each of the ordered times to tumor by increments of the reciprocal of the number of animals in the group. When the observation times are not unique and there is censoring, the step size is modified appropriately, so that steps occur only when new skin-tumor-bearing animals are observed.

The Kaplan-Meier estimates were used to visually compare dose groups for each material without assumption of a distribution for the data. These curves are represented in Figures 1–4 by solid lines. To assess the explicit dose-response relationship and, subsequently, to compare these estimates for purposes of establishing relative potency, we have assumed a parametric form for the distribution. The model we have chosen is the three-parameter Weibull model, under which the survivor function is $\exp[-b(t - w)^k]$, where $t>w$, $b>0$, $k>0$. The parameters k, w, and b are the shape, location, and scale parameters, respectively, and t is the time (days), to initial observation of a skin tumor. The survivor function gives the probability that the time to tumor exceeds t; w being a minimum latency period before which no tumor can occur. Pike (1) suggested that for a particularly pure strain of animal the k and w would be independent of the treatment. Maximum likelihood estimates of a common k and a common w for all groups in which two or more skin tumors were observed gave values of 5.97 for k and 43.4 for w. Using these values for k and w, it was

possible to estimate the parameter b for each material—dose combination. The estimated Weibull time distributions for skin tumors are also shown in Figures 1—4 by dashed lines superimposed over the corresponding Kaplan-Meier nonparametric estimates. The observed agreement between these two methods is remarkable given the wide variation in tumor frequencies and temporal distributions across the different treatment groups.

Estimation of Relative Potency

The calculated scale parameter b, derived from the Weibull fit to each material—dose combination, is used to obtain estimates of relative carcinogenic potency for materials tested in a common inbred mouse strain under reasonably stable and reproducible environmental conditions. A weighted linear regression of the logarithm of b on the logarithm of dose in mg/week is performed for each material for which responses were obtained in two or more dose groups. Weighting for each data point was the reciprocal of the number of tumors for that material—dose combination. The additional constraint that the regression lines be parallel for all test materials and the reference carcinogen, an assumption that appears to be reasonable when compounds are compared in the same strain, makes it possible to obtain estimates of relative potency, even when responses are limited to occurrences in one group or are low in frequency. Only data sets for materials with responses in two or more dose groups contribute to the common slope of the regression lines.

The dose-response lines for these materials and their respective tumor-inducing PNA fractions are shown graphically in Figure 5. Open circles correspond to the logs of the estimated b's, and squares are the

corresponding values on the regression lines. The potency of a syncrude relative to BP is the antilog of the absolute difference between the synthetic petroleum's log b intercept and the BP's log b intercept divided by the common slope, these values coming from the fitted regression lines shown in Figure 5. Approximate 95% confidence limits on the relative potencies were calculated by use of Fieller's theorem (11). For comparisons in which the unknown material is unlikely to be as potent as BP, only upper 95% confidence limits are provided. However, when there is no justification for this both the upper and lower limits are provided. By these procedures the carcinogenic potencies of the three syncrudes and their respective PNA isolates were determined relative to BP. The results are summarized in Table 5.

In the context of the original objectives of this experiment, these observations permit several conclusions: (1) Diluting the crude materials so that dosages of total PNA's were approximately equal failed to equilize the skin tumorigenic potency of the syncrudes, therefore factors other than PNA's were contributing to the response, or qualitative and quantitative differences in the composition of the PNA class affected the response. (2) None of the crudes were more than 1/500 as active as BP, and the single coal liquid that had been upgraded by hydrotreatment (COED syncrude) was 1/1400 as potent a skin carcinogen as an equivalent amount of BP. (3) The composite sample of natural petroleums, while containing materials recoverable in a PNA fraction, failed to elicit a measurable response under the conditions of this bioassay. (4) A 3-fold difference in the skin carcinogenic potency of this set of three active syncrudes was demonstrated. (5) The activity of PNA isolates was substantially greater

than that of the parent mixture. This is consistent with the view that skin carcinogenic principles present within these complex materials are concentrated within this chemical class.

Comparison of the potency of the parent mixture with that of the respective PNA isolate will reveal the likelihood that components present within this fraction determine the mixture's activity. Table 6 gives these comparisons for both synthoil and shale oil versus their respective PNA isolates. In this instance the relative potency of the synthoil crude is 1/4 that of its isolate, and shale oil is 1/27 as potent as its isolate. This suggests that components present within the PNA fraction are accountable for most of the skin carcinogenicity of the crude and that differences that persist after normalization reflect qualitative or quantitative differences in the composition of this fraction.

The question of whether differences in the composition of the PNA isolates were responsible for differences in the activity of the whole mixture could be addressed by comparing the potency of the two isolates. In this case the shale oil PNA fraction was observed to be 2.3 times more potent than the synthoil PNA fraction but the 95% confidence limits on this estimate were 0.3—22.3. Thus the data were inadequate to establish a statistically significant difference between these fractions.

This study provides a basis for comparison of other mixtures and chemical fractions of these mixtures with the current materials. We anticipate that as our quantitative data base expands for a wide variety of contemporary synthetic fossil liquids, it will become feasible to search for short term *in vitro* and *in vivo* biochemical correlates of both exposure and effect. If these efforts are successful, then an approach toward

direct estimation of human exposure risk may be developed with organ-cultured human skin or human skin grafts maintained on athymic nude mice.

REFERENCES

1. Pike, M. A. A suggested method of analysis of a certain class of experiments in carcinogenesis. Biometrics 22: 142-161 (1966).
2. Peto, R., and Lee, P. N. Weibull distributions for continuous-carcinogenesis experiments. Biometrics 29: 457-470 (1973).
3. Peto, R., Lee, P. N., and Paige, W. S. Statistical analysis of the bioassay of continuous carcinogens. Br. J. Cancer 26: 258-261 (1972).
4. Lee, P. N., and O'Neill, J. A. The effect both of time and dose applied on tumor incidence rate in benzopyrene skin painting experiments. Br. J. Cancer 25: 759-770 (1971).
5. Holland, J. M., Gosslee, D. G., and Williams, N. J. Epidermal carcinogenicity of bis(2,3-epoxycyclopentyl)ether, 2,2-bis(p-glycidyloxyphenyl)propane, and m-phenylenediamine in male and female C3H and C57BL/6 mice. Cancer Res. 39: 1718-1725 (1978).
6. Thomas, D. G., Breslow, N., and Gart, J. J. Trend and homogeneity analyses of proportions and life table data. Comput. Biomed. Res. 10: 373-381 (1977).
7. Guerin, M. R., Epler, J. L., Griest, W. H., Clark, B. R., and Rao, T. K. Polycyclic aromatic hydrocarbons from fossil fuel conversion processes. In: Carcinogenesis: Polyaromatic Hydrocarbons, P. W. Jones and R. I. Freudehthal, eds., Raven Press, New York, 1978, pp. 21-33.

8. Griest, W. H., Guerin, M. R., Clark, B. R., Ho, C., Rubin, I. B., and Jones, A. R. Relative chemical composition of selected synthetic crudes. In: Symposium on Assessing the Industrial Hygiene Monitoring Needs for the Coal Conversion and Oil Shale Industries, Brookhaven National Laboratory, Upton, New York, November 1978.

9. Rubin, I. B., Guerin, M. R., Hardigree, A. A., and Epler, J. L. Fractionation of synthetic crude oils from coal for biological testing. Environ. Res. 12: 358-365 (1976).

10. Kaplan, E. L., and Meier, P. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc. 53: 457-481 (1958).

11. Fieller, E. C. A fundamental formula in the statistics of biological assay, and some applications. Q. J. Pharm. Pharmacol. 17: 117-123 (1944).

Table 1. Materials tested for relative skin carcinogenicity

Material	Treatment group	Weekly dose,[a] mg
Synthoil crude	A	3.0,0.6,0.12,0.9
Shale oil crude	B	7.5,1.5,0.3,0.9
COED syncrude	C	2.5,0.5,0.1,0.9
Natural petroleum blend	D	6.0,1.2,0.24,0.9
Synthoil PNA isolate	AN	0.15,0.03,0.006
Shale oil PNA isolate	BN	0.15,0.03,0.006
COED PNA fraction	CN	0.15,0.03,0.006
Petroleum PNA fraction	DN	0.15,0.03,0.006
Benzo(a)pyrene	BP	0.15,0.03,0.006
Acetone–cyclohexane solvent control	S	0.15

[a]Applied in three increments on Monday, Wednesday, and Friday.

Table 2. Characteristics of materials tested for skin carcinogenicity

Material	Type	Specific gravity (g/cm^3)	Viscosity, (sec at 100°F)	Pour point, °F	Color	Weight % sulfer	Weight % nitrogen
Synthoil crude[a]	centrifuged crude product	1.136	80(180°F)	-	brownish-black	0.52	1.30
Shale oil crude[b]	centrifuged crude product	0.909	66	30	brownish-black	0.93	1.14
COED syncrude[c]	hydrotreated product oil	0.940	48	43	pale yellow	0.05	0.05
Wilmington, California[d] (10%)	natural	0.938	470	< 5	brownish-black	1.59	0.631
South Swan Hills, Alberta, Canada[d] (20%)	natural	0.826	37	< 5	brownish-green	0.11	0.056
Prudhoe Bay, Alaska[d] (20%)	natural	0.893	84	15	brownish-black	0.82	0.230
Gach Saran, Iran[d] (20%)	natural	0.880	72	< 5	brownish-black	1.57	0.226
Louisiana–Mississippi sweet[e] (10%)	natural	0.825	50	< 5	brownish-green	0.17	0.067
Arabian light[e] (20%)	natural	0.858	46	<30	brownish-black	1.80	0.1–0.2

continued on the next page

Table 2 continued

[a]Pittsburgh Energy Technology Center. From Run FB46 of western Virginia coal, Pittsburgh seam, Ireland mine; 1/2 ton/day, 24.5-ft catalytic reactor (450°C, 4000 psi), feed rate 25 lb/hr of 35% solid slurry in equilibrium coal-derived oil. Data provided by Nestor Mazzoco, Pittsburgh Energy Technolgoy Center.

[b]Laramie Energy Technology Center. Run No. 14, Colorado shale, Rifle, Colorado; Fisher assay 24.4 gal/ton from a 150-ton above-ground simulated *in situ* retort. Data provided by John McKay, Laramie Energy Technology Center.

[c]FMC Corporation, Princeton, New Jersey, from western Kentucky coal. Analysis is not based upon this specific sample but is valid for generically similar material; therefore, the data are prsented for comparison purposes only. Data provided by Dr. C. A. Hochwalt, Jr., Cogas Development Co., Princeton, New Jersey.

[d]Bureau of Mines routine crude oil analysis. Data provided by J. Dooley, Bartelsville Energy Technology Center, Bartlesville, Oklahoma. Numbers in parentheses refer to the volume percent of each component in the composite sample.

[e]Analysis is not based on these specific samples, therefore the data are approximate and given for comparison purposes only; data provided by J. Dooley. Numbers in parentheses as in footnote *a*.

Table 3. Summary of the skin tumor response obtained in mice exposed repetitively to synthetic fossil liquids

Material	Weekly dose, mg	No. with skin tumors	Mean time to tumor, days (±S.E.)
Synthoil crude	3.0	46	490 (12.7)
	0.9	13	569 (17.8)
	0.6	1	596
Shale oil crude	7.5	45	482 (14.8)
	1.5	1	315
COED syncrude	2.4	4	549 (45.8)
	0.9	1	494
	0.5	1	658
Synthoil PNA isolate	0.15	4	573 (38.9)
Shale oil PNA isolate	0.15	2	541 (55.5)
	0.03	1	485
	0.006	1	392
Benzo(a)pyrene	0.15	48	146 (2.6)
	0.03	49	216 (5.2)
	0.006	43	513 (14.3)
Solvent control	0.15	0	-

Table 4. Differences in survival rate between mice treated continuously with either fossil liquids or vehicle alone

Material	Weekly dose, mg	No. of deaths	Chi square	Exact *P* (Cox's test)
Synthoil crude	3.0	38	24.6	<0.01
	0.9	19	0.8	0.36
	0.6	18	0.8	0.37
	0.12	13	0.04	0.85
Shale oil crude	7.5	32	13.1	<0.01
	1.5	20	1.2	0.26
	0.9	20	1.7	0.20
	0.3	11	0.08	0.77
COED syncrude	2.5	22	2.4	0.12
	0.9	18	1.0	0.32
	0.5	23	3.8	0.05
	0.1	22	3.0	0.08
Petroleum blend	6.0	10	0.2	0.68
	1.2	14	0.02	0.89
	0.9	11	0.4	0.50
	0.24	15	0.02	0.89
Benzo(*a*)pyrene	0.006	28	9.1	<0.01
Solvent control	0.15	14		

Table 5. Skin carcinogenic potency of synthetic petroleums and PNA fractions relative to BP

Material	Relative potency	Upper 95% confidence limit
Synthoil crude	1/503	1/128
Shale oil crude	1/1380	1/275
COED syncrude	1/1440	1/263
Synthoil PNA isolate	1/119	1/27
Shale oil PNA isolate	1/51	1/13

Table 6. Comparison of the potency of the parent mixture relative to that of the respective PNA isolate

Comparison	Relative potency	95% Confidence limits
Synthoil	1/4	1/25—1/0/5
Shale oil	1/27	1/274—1/4

Figure Legends

FIGURE 1. Proportions of mice without tumor associated with continuous exposure to synthoil crude. Dosages were approximately 3.0 (□), 0.9 (0), and 0.6 (Δ) mg/week. Solid lines represent the Kaplan-Meier estimate; dashed lines, the Weibull fitted curve.

FIGURE 2. Proportions of mice without tumor associated with continuous exposure to shale oil at approximately 7.5 (□) and 1.5 (Δ) mg/week. Solid lines, Kaplan-Meier; dashed lines, fitted Weibull.

FIGURE 3. Proportions of mice without tumor associated with continuous exposure to COED process coal liquid at approximately 2.4 (□) and 0.9 (0) mg/week. Solid lines, Kaplan-Meier; dashed lines, fitted Welbull.

FIGURE 4. Proportions of mice without tumor associated with continuous exposure to BP at 0.15 (□), 0.03 (Δ), and 0.006 (0) mg/week. Solid lines, Kaplan-Meier; dashed lines, fitted Weibull.

FIGURE 5. The relationship between log b, a scale parameter obtained from the Weibull fits with a constant k and w for all comparisons, and the log dose rate. 0, log b from Weibull; □, weighted, linear-parallel, least-squares fitted values at each data point. Parallel lines drawn through the latter points are the basis for the calculation of relative potency. Treatment groups are identified by the letter codes in Table 1.

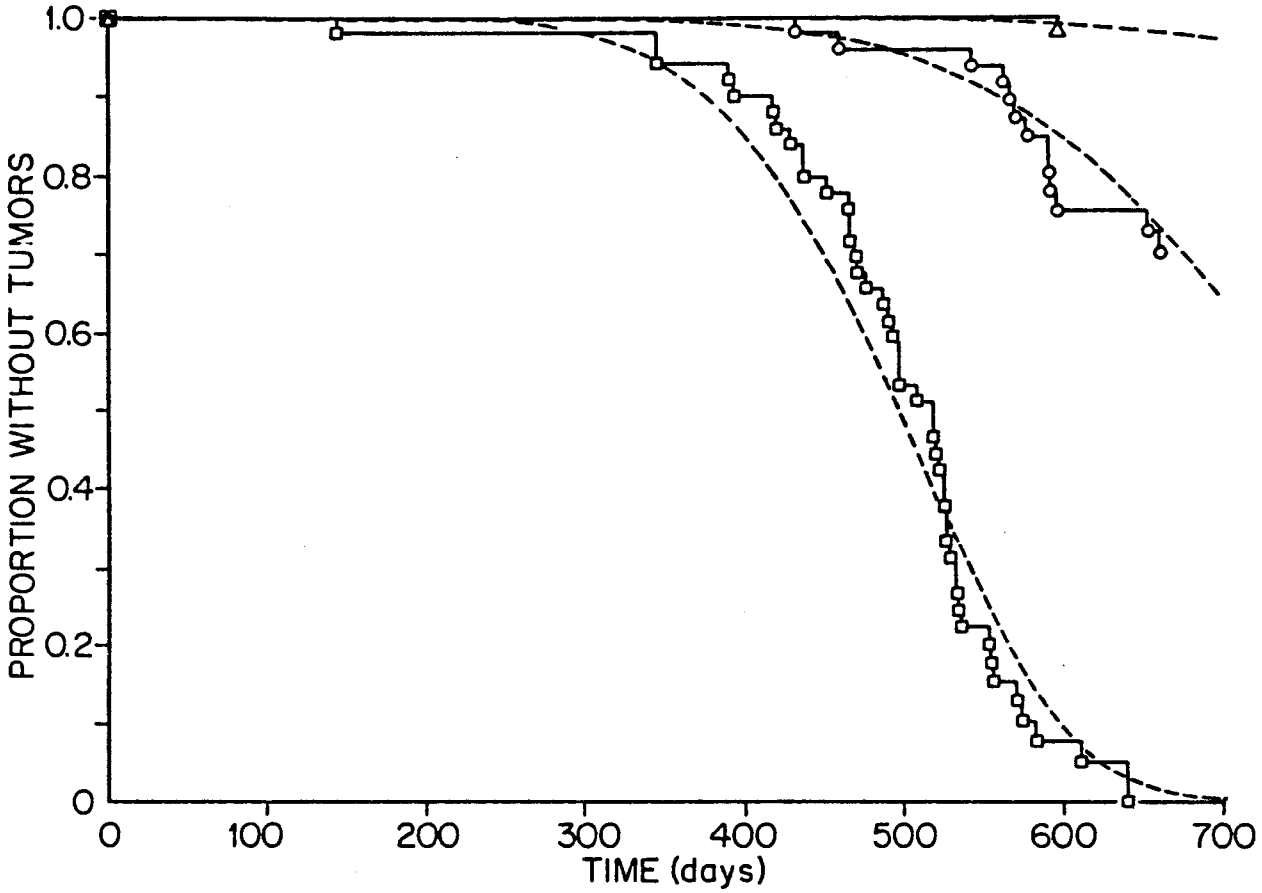

1.0
0.8
0.6
0.4
0.2
0
PROPORTION WITHOUT TUMORS
0
100
200
300
400
500
600
700
TIME (days)

Fig. 1

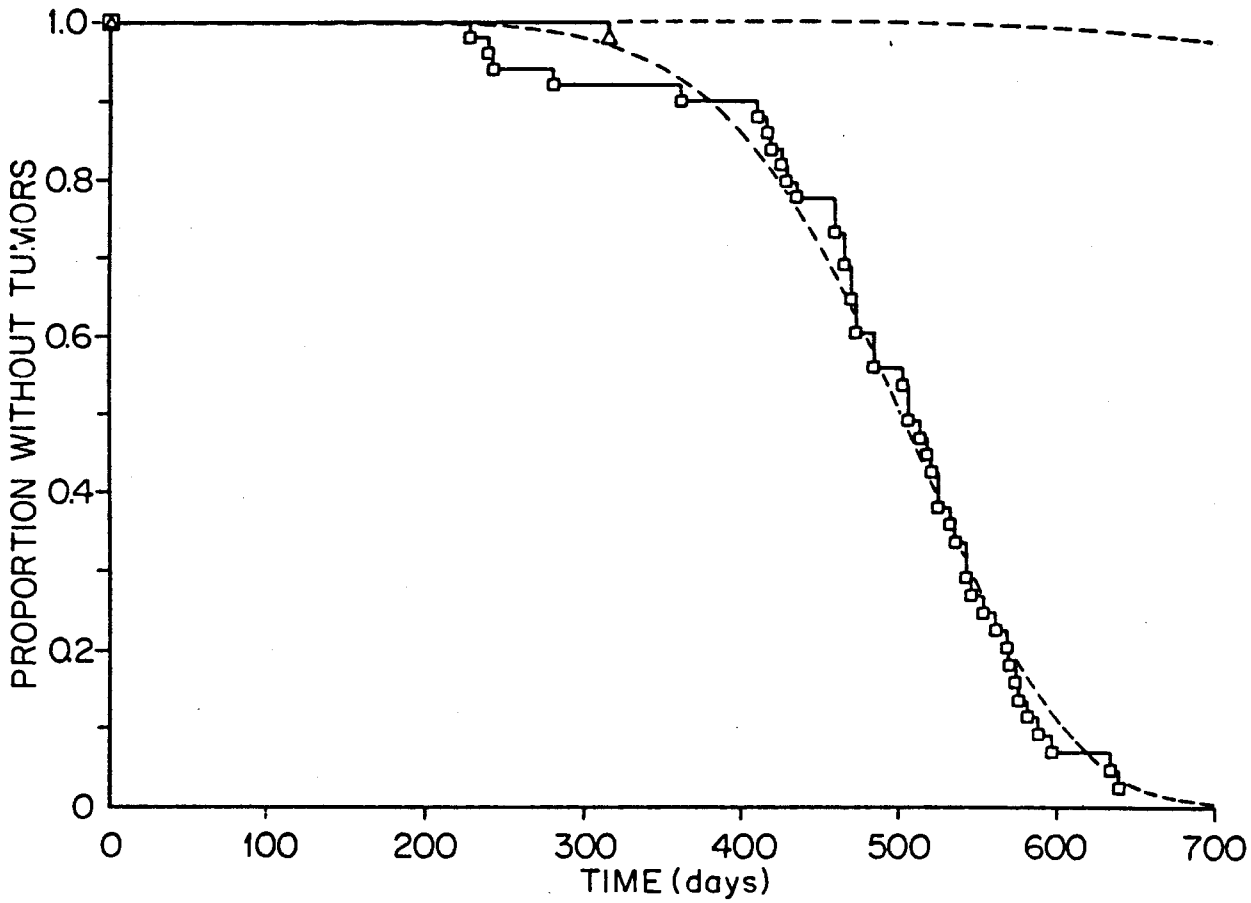

1.0
0.8
0.6
0.4
0.2
0
PROPORTION WITHOUT TUMORS
0
100
200
300
400
500
600
700
TIME (days)

Fig. 2

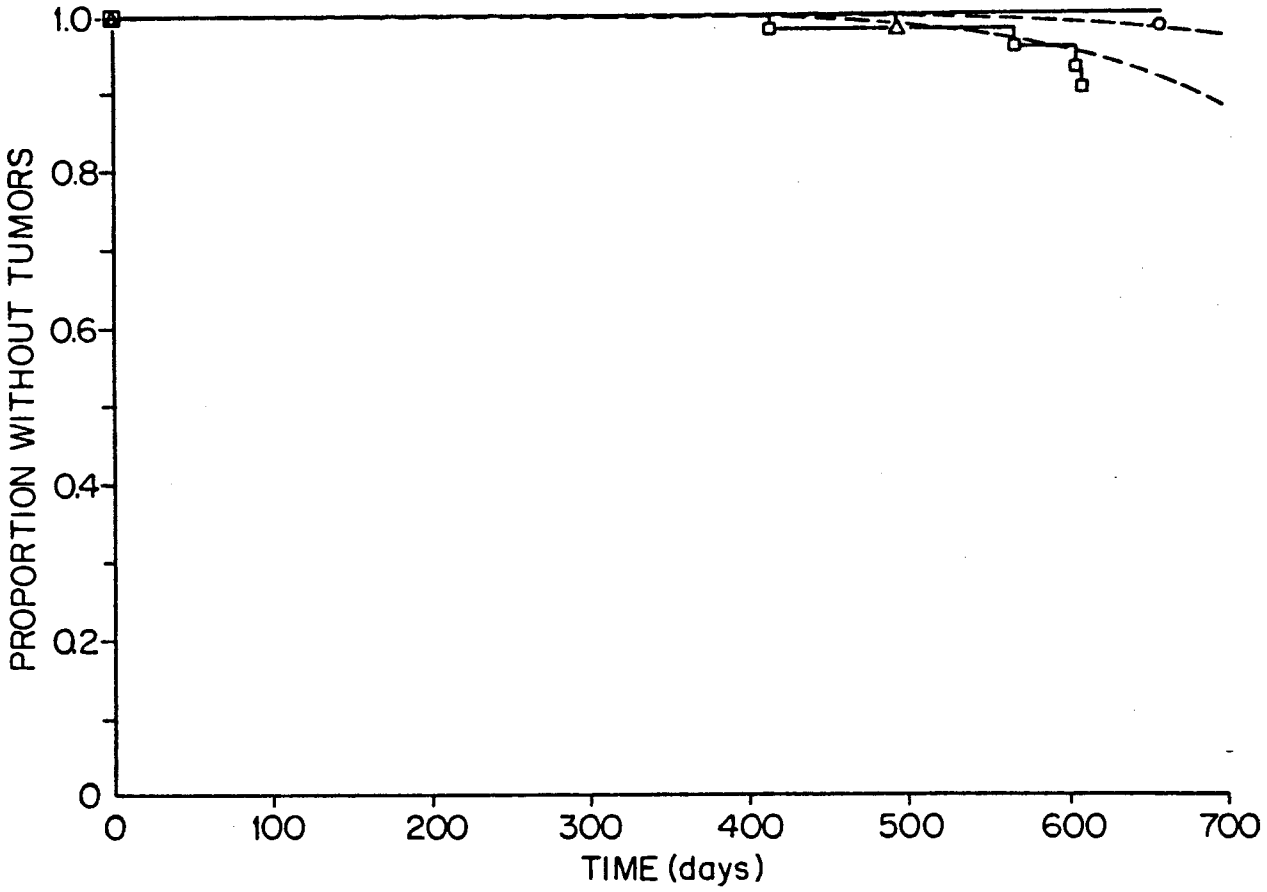

1.0
0.8
0.6
0.4
0.2
0
PROPORTION WITHOUT TUMORS
0
100
200
300
400
500
600
700
TIME (days)

Fig. 3

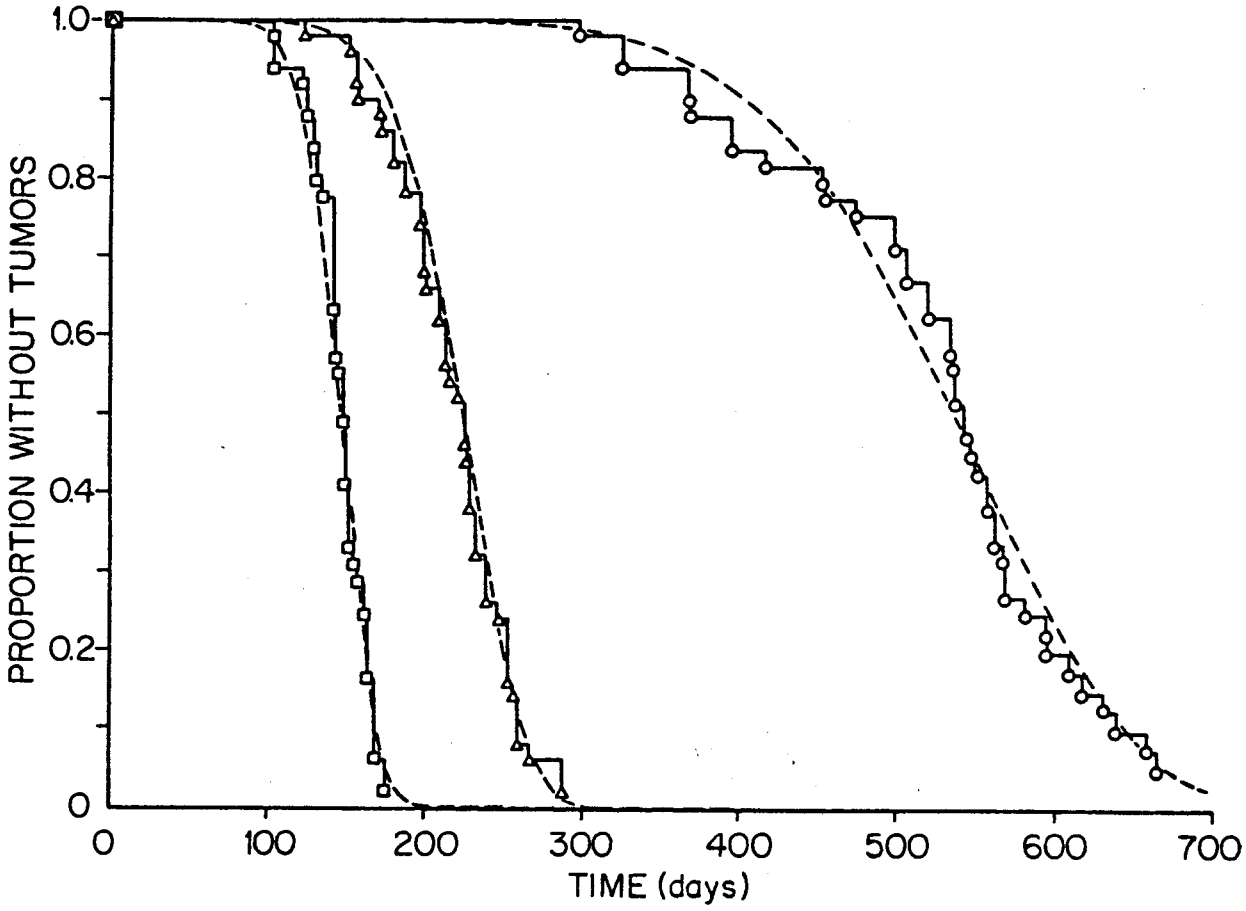

1.0
0.8
0.6
0.4
0.2
0
PROPORTION WITHOUT TUMORS
0
100
200
300
400
500
600
700
TIME (days)

Fig. 4

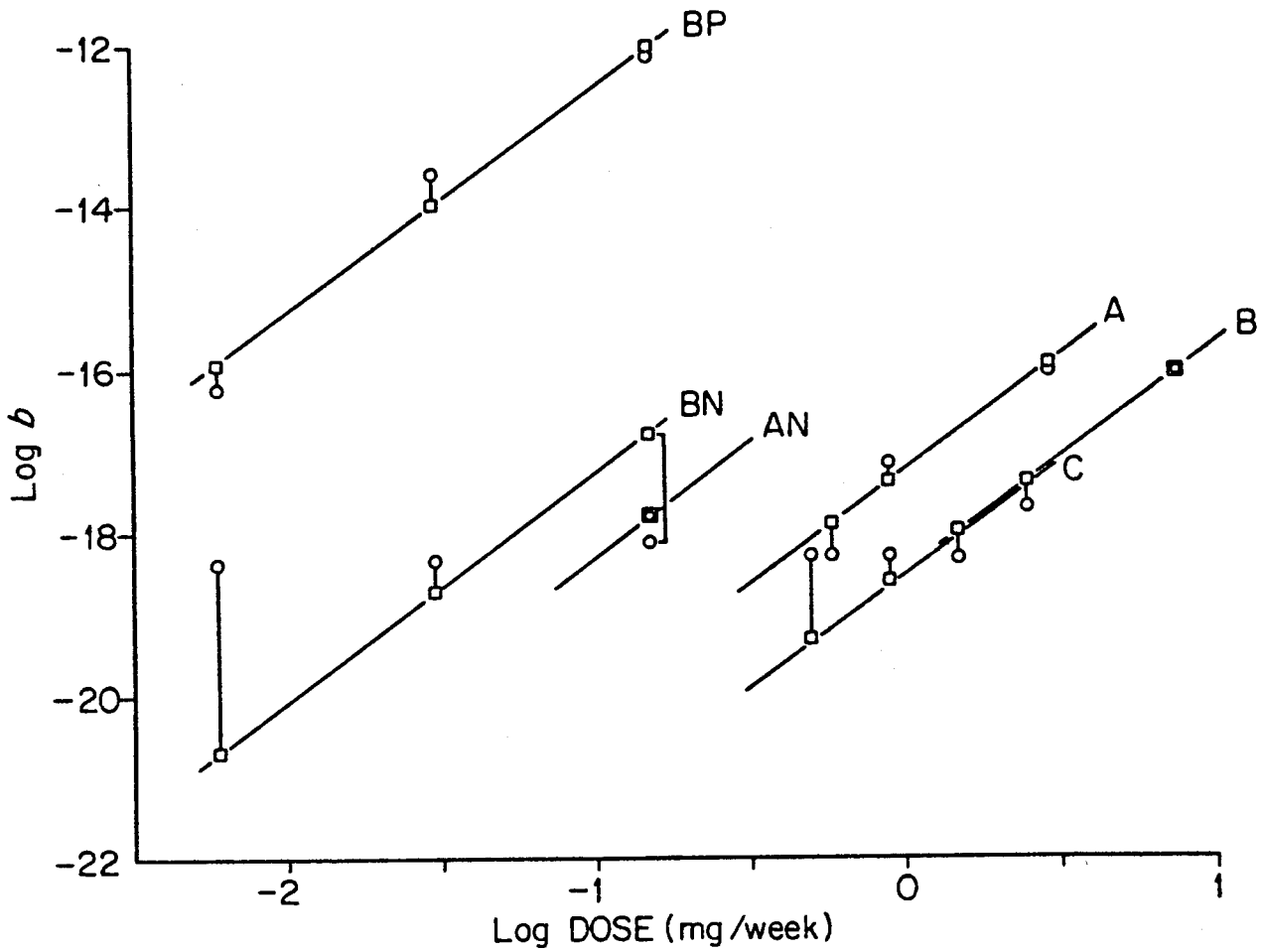


Fig. 5

DETERMINE FORMATION KINETICS OF ACTIVATION AND DETOXIFICATION PRODUCTS IN SKIN CELLS

J. K. Selkirk, Principal Investigator

Oak Ridge National Laboratory
Biology Division, Post Office Box Y
Oak Ridge, Tennessee 37830

EPA-IAG-D5-E681-AT
DOE-40-516-75

Project Contact: DOE/Biomedical Programs
EPA Coordination: EPA/ORD

Office of Research and Development
U.S.E.P.A.
Washington, D.C. 20460

Determine Formation Kinetics of Activation and Detoxification Products in Skin Cells

J. K. Selkirk

I. PROGRESS

The last few months of this project were concerned with confirmation of the metabolic data derived from the epithelial liver cell lines. In addition to the metabolic profile from benzo(a)pyrene derived by high-pressure liquid chromatography, we studied the interaction of the activated carcinogen with cellular macromolecules. We hope to determine if there was a direct quantitative relationship between the amount of metabolism of benzo(a)pyrene in culture with the amount of the activated compound bound to DNA. The results showed for both the nonmalignant and malignant cell lines that its epithelial type had all the metabolic components of activation and detoxification present in their proper ratios and that these cells were capable of producing mutagenic and carcinogenic metabolites of B(a)P. This final portion of the work was done in collaboration with the National Cancer Center in Tokyo and a manuscript is in preparation.

Final Report, December, 1980