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Attn: Section 8(e) Coordinator
(CAP Agreement)

Gentlemen:

Phillips Petroleum Company is submitting the enclosed sixty (60) reports (two boxes, numbered 1 and 2) of toxicological studies pursuant to catagory II.B.2.b of the CAP Agreement 8ECAP-0075 Reports. Reports being submitted contain no confidential business information.

We are sending an additional five boxes (box numbers 3-7) of reports of studies that have, previously, been submitted to the FYI coordinator of the Office of Pollution Prevention and Toxics by the American Petroleum Institute (API). These are being provided solely for the Agency's convenience.

For questions concerning this correspondence, plese contact Fred Marashi at 918-661-8153.

Very truly yours,

Barbara J. Price

Barbara J. Price
Vice President
Health, Environment & Safety

Enclosure (Seven Boxes)

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Phillips Petroleum Company

CAP Identification Number: 8ECAP-0075
Pursuant to Category: II.B.2.b

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Title of Study: In Vivo and In Vitro Mutagenicity Studies for Petroleum Coking Sample 4-1-140

Name of Chemical: Petroleum Coking Sample 4-1-140

CAS#: 64741-79-3

Summary: The results demonstrate that in hte microbial system (Ames Assay) and the mammalian cell assay (Mouse Lymphoma), Petroleum Coking Sample 4-1-140 is not mutagenic. However, in the In Vivo Cytogenetics Test, this compound was found to produce structural mutations after five days of inhalation exposure at 40 μg/liter.

Fiche # 2136

Contact:

Fred Marashi
Phillips Petroleum Company
13 D2 PB
Bartlesville, OK 74004
Phone: 918/661-8153
Fax: 918/661-5664

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IN VIVO AND IN VITRO MUTAGENICITY STUDIES

PETROLEUM COKING SAMPLE 4-1-140

FINAL REPORT

Submitted to

American Petroleum Institute
Washington, D.C.

HAZLETON
LABORATORIES AMERICA, INC.
[illegible]

February 27, 1980

HAZLETON

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SUBJECT: In Vivo and In Vitro Mutagenicity Studies
Project No. 306-110

We, the undersigned, hereby declare that the work was performed under our supervision, according to the procedures herein described.

Study Director and
In Vitro Laboratory Supervision:

Charles E. Piper

CHARLES E. PIPER, Ph.D.
Senior Staff Scientist
Mutagenicity Section
Toxicology Department

Inhalation:

WILLIAM COATE, Ph.D.
Director
Inhalation Department

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LABORATORIES AMERICA, INC.

SPONSOR American Petroleum Institute

DATE [illegible]

MATERIAL Petroleum Coking Sample 4-1-140

SUBJECT FINAL REPORT
In Vivo and In Vitro Mutagenicity Studies
Project No. 596-110

I. INTRODUCTION

The purpose of this study was to evaluate the mutagenic potential of the test substance, Petroleum Coking Sample 4-1-140, using three test systems; the Ames Test, the Mouse Lymphoma Test, and the In Vivo Cytogenetics Assay.

The Ames Test is a bacterial mutagenicity assay utilizing five selected strains of Salmonella typhimurium. The organisms are exposed to varying concentrations of the test agent both with and without metabolic activation.

The Mouse Lymphoma Test is a mammalian assay utilizing the L5178Y mouse lymphoma cell line. The cells are exposed to varying concentrations of the test agent both with and without metabolic activation. The assay measures the mutation frequency at the thymidine kinase locus (TK).

The In Vivo Cytogenetics Assay evaluates the mutagenic potential of a test material as manifested by the production of cytogenetic abnormalities such as deletions, exchanges, rings, and breaks in the bone marrow chromosomes of rats receiving the test material.

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The following report includes results of testing Petroleum Coking Sample 4-1-140 utilizing the three test systems described above.

II. TEST MATERIAL

The test material, Petroleum Coking Sample 4-1-140 is a black powder which was received from the sponsor on March 19, 1979. The sample was coded and stored in a tightly sealed container at room temperature. Dosage calculations were based upon a purity of 100% active ingredient.

III. METHODS

A. *Salmonella typhimurium* Mammalian Microsome Plate Incorporation Assay (Ames Test)

The experimental compound was investigated in accordance with the method described by Ames et al. (1975), using the preincubation modification of Yahagi et al. (1977). A detailed description of the methodology is presented in Appendix 1. Two hundred and ninety-nine mg of the compound were found to dissolve in 2.99 ml of dimethylsulfoxide (DMSO). The primary concentration, 100 mg/ml, was then used for the toxicity evaluation.

Toxicity Test

The concentration of Petroleum Coking Sample 4-1-140 prepared as described above, was further diluted in DMSO to contain 100, 10, 1, and 0.1 µg per one tenth ml. The initial concentration and the five

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tenfold serial dilutions were then used to establish the level at which approximately 50 percent of the bacterial cells would survive.

To each two ml of complete top agar, 0.1 ml of an overnight broth culture of S. typhimurium, TA100, and 0.1 ml of the appropriate concentration of the test compound or diluent were added. The contents of the tube were mixed and poured onto Vogel-Bonner Medium E minimal agar plates. The plates were gently rotated to assure uniform distribution of the top agar, allowed to harden on an even surface for one hour, inverted and put in a dark 37°C incubator.

Following forty-eight hours of incubation, heavy chemical precipitation at the highest concentration completely masked the background lawn of growth; however, no reduction in revertants of TA100 was observed at this concentration.

Based on the results of the toxicity study, 10,000 ug/plate was chosen as the starting concentration for the mutational assay.

Mutational Test

Exposure of the auxotrophic S. typhimurium strains to the test compound was accomplished by the preincubation suspension assay. Five concentrations of the test chemicals were evaluated in triplicate against the five S. typhimurium strains with and without a metabolic activation system.

To ensure uniformity in all experiments, the media were prepared in bulk volumes. Twenty-ml aliquots were aseptically dispensed,

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using a Petrimat, into 100 x 15 mm disposable petri dishes and stored at room temperature in the same location until required. All other media and reagents were prepared in a similar manner.

A sample of Petroleum Coking Sample 4-1-140 weighing 1090 mg was suspended and further diluted in 30% DMSO to contain 10,000, 3333.3, 1111.1, 370.4 and 123.5 µg/0.1 ml. To insure a homogeneous suspension, each dilution of the test compound was vigorously mixed prior to use. To each series of sterile 13 x 100 mm test tubes inoculated with 0.1 ml of an overnight broth culture of either S. typhimurium, 1535, 1537, 1538, TA98, or TA100 and 0.5 ml of the S-9 mix for the activated test, 0.1 ml of the test compound or diluent were added. Ten sets of tubes were prepared in this manner. In groups of two, a series of tubes containing each organism with and without the metabolic activation mix were simultaneously incubated at 37°C for twenty minutes. At the end of the preincubation interval, 2.0 ml of complete top agar were aseptically added to each tube, mixed and poured onto minimal agar plates. Positive control chemicals were treated similarly.

An additional 396 mg sample was prepared and diluted for repeat testing with S. typhimurium TA100 with and without metabolic activation. Following 48 hours of incubation, the revertants to histidine prototrophy in both test and control plates were counted.

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B. Mouse Lymphoma Assay

The experimental compound was investigated in accordance with the method described by Clive et al. (1972, 1975). A detailed description of the methodology is presented in Appendix 2.

Toxicity Testing

Prior to the mutational assay, preliminary toxicity tests were performed to determine the appropriate test doses. The levels tested in the mutational assay are governed by compound solubility and the toxicity determination.

Petroleum Coking Sample 4-1-140 was prepared at a concentration of 100,000 μg/ml by adding 372 mg to 3.72 ml of DMSO. This concentration was determined to be the limit of solubility. Additional concentrations were prepared in the same solvent to contain 50,000, 10,000 and 1000 μg/ml.

To each tube containing approximately 6 x 10^6 TK +/- cells suspended in ten ml of $F_{10}p$, 0.1 ml of the appropriate concentration of the test compound, diluent or positive control (620 μg/ml EMS) were added. The tubes were mixed, gassed with CO_2, tightly sealed and incubated at 37°C on a roller drum at 60 rpm for four hours in an atmosphere of 5% CO_2 and air. Following exposure, the cells were washed twice, resuspended in 20 ml $F_{10}p$ and incubated.

After 24 and 48 hours incubation, no significant toxicity as indicated by a decrease in viable cells was observed. Based on these

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observations, 2000 µg/ml was chosen as the starting dose for mutational studies.

Mutagenicity Assay

The experimental design for the first mutagenicity assay performed is shown in Table 1 (Part B).

Petroleum Coking Sample 4-1-140 (874 mg) was suspended into DMSO (8.74 ml) to yield a concentration of 100,000 µg/ml. As shown in Table 1 (Part B), eight increasing volumes of the stock solution of the experimental compound were added to individual tubes containing 6×10^6 precleansed TK +/- cells suspended in 10 ml $F_{10}P$. For the activated test, precleansed TK +/- cells suspended in 6 ml $F_{10}P$ and 4.0 ml of the S-9 mix were exposed to the eight test doses. Exposure to the test chemical during the forty-eight hour expression time was conducted as described for toxicity testing.

At the end of the expression period, a sample from each of the cultures was centrifuged, and the cells were resuspended at 1.0×10^6 viable cells/ml in $F_{10}P$. The concentrated cells were serially diluted and appropriate dilutions were plated in triplicate in cloning medium (see appendix) with and without TFT. The plates were incubated for 12 $\pm$ 2 days. The mutant colonies (TK -/-) were counted on the selective TFT containing plates and the survivors (TK +/- and TK -/-) were counted on the nonselective medium plates.

Media, solvent and positive controls as shown in Table 1 (Part B) were treated in a similar manner.

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1. In Vivo Bone Marrow Cytogenetic Assay

Test Animals

Twenty-seven sexually mature male albino rats (Sprague-Dawley CR-1: COB® CD® [SD] BR Strain) were received from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts, on July 18, 1979.

The animals were acclimated to the laboratory conditions for ten days prior to initiation of the study. Throughout the course of the study a diurnal cycle of 12 hours light and 12 hours dark (6:00 PM to 6:00 AM) was maintained (exposures were conducted during the light phase).

Assignment to Groups

Following quarantine, the animals were randomly subdivided after culling of weight outliers and unhealthy animals, into three groups of eight animals each weighing between 200 to 300 grams as follows:

Group Number	No. of Animals	Treatment	Target Concentration µg/liter	No. of Exposures
1	8	Air Control	0	20
2	8	PCS 4-1-140	40	5
3	8	PCS 4-1-140	10	20

The animals in Group 2 started exposures on July 30, 1979, those in Groups 1 and 3 started August 4, 1979. Group 2 animals completed exposures on August 2, 1979, Groups 1 and 3 animals completed exposures on August 23, 1979.

Animal Housing and Care

During the rest periods, the animals were housed individually in stainless steel wire mesh cages, food (Wayne Lab Blox® 6%) and water were available ad libitum. Prior to each daily exposure, the animals were transferred into stainless-steel wire mesh cages measuring 20"L X 10½"W X 4"H, divided into 10 equal compartments. No food or water were available during exposure periods.

Exposure Conditions

The animals in the treatment groups were exposed to the test material in 100-liter Plexiglas® inhalation chambers operated dynamically with dried filtered air. Total airflow into the chambers was monitored by flowrators and held constant at 30 liters per minute. Each exposure lasted for six hours per day and exposures were conducted in blocks of five consecutive days per week. Temperature and relative humidity in the control chamber were monitored at hourly intervals. The mean (±S.D.) temperature for the entire study was 81.5 (±2.48)°F; the mean (±S.D.) relative humidity for the entire study was 7.95 (±12.2)%.

Exposure Methods

Compound Generation

Two samples of a black particulate matter designated Petroleum Coking Sample 4-1-140 were generated as dusts using Wright Dust Feed mechanisms. Ten liters of air/minute were

passed through each dust feed to pick up the sample. The aerosols were combined with an accessary airflow of 20 liters/minute to transfer them into the chamber. The aerosolizing apparati were packed daily with fresh supplies of compound. Wright Dust Feed gear ratios were adjusted as necessary to best achieve the 40 and 10 µg/liter concentrations.

The animals in the control group were exposed to filtered air in an identical chamber operated to 30 liters of air/minute.

Chamber Monitoring

Throughout the study, two daily gravimetric filter (Gelman DM-450) samples were obtained from each of the chambers housing animals from Groups 2 and 3. At two and four hours post-generation, preweighed filters, were introduced into the exposure environment via a stainless-steel probe positioned in the approximate center of the chamber. The treated filters were weighed and the gravimetric concentration was calculated as the weight of material collected per total volume of air sampled. Daily average concentrations and overall means (±S.D.) for the test chambers are presented in Table 1 (Part C). While the chamber concentration for the test groups were slightly higher than expected, the overall mean concentrations were within 16% of the target level.

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Particle Size Distribution

Aerodynamic particle size measurements of the test agent were made prior to the animal exposures. Using an Andersen 8-stage Cascade Impactor equipped with a back-up (glass fiber) filter operating at 28.4 liter/minute, the 40 µg/liter sample was collected for 60 minutes and the 10 µg/liter sample's collection time was extended to 120 minutes. Weight gains on each stage and back-up filters were converted to percentages of the total weight of each collected sample and were then cumulatively plotted on log-normal paper. Results of these particle size distribution determinations are presented in Table 2 (Part C) for Groups 2 and 3. These data indicate that the majority of the particles sampled were within the respirable range (<5.00 microns) for both groups.

Biological Measurements

Pharmacotoxic Signs

All animals were observed prior to, at hourly intervals during, and after each daily exposure session for evidence of unusual physical appearances and/or behavior. Observations were recorded.

Body Weights

All animals were individually weighed prior to the start of exposures and at weekly intervals thereafter until time of sacrifice.

Administration of Colchicine

The day after the last exposure of the test gr[illegible] to Petroleum Coking Sample 4-1-140, the test groups and the negative control group animals all received an intraperitoneal injection of colchicine (2.0 mg/kg body weight) to inhibit mitosis in dividing cells.

Sacrifice of Animals

Approximately two hours after injection of colchicine, the animals were sacrificed using carbon dioxide (CO_2) anesthesia followed by cervical dislocation.

Preparation of Slides

Immediately following sacrifice, bone marrow cells were collected from both femurs of each animal by aspiration into a 12 ml syringe filled with 5 ml of pre-warmed (37°C) Hank's balanced salt solution at pH 7.4 (GIBCO). The aspirate was transferred to a centrifuge tube and centrifuged for five minutes at 100 x g. The supernate was decanted and 5.0 ml of 0.075 M KCl were added to each tube. After standing at room temperature for 25 minutes, each tube was centrifuged for five minutes at 100 x g. The supernate was decanted and 5 ml of freshly prepared Carnoy's fixative (3:1, methanol:glacial acetic acid vol:vol) were added to each tube. They were then centrifuged for five minutes at 100 x g. The supernate was discarded and the cells were resuspended in 5 ml of fresh fixative. This process was repeated two more times. Each tube was sealed and

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refrigerated overnight (4°C). The next day the tubes were centrifuged at 100 x g for five minutes. The supernate was decanted and the cells were suspended in 2-3 ml fresh Carnoy's fixative. Three drops of this final cell suspension were dropped onto pre-cleaned glass microscope slides which were then air dried. Three slides were made for each animal. Each slide was identified with the animal's number.

Stain Preparation

Stock Giemsa was prepared from 1.52 g Giemsa powder (Polyscience) added to 100 ml glycerin and 100 ml absolute methanol. Twenty-four ml of this stock solution was then added to 18 ml acetone and 198 ml Harleco buffer phosphate (pH 6.8).

Slide Staining

Upon completion of all preparations, the slides were stained for 16 minutes in the Giemsa stain preparation (described above). Slides were rinsed twice in tap water, air dried, and subsequently mounted with glass cover slips in CoverBond® mounting media.

Cytogenetic Analysis

After all slides were prepared and stained, the animal number on each slide was covered with tape and temporarily assigned a slide code number. The numbers were not decoded until all slides had been analyzed. The evaluation sequence of pre-numbered slides was

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determined from a table of random numbers. All slides were scored without reference to group identification. Upon completion of all scoring, the data sheets were placed into the appropriate groups and statistical analyses were performed. The metaphases were observed for the presence of cytogenetic abnormalities, and mitotic index (number of cells undergoing mitosis per 100 cells counted) was recorded for each animal.

Scanning Slides for Cytogenetic Analysis

Fifty cells in the metaphase state of mitosis were examined from each rat that provided analyzable cells. The slides were scanned with a low power objective (10x) to find metaphases and, once found, the metaphases were analyzed via high power oil immersion lens (100x). Identification numbers for each slide as well as stage vernier settings for each metaphase were recorded on standardized scoring sheets. In order to reduce scorer bias, once a cell was located for analysis on high power, it was not discarded from analysis. The only exception was where a metaphase was damaged during coverslipping and mechanical tearing or shearing were visible in the cell.

Classification of Cytogenetic Aberrations

Chromosomal aberrations were classified into one of four basic groups: (1) chromatid and (2) chromosome breaks; (3) markers which involve dicentrics, exchanges, rings and other miscellaneous

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configurations; and (4) severely damaged cells. Chromatid aberrations involve only one arm of the chromosome, whereas chromosomal aberrations involve both arms at identical loci. A break is defined as any separation that exceeds the width of the chromatid arm, or a discontinuity accompanied by disturbance of the axial integrity. A break may or may not be associated with an identifiable piece of displaced chromatid material.

Aberrant configurations which altered the structural appearance of a single chromosome or involved the joining together of more than one chromosome were categorized as markers. Markers consist of exchanges, dicentrics, rings, and translocations. An exchange is a shift of a segment of a chromosome to a new position on a non-homologous chromosomal structure. A dicentric is a chromosome which possesses two centromeres. A ring chromosome is one in which the ends have joined to form a circle. A translocation involves the transfer of chromosomal material between two or more chromosomes. Those cells which had more than ten scorable aberrations were classified as severely damaged. Therefore, aberrant cells consisted of those cells showing chromatid breaks, chromosome breaks, or markers, or those cells classified as severely damaged.

Photographic Documentation

A minimum of ten representative photographs were taken for each animal exhibiting characteristic aberrations as well as normal

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cells. Karyotype analysis was not made since this type of evaluation is not appropriate in the rat because most of the chromosomes are morphologically similar (telocentric) and pairs are not easily identified without more sophisticated staining procedures.

Statistical Analyses

The mean changes in body weights, and the mean mitotic index were analyzed using Bartlett's test for equality of variance (Bartlett, 1937) and the one-way classification of analysis of variance (ANOVA) (Snedecor and Cochran, 1967). If a significant result were obtained from both analyses, group mean values were compared using the multiple comparison procedure of Games and Howell (1976). If only the ANOVA was significant, Scheffe's multiple comparison procedure (Scheffe, 1953) was used to compare group means.

The percentages of cells containing chromatid breaks, chromosome breaks, markers, severely damaged cells and total aberrant cells for each group of animals were evaluated by the Wilcoxon nonparametric comparison of group means (Snedecor and Cochran, 1967).

Storage of Raw Data

At the completion of this study, all raw data will be stored in the Archives of Hazleton Laoratories America, Inc. All slides will be held by the Cytogenetics Group of the Mutagenicity Department.

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RESULTS

A. Ames Assay

S. typhimurium revertants to histidine prototrophy on test and control plates were counted with a New Brunswick Scientific Electronic Colony Counter.

Heavy chemical precipitation at the two highest test concentrations necessitated manual counting of revertants at these levels.

As indicated in Tables 1 and 2 (Part A), S. typhimurium strains 1535, 1537, 1538, TA98, and TA100 show little or no incease in histidine reversion following exposure to graded doses of Petroleum Coking Sample 4-1-140 in the presence or absence of a metabolic activation system. While the highest dose tested did not clearly show bacterial inhibition, the limits of solubility of Petroleum Coking Sample 4-1-140 were clearly reached.

Diagnostic mutagen controls are included at the end of Tables 1 and 2 (Part A), and confirm the sensitivity and responsiveness of the tester strains to mutagenic action. Spontaneous reversion rates of the tester strains with and without the solvents are similarly listed on these tables.

B. Mouse Lymphoma Assay

As shown in Table 2 (Part B), exposure to 2,000 µg/ml of Petroleum Coking Sample 4-1-140 did not cause an appreciable loss in viability of L5178Y mouse lymphoma cells. The insolubility of the experimental compound precluded the testing of higher concentrations.

Cloning results and calculated mutation frequencies presented in Table 3 (Part B) indicate that little or no increase in the mutation frequency of L5178Y cells occurred following exposure to eight graded doses of Petroleum Coking Sample 4-1-140 both in the presence and absence of metabolic activation. The values for spontaneous mutation frequencies and induced mutation frequencies for the positive controls were within acceptable limits (Table 3).

C. In Vivo Bone Marrow Cytogenetics Study

Pharmacotoxic Observations and Mortality

Group 1

All animals in Group 1 exhibited normal appearance and behavior throughout the study.

Group 2 - (40 µg/l)

The animals in Group 2 exhibited normal appearance and behavior during exposures. When removed from the chamber each day the animals appeared to have normal behavior and physical appearance except for black stained fur (compound buildup) which was observed on all animals on each of the five exposure days.

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Group 3 - (10 µg/l)

The animals in Group 3 exhibited normal appearance and behavior during exposures except for black stained fur (compound buildup) which was observed on all animals on each of the twenty exposure days.

No animals died in any groups during the course of this study.

Body Weights

Individual body weights with group means (±S.D.) measured prior to exposure and at termination are presented on the following page for Groups 1, 2, and 3. Statistical analysis indicated no significant differences in weights of animals from Groups 1 and 3 at any interval. Group 2 weight data for termination was measured five days after the pre-exposure measurement, not twenty as in Groups 1 and 3, and therefore, could not be statistically compared to those groups. All Group 2 animals gained weight.

Group	Initial Body Wt. (g)	Terminal Body Wt. (g)	Body Wt. Changes (g)
1	256 ± 5.2	390 ± 13.5	134.5 ± 15.3
2	257 ± 7.4	287 ± 9.4	28.5 ± 4.5
3	259 ± 9.5	411 ± 29.6	152.0 ± 23.6

Cytogenetic Analysis

Each group was composed of eight males which were administered the control or test substances. Each animal was to provide 50 analyzable metaphases, however, in Groups 1 (negative control) and 2 (40 µg/l x 5 days), a total of 350 metaphases were examined for the

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presence of chromosomal aberrations. In Group 3 (10 µg/l x 20 days), 300 metaphases were examined. Those animals not providing analyzable metaphases, did so due to a failure in the technical procedure.

The number of cells exhibiting aberrations and the specific types of aberrations observed within each metaphase for the individual animals are presented in Tables 3-5 (Part C).

The percent of total aberrant cells per group and the percent of the four categories of aberrations observed for each group is presented below:

Group	% Total Aberrations	% Chromatid Breaks	% Chromosome Breaks	% Markers	% Severely Damaged
1	1.7	0.6	0	1.1	0
2	15.7	9.1	0	5.4	1.1
3	3.3	1.3	0.3	1.6	0

The two test groups exhibited elevations in the incidences of chromatid breaks, markers, and total aberrations when compared to the negative control (Group 1).

The differences between the test groups and negative control group for the four categories of chromosome aberrations were statistically evaluated using the Wilcoxon method of analysis, the results are presented in Tables 6-8 (Part C). There were no significant differences among any of the groups when comparing the percentages of chromosome breaks and severely damaged cells. However, the differences between Groups 1 and 2 for chromatid breaks,

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markers, and total aberrations are significant. Group 3 did not demonstrate any significant differences from Group 1 for any of the categories of chromosomal aberrations.

Table 9 expresses the cytogenetic results as the number of animals with increasing (0 ≥ 10) numbers of abnormal cells containing the following categories of aberrations: chromatid breaks, chromosome breaks, markers, severely damaged cells. As can be observed in this table, the general trend is for the treated groups to have more animals with greater numbers of chromosomal aberr tions when compared to the negative control. Group 2 has more animals with increased numbers of chromatid breaks, markers, severely damaged cells, and total aberrations when compared to the Group 3.

Mitotic Indices

The mitotic index was also examined for each animal. The mean values of the mitotic indices for each group is presented below.

Group	Mean Mitotic Index	Range
1	3.14 ± 1.35	1-5
2	2.71 ± 1.11	1-4
3	3.00 ± 1.41	1-5

When statistically analyzing the differences among the groups there were no significant changes found.

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Photographic Documentation

A minimum of ten photographs of bone marrow cells was taken of each animal that was analyzable. The negatives and contact proofs as well as the vernier location of each photograph are on file at HLA.

Representative photographs from each group have been selected for inclusion in this report.

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Group 1 - Page 23

Slide No.	Vernier Location	Observation
318 B	475 - 1183	Normal
300 B	269 - 1212	Acentric Fragments
314 B	483 - 1182	Exchange
314 A	218 - 1240	Normal

Group 2 - Page 24

Slide No.	Vernier Location	Observation
311 B	475 - 1108	Severely Damaged
311 A	253 - 1282	Dicentric Exchange
303 A	301 - 1108	Normal
320 A	445 - 1098	Multiple Aberrations

Group 3 - Page 25

Slide No.	Vernier Location	Observation
319 B	485 - 1182	Exchanges
319 B	459 - 1122	Normal
302 C	438 - 1118	Chromatid Breaks, Exchange
304 A	468 - 1135	Normal

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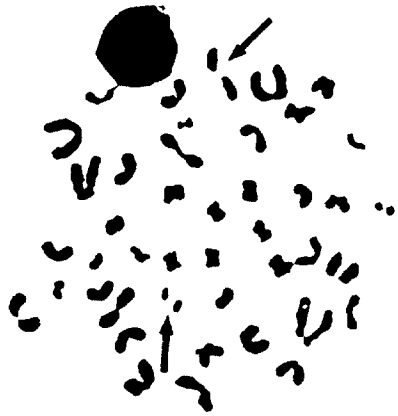
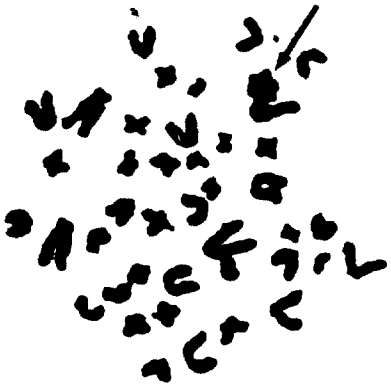
GROUP 1

A

B



C



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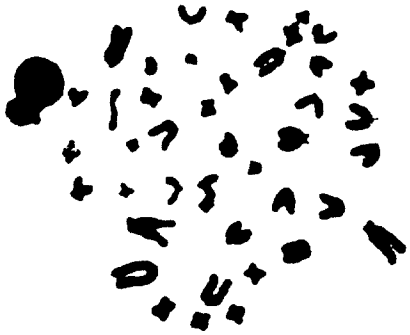

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GROUP 2

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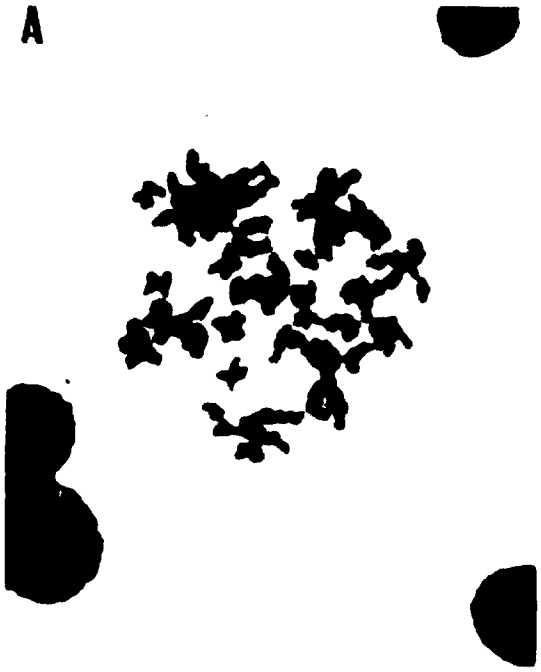

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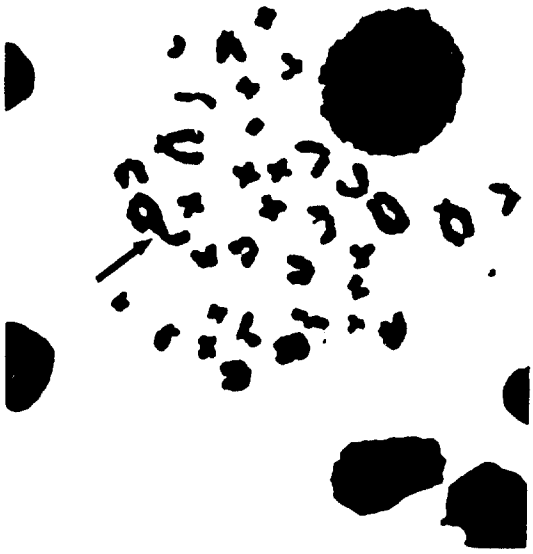

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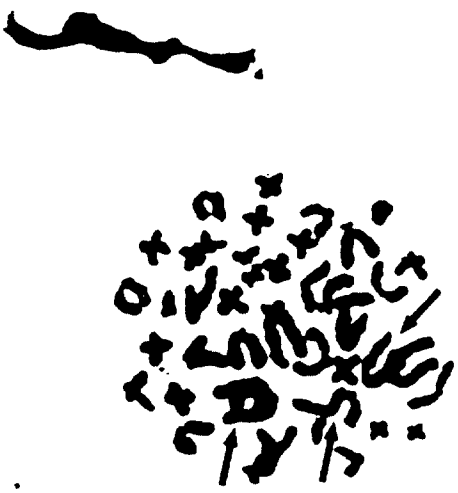

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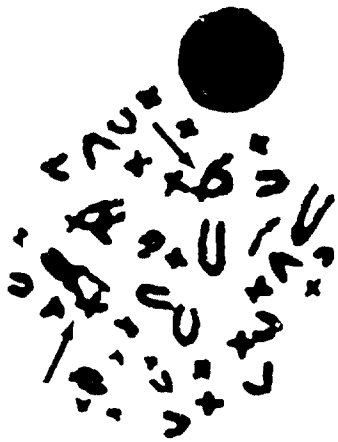
GROUP 3

A



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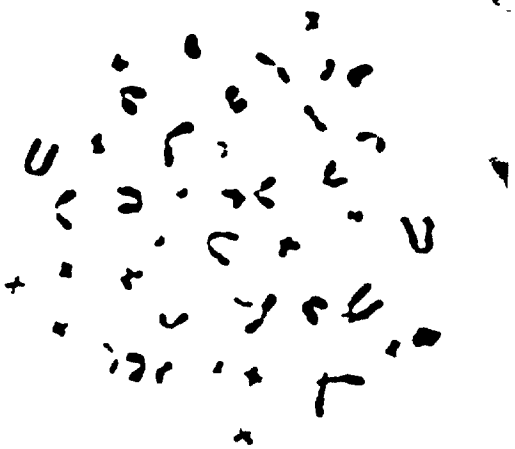
C

D



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V. CONCLUSIONS

A. Ames Assay

The criteria outlined below have been established in our laboratory for evaluation of the Ames Assay:

For a test to be considered valid, the following conditions are required:

1. Demonstration of toxicity of the chemical for the *S. typhimurium* strain(s), unless this is not possible due to limited solubility of the test compound.
2. The solvent controls are within normal range.
3. Confirmation of sensitivity and responsiveness of the tester strains to mutagenic action.

If the above criteria are met, a chemical that exhibits a positive dose response over three concentrations with the baseline increase equal to twice the solvent control is considered to be mutagenic.

The above criteria were satisfied, and under these conditions, the experimental compound, Petroleum Coking Sample 4-1-140 did not exhibit a positive response and is, therefore, not mutagenic in this test system.

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B. Mouse Lymphoma Assay

The criteria outlined below have been established in our laboratory for evaluation of the Mouse Lymphoma Assay. For a test to be considered valid, the following conditions are required:

1. A dose-related response is observed
2. The mutation frequencies at two or more test concentrations are at least two to three fold higher than the mutation frequency of the solvent control
3. The solvent and positive control responses are within acceptable ranges.

By these criteria, Petroleum Coking Sample 4-1-140 does not demonstrate the potential to induce forward mutations at the thymidine kinase (TK) locus in L5178Y, clone 3.7.2, Mouse Lymphoma cells with or without metabolic activation when tested under the conditions described.

C. In Vivo Cytogenetics Study

Albino male rats were administered Petroleum Coking Sample 4-1-140 via inhalation at 10 and 40 µg/liter for 20 and 5 days, respectively. No physical changes or unusual behavior was observed in any animals among the test or control groups. No deaths were observed in any group.

There were no significant differences among the groups when mean body weight changes or mean mitotic indices were statistically

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compared. Statistical analyses of chromosomal aberrations revealed no significant differences for any of the categories when comparing Group 3 anmals to the negative control group (Group 1). However, when comparing Group 2 animals to the negative control animals, the Group 2 animals had significantly greater frequencies of chromatid breaks, marker type chromosomes and total aberrations. Therefore, under conditions of this study, Petroleum Coking Sample 4-1-140 had the ability to produce structural mutations in bone marrow cells of rats when exposed to 40 µg/liter via inhalation for five consecutive days.

VI. SUMMARY

The mutagenic potential of Petroleum Coking Sample 4-1-140 was evaluated using three test systems, the Ames Test, the Mouse Lymphoma Test, and the In Vivo Bone Marrow Cytogenetics Test (via inhalation).

The results demonstrate that in the microbial system (Ames Assay) and the mammalian cell assay (Mouse Lymphoma), Petroleum Coking Sample 4-1-140 is not mutagenic. However, in the In Vivo Cytogenetics Test, this compound was found to produce structural mutations after five days of inhalation exposure at 40 µg/liter.

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PART A

AMES ASSAY

TABLES 1 - 2

Table 1
Number of Revertants/Plate Following Twenty Minutes Preincubation to Graded Doses of
Petroleum Coking Sample 4-1-140, Without Metabolic Activation
In Vitro and In Vivo Mutagenicity Studies

Strain	Dose (µg/plate)														
	123.5			370.4			1111.1			3333.3*			10,000*		
TA35	32	30	41	18	26	22	29	24	35	21	20	16	27	18	20
TA37	11	8	6	8	7	8	9	5	8	8	8	10	9	9	10
TA38	39	20	25	23	30	34	28	27	28	18	21	13	21	21	15
TA98	33	26	27	37	26	30	32	33	27	27	27	23	26	22	31
TA100	163	136	136	130	150	147	147	147	139	131	138	164	149	161	157

Controls:

Strain	Negative									Positive (µg/plate)											
	Organism			14% DMSO			3% ETOH			30% DMSO			HMNG 1.25			4 NPA 100			9-AA 15.6		
TA35	32	29	31	31	29	36				35	31	36	1912	1883	2057						
TA37	12	9	13				6	9	10	6	11	6							1208	1302	1647
TA38	24	18	21	14	18	18				22	19	16				1178	1285	1283			
TA98	27	17	26	24	38	28				20	23	20				1486	1507	1646			
TA100	167	158	169	159	144	138				130	151	146	2441	2212	2472						

*: Manually counted due to chemical precipitation.

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Table 2
Number of Revertants/Plate Following Twenty Minutes Preincubation to Graded Doses of Petroleum Coking Sample 4-1-140, With Metabolic Activation
In Vitro and In Vivo Mutagenicity Studies

Strain	Dose (µg/plate)														
	123.5			370.4			1111.1			3333.3*			10,000*		
1535	22	19	23	26	18	20	20	20	17	13	28	12	18	14	20
1537	13	17	6	12	13	7	9	9	10	11	18	11	14	11	6
1538	40	44	30	47	47	39	35	38	29	35	34	31	29	18	35
TA98	48	48	45	43	49	34	50	41	51	42	49	54	44	45	48
TA100	127	132	153	147	140	149	149	130	137	159	154	146	139	157	153

Controls:

Strain	Negative									Positive (µg/plate)					
	Organism			Organism + S-9			14% DMSO			30% DMSO			2-AA 0.625		
1535	32	29	31	18	19	21	19	14	18	18	15	17	102	112	118
1537	12	9	13	7	12	12	11	11	16	10	16	9	35	43	31
1538	24	18	21	37	36	29	25	36	28	21	31	19	1000	1036	951
TA98	27	17	26	47	46	44	34	48	39	44	27	45	1484	1380	1461
TA100	167	158	159	163	160	169	138	146	159	151	143	130	1418	1294	1385

** Manually counted due to chemical precipitation.

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PART B

MOUSE LYMPHOMA ASSAY

TABLES 1 - 3

Table 1
Experimental Design
In Vitro and In Vivo Mutagenicity Studies with Petroleum Coking Sample 4-1-140
Mouse Lymphoma Assay

Culture Tube #	Medium	S-9 Mix	Contents Cells (1×10^6/ml)	Chemical
1	$F_{10}P$	None	6 ml	Solvent (0.2 ml DMSO)
2	$F_{10}P$	None	6 ml	620 µg/ml EMS (0.1 ml Stock 100x)
3	$F_{10}P$	None	6 ml	2000 µg/ml PCS 4-1-140 (.2 ml Stock A)
4	$F_{10}P$	None	6 ml	1800 µg/ml PCS 4-1-140 (.18 ml Stock A)
5	$F_{10}P$	None	6 ml	1600 µg/ml PCS 4-1-140 (.16 ml Stock A)
6	$F_{10}P$	None	6 ml	1400 µg/ml PCS 4-1-140 (.14 ml Stock A)
7	$F_{10}P$	None	6 ml	1200 µg/ml PCS 4-1-140 (.12 ml Stock A)
8	$F_{10}P$	None	6 ml	1000 µg/ml PCS 4-1-140 (.10 ml Stock A)
9	$F_{10}P$	None	6 ml	800 µg/ml PCS 4-1-140 (.08 ml Stock A)
10	$F_{10}P$	None	6 ml	600 µg/ml PCS 4-1-140 (.06 ml Stock A)
11	None	4 ml	6 ml	Solvent (0.2 ml DMSO)
12	None	4 ml	6 ml	50 µg/ml 2AAF (0.1 ml Stock 100x)
13	None	4 ml	6 ml	2000 µg/ml PCS 4-1-140 (.2 ml Stock A)
14	None	4 ml	6 ml	1800 µg/ml PCS 4-1-140 (.18 ml Stock A)
15	None	4 ml	6 ml	1600 µg/ml PCS 4-1-140 (.16 ml Stock A)
16	None	4 ml	6 ml	1400 µg/ml PCS 4-1-140 (.14 ml Stock A)
17	None	4 ml	6 ml	1200 µg/ml PCS 4-1-140 (.12 ml Stock A)
18	None	4 ml	6 ml	1000 µg/ml PCS 4-1-140 (.10 ml Stock A)
19	None	4 ml	6 ml	800 µg/ml PCS 4-1-140 (.08 ml Stock A)
20	None	4 ml	6 ml	600 µg/ml PCS 4-1-140 (.06 ml Stock A)
21	Media only	None	6 ml	None
22	Media + S-9			None

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Table 2
Summary of Growth Data
In Vitro and In Vivo Mutagenicity Studies with Petroleum Coking Sample 4-1-140
Mouse Lymphoma Assay

Tube No.	Chemical Contents	Daily Cell Counts x 10^6 24 Hours[1]	48 Hours[2]
1	Solvent DMSO	1.59	0.905
2	620 µg/ml EMS	0.54	0.705
3	2000 µg/ml PCS 4-1-140	0.99	1.06
4	1800 µg/ml PCS 4-1-140	0.80	1.145
5	1600 µg/ml PCS 4-1-140	0.93	1.36
6	1400 µg/ml PCS 4-1-140	1.43	0.985
7	1200 µg/ml PCS 4-1-140	1.49	0.90
8	1000 µg/ml PCS 4-1-140	1.3	1.01
9	800 µg/ml PCS 4-1-140	1.26	1.17
10	600 µg/ml PCS 4-1-140	1.62	0.705
11	Solvent + S-9 DMSO	0.84	1.285
12	50 µg/ml 2AAF + S-9	0.20	0.405
13	2000 µg/ml PCS 4-1-140 + S-9	0.59	1.09
14	1800 µg/ml PCS 4-1-140 + S-9	0.85	1.11
15	1600 µg/ml PCS 4-1-140 + S-9	0.80	1.165
*16	1400 µg/ml PCS 4-1-140 + S-9	0.10	NA
17	1200 µg/ml PCS 4-1-140 + S-9	0.65	0.965
18	1000 µg/ml PCS 4-1-140 + S-9	0.55	1.055
19	800 µg/ml PCS 4-1-140 + S-9	0.53	1.15
20	600 µg/ml PCS 4-1-140 + S-9	0.74	1.135
21	Media Control	1.29	0.99
22	Media Control + S-9	0.60	1.705

* Culture terminated due to severe toxicity unrelated to dose response.

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Table 3
Cloning Results and Mutation Frequencies
In Vitro and In Vivo Mutagenicity Studies with Petroleum Coking Sample 4 1-150
Mouse Lymphoma Assay

Tube No.	Contents	No. of Colonies on Select. Plates (TFT)	Column A Mean	No. of Colonies on Non-Select. Plates	Column B Mean	Column C[1] Non-Select. CFC/ml (10^5)	Column D[2] Select. CFC Plated ($x10^5$)	Mutation Frequency[3] (per 1×10^5 cells)
1	Solvent DMSO	56, 56, 53	55	80, 100, 103	94	9.4	4.7	11.7
2	620 µg/ml EMS	103, 104, 135	114	19, 18, 13	17	1.7	0.85	134.1
3	2000 µg/ml PCS 4-1-140	42, 36, 39	39	84, 99, 92	92	9.2	4.6	8.5
4	1800 µg/ml EMS PCS 4-1-140	41, 39, 33	38	93, 72, 69	78	7.8	3.9	9.7
5	1600 µg/ml EMS PCS 4-1-140	37, 41, 27	35	58, 56, 44	53	5.3	2.65	13.2
6	1400 µg/ml EMS PCS 4-1-140	43, 52, 34	43	90, 75, 90	85	8.5	4.25	10.1
7	1200 µg/ml EMS PCS 4-1-140	48, 47, 53	49	72, 115, 79	89	8.9	4.45	11.0
8	1000 µg/ml EMS PCS 4-1-140	45, 51, 46	47	83, 106, 92	94	9.4	4.7	10.0
9	800 µg/ml EMS PCS 4-1-140	44, 44, 38	42	90, 66, 70	75	7.5	3.75	11.2
10	600 µg/ml EMS PCS 4-1-140	63, 58, 58	60	90, 83, 102	92	9.2	4.6	13.0
11	Solvent + S-9 DMSO	42, 29, 37	36	65, 70, 74	70	7.0	3.5	10.3
12	50 µg/ml 2AAF + S-9	75, 85, 75	78	51, 60, 66	59	5.9	2.95	26.4
13	2000 µg/ml PCS 4-1-140 + S-9	37, 43, 42	41	92, 100, 116	103	10.3	5.15	8.0
14	1800 µg/ml PCS 4-1-140 + S-9	40, 48, 49	46	66, 73, 70	70	7.0	3.5	13.1
15	1600 µg/ml PCS 4-1-140 + S-9	43, 41, 30	38	50, 40, 56	49	4.9	2.45	15.5
16	1400 µg/ml PCS 4-1-140 + S-9	-	-	-	-	-	-	-
17	1200 µg/ml PCS 4-1-140 + S-9	36, 39, 27	34	144, 126, 124	131	13.1	6.55	5.2
18	1000 µg/ml PCS 4-1-140 + S-9	47, 36, 42	42	78, 77, 89	81	8.1	4.05	10.4
19	800 µg/ml PCS 4-1-140 + S-9	32, 42, 30	35	68, 77, 62	69	6.9	3.45	10.1
20	600 µg/ml PCS 4-1-140 + S-9	35, 32, 36	34	63, 58, 74	65	6.5	3.25	10.5
21	Media Control	37, 31, 36	35	71, 71, 76	73	7.3	3.65	7.6
22	Media Control + S-9	32, 30, 42	35	74, 68, 69	70	7.0	3.5	10.0

[1] Column C shows the number of colony forming cells (CFC) per ml of the concentrated cell suspensions after the two day expression time. This number equals the mean number of colonies on the non-selective plates (Column B) multiplied by 10^4 (dilution factor).

[2] Column D shows the number of CFC plated into selective medium (0.5 ml of the concentrated cell suspensions was plated). This number equals the number of CFC per ml (Column C), divided by 2.

[3] The Mutation Frequency is the number of TK-/- mutants per 1×10^5 CFC. This number equals the mean number of TK -/- colonies on the selective plates (Column A), divided by the number of CFC plated (Column D).

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PART C

IN VIVO CYTOGENETICS ASSAY

TABLES 1 - 9

Table 1
Daily Me : Gravimetric Chamber Concentrations with Grand Means (±S.D.) for Groups 2 and 3
In 'itro and In Vivo Mutagenicity Studies with Petroleum Coking Sample 4-1-140
Bone Marrow Cytogenetics Study
(μg/liter)

Day	Group 2 Mean (ppm)	Group 3 Mean (ppm)
1	33.60	9.9
2	47.50	10.95
3	43.70	8.25
4	50.30	8.00
5	51.60	8.60
6		11.35
7		16.05
8		9.80
9		10.55
10		13.35
11		17.20
12		8.50
13		11.15
14		14.00
15		12.60
16		9.65
17		11.20
18		10.40
19		11.55
20		11.25
Mean	45.34	12.45
± S.D.	8.22	2.56

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Table 2
Particle Size Data
In Vitro and In Vivo Mutagenicity Studies with Petroleum Coking Sample 4-1-140
Bone Marrow Cytogenetics Study

Stage Number	Stage Cutoffs (μ)	Actual Weight Collected (mg) Group 2	Group 3
0	10	2.25	1.04
1	9	4.18	1.28
2	5.8	5.29	1.00
3	4.7	18.38	3.20
4	3.3	17.72	4.24
5	2.1	12.60	3.68
6	1.1	5.39	1.92
7	0.7	0.78	1.10
Filter	0.4	0.35	1.00
Total		66.94	18.46

Mass Median Diameter (μ) = 3.10 2.54
Geometric Standard Deviation (σg) = 2.09 2.99

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Table 3
Incidence of Chromosome Aberrations of Bone Marrow Cells from Male Rats
Treated with Normal Air for Twenty Consecutive Days
In Vitro and In Vivo Mutagenicity Studies with Petroleum Coking Sample 4-1-140

Group Number	Animal Number	Number of Cells Examined	Number of Aberrant Cells	Number of Normal Cells	% of Aberrant Cells	Number of Aberrations/Animal			
						Chromatid Breaks	Chromosome Breaks	Marker	Severely Damaged
1	48201	50	1	49	2	0	0	1	0
	48202	50	3	47	6	2	0	1	0
	48203	50	0	50	0	0	0	0	0
	48204	50	1	49	2	0	0	1	0
	48205	50	0	50	0	0	0	0	0
	48206	50	1	49	2	0	0	1	0
	48207	0	NA	NA	NA	NA	NA	NA	NA
	48208	50	0	50	0	0	0	0	0
	Totals	350	6	344	1.7	2	0	4	0

KEY: NA = Not applicable.

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Table 4
Incidence of Chromosome Aberrations of Bone Marrow Cells from Male Rats
Treated with 40 μg/liter of Petroleum Coking Sample 4-1-140 for Five Consecutive Days
In Vitro and In Vivo Mutagenicity Studies with Petroleum Coking Sample 4-1-140

Group Number	Animal Number	Number of Cells Examined	Number of Aberrant Cells	Number of Normal Cells	% of Aberrant Cells	Number of Aberrations/Animal			
						Chromatid Breaks	Chromosome Breaks	Marker	Severely Damaged
2	48221	50	8	42	16	6	0	2	0
	48222	50	10	40	20	6	0	3	1
	48223	50	7	43	14	4	0	2	1
	48224	0	NA	NA	NA	NA	NA	NA	NA
	48225	50	6	44	12	4	0	2	0
	48226	50	12	38	24	6	0	4	2
	48227	50	5	45	10	2	0	3	0
	48228	50	7	43	14	4	0	3	0
	Totals	350	55	295	15.7	32	0	19	4

KEY: NA = Not applicable.

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Table 5
Incidence of Chromosome Aberrations of Bone Marrow Cells from Male Rats
Treated with 10 µg/liter of Petroleum Coking Sample 4-1-140 for Twenty Consecutive Days
In Vitro and In Vivo Mutagenicity Studies with Petroleum Coking Sample 4-1-140

Group Number	Animal Number	Number of Cells Examined	Number of Aberrant Cells	Number of Normal Cells	% of Aberrant Cells	Number of Aberrations/Animal			
						Chromatid Breaks	Chromosome Breaks	Marker	Severely Damaged
3	48211	50	1	49	2	0	0	1	0
	48212	50	3	47	6	2	1	0	0
	48213	50	1	49	2	0	0	1	0
	48214	50	1	49	2	0	0	1	0
	48215	50	1	49	2	0	0	1	0
	48216	50	3	47	6	2	0	1	0
	48217	0	NA	NA	NA	NA	NA	NA	NA
	48218	0	NA	NA	NA	NA	NA	NA	NA
	Totals	300	10	290	3.3	4	1	5	0

KEY: NA = Not applicable.

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Table 6
Wilcoxon Nonparametric Comparison of the Percent of
Chromatid Breaks in the Bone Marrow of Rats Treated with
Petroleum Coking Sample 4-1-140
In Vitro and In Vivo Mutagenicity Studies with Petroleum Coking Sample 4-1-140

Groups Compared	Average Rank	Calculated Test Statistic	Tabled Value	Result
Negative Control - Group 2	4.1,10.9	T = 28.5	36	SIGNIFICANT
Negative - Group 3	6.4, 7.8	T = 37.5	27	NOT SIGNIFICANT
Group 2 - Group 3	9.0, 3.7	T = 22.0	27	SIGNIFICANT

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Table 7
Wilcoxon Nonparametric Comparison of the Percent of
Markers in the Bone Marrow of Rats Treated with
Petroleum Coking Sample 4-1-140
In Vitro and In Vivo Mutagenicity Studies with Petroleum Coking Sample 4-1-140

Groups Compared	Average Rank	Calculated Test Statistic	Tabled Value	Result
Negative Control - Group 2	4.0,11.0	T = 28.0	36	SIGNIFICANT
Negative - Group 3	5.9, 8.3	T = 34.5	27	NOT SIGNIFICANT
Group 2 - Group 3	10.0, 3.5	T = 21.0	27	SIGNIFICANT

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Table 8

Wilcoxon Nonparametric Comparison of Two Percent of
Total Aberrations in the Bone Marrow of Rats Treated with
Petroleum Coking Sample 4-1-1.0
In Vitro and In Vivo Mutagenicity Studies with Petroleum Coking Sample 4-1-1.0

Groups Compared	Average Rank	Calculated Test Statistic	Tabled Value	Result
Negative Control - Group 2	4.0, 11.0	T = 28.0	36	SIGNIFICANT
Negative - Group 3	5.5, 8.3	T = 31.5	27	NOT SIGNIFICANT
Group 2 - Group 3	10.0, 3.5	T = 21.0	27	SIGNIFICANT

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Table 9
Bone Marrow Data
Number of Animals with Increasing Numbers of Abnormal Cells
In Vivo Bone Marrow Cytogenetics Study of Petroleum Coking Sample 4-1-140 in the Rat

Group	0	1	2	3	4	5	6	7	8	9	≥10	Total No. of Animals
Chromatid Breaks												
Negative Control	6	0	1									7
Group 2	0	0	1	0	3	0	3					7
Group 3	4	0	2									6
Chromosome Breaks												
Negative Control	7											7
Group 2	7											7
Group 3	5	1										6
Marker Aberrations												
Negative Control	3	4										7
Group 2	0	0	3	3	1							7
Group 3	1	5										6
Severely Damaged												
Negative Control	7											7
Group 2	4	2	1									7
Group 3	6											6
Aberrant Cells												
Negative Control	3	3	0	1								7
Group 2	0	0	0	0	0	1	1	2	1	0	2	7
Group 3	0	4	0	2								6

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APPENDICES

1. TECHNICAL PROCEDURES - AMES TEST

2. TECHNICAL PROCEDURES - MOUSE LYMPHOMA

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TEST FOR MICROBIAL MUTAGENESIS
Salmonella typhimurium Assay

I. BACKGROUND

Microbial mutagenesis testing provides a mechanism whereby relatively large numbers of chemicals can be screened to identify potentially dangerous substances among the thousands of chemicals to which humans are exposed. In vitro microbial tests are available which are reliable, rapid, sensitive and relatively inexpensive prescreen procedures. Using this type of test, it would be practical to evaluate a new compound for mutagenicity, during the course of its development, without significantly affecting either the program or the cost of development. The microbial assay using *Salmonella typhimurium* devised by Ames and his associates (1) appears to be ideally suited for this purpose. When used in conjunction with microsomal preparations which incorporate aspects of mammalian metabolic activation into the *in vitro* test, the Ames test has proven to be effective in detecting mutagens from a wide variety of chemical classes.

II. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of experimental compounds in strains of *Salmonella typhimurium*.

III. MATERIALS AND METHODS

A. Chemicals

1. Supply and Storage of Test Chemicals

Chemicals to be tested will be supplied by the sponsor. Approximately five grams of a sample from a single manufactured lot of each chemical will be required. Chemicals will be stored at Hazleton Laboratories America, Inc. (HLA) under conditions compatible with safety and stability until required for use. After completion of the tests, and the sponsor's acceptance of the final report, the excess samples will be returned to the sponsor or discarded.

2. Handling of Toxic Materials

Preparation of test chemicals for assay will be performed using precautions for handling toxic substances which conform to the safety standards of the Toxicologic Subcommittee for Carcinogen Standards, U.S. Department of Health, Education and Welfare.

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B. Indicator Organisms

Histidine deficient Salmonella typhimurium strains TA1535, TA1537, TA1538, TA98 and TA100 have been obtained directly from the laboratory of Dr. Bruce Ames.

These strains were derived from strain TA1530 which is characterized by deletions through the gal-bio-uvrB region of the chromosome and are further delineated by having the deep rough (rfa) character. The effect of these mutations is the elimination of the DNA excision-repair system and a substantial loss of the lipopolysaccharide barrier that coats the surface of the bacteria. Due to the uvrB deletion biotin as well as histidine is required for growth. Strain TA1535 has a hisG-46 base-pair-substitution mutation. Strain TA1535 has a hisG-3076 frameshift mutation and strain TA1538 has a hisD-3052 frameshift mutation. These strains are used to detect the effect of mutagens on DNA in the absence of histidine and an excision-repair system. TA98 was derived from TA1538 and TA100 was derived from TA1535. These strains contain a plasmid that conveys resistance to ampicillin and are more effective in detecting some classes of mutagens than the original tester strains.

C. Maintenance of Stock Cultures

Upon arrival the five S. typhimurium tester strains were inoculated to 5 ml of nutrient broth and incubated overnight at 37 ± 0.5°C. A 0.8 ml portion of the culture was mixed with 0.07 ml DMSO in each of several 2 ml (0.5 dram) sterile, glass, screw-capped vials. The mixtures are stored frozen.

D. Verification of Tester Strains

1. Spontaneous Reversion

When the frozen permanent stock cultures were prepared, one tenth ml of each of the overnight broth cultures was inoculated on a nutrient agar plate and incubated at 37 ± 0.5°C for 18 to 24 hours. Isolated colonies were transferred to fresh nutrient broth. The suspensions were inoculated to minimal agar medium, Vogel-Bonner Medium E (VBE) (see appendix) with and without 0.1M L-histidine HCl and with 0.5mM biotin. The rate of spontaneous reversion to histidine prototrophy was noted.

Expected rates of spontaneous reversion per agar plate for bacterial strains are presented in the following table.

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Strain	Without Microsomes	With Microsomes
S. typhimurium		
TA1535	15-30	15-45
TA1537	6-13	6-18
TA1538	15-30	20-60
TA98	25-50	30-70
TA100	140-250	150-275

Spontaneous reversion rates will be determined in each experiment and the presence of the specific genetic markers will be confirmed periodically.

2. Genetic Characterization

Strains having the deep rough, *rfa*, characteristic (*S. typhimurium* strains TA1535, TA1537, TA1538, TA100 and TA98) will be inoculated to medium which contains 10 ± 1 of 2 mg/ml crystal violet solution. Inhibition of growth should be noted following approximately 12 hours of incubation at 37 ± 0.5°C.

To test strains TA100 and TA98 for the presence of the ampicillin R factor, 10 ± 1 of ampicillin solution (8 mg/ml 0.02 N NaOH) will be streaked across the surface of a nutrient agar plate and allowed to dry. The cultures to be tested will be cross-streaked against the ampicillin. After incubation for 12 to 24 hours at 37 ± 0.05°C, strains which have the R factor will show no zone of inhibition around the ampicillin streak.

E. Metabolic Activation

1. Preparation of Liver Homogenate (S-9)

Enzymes will be induced in male Sprague Dawley rats by a single intraperitoneal injection of polychlorinated biphenyl mixture (200 mg Monsanto Aroclor 1254/ml corn oil) at a dosage of 500 mg/kg five days before sacrifice. The animals will be deprived of food and water prior to sacrifice.

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The rats will be sacrificed by decapitation, the livers removed, placed in sterile ice-cold beakers, and washed in an equal volume of 0.15M KCl. All livers will be minced with sterile scissors in three volumes of the sterile KCl rinsing solution and homogenized. The homogenates will be centrifuged at 9000xg in a refrigerated centrifuge and the supernate (S-9 fraction) retained. The S-9 fraction will be distributed in aliquots, quickly frozen on dry ice, and stored in a freezer. As needed, sufficient S-9 fraction will be thawed and kept on ice until incorporated into the S-9 mix. The unused portion will be discarded at the end of the day. Optimum S-9 concentration will be verified with benzo(a)pyrene, an aromatic amine 2-acetylaminofluorene and 2-aminoanthracene.

2. Preparation of S-9 Mix

The S-9 mix contains per ml:

a.	S-9	0.04 to 0.15 ml
b.	$MgCl_2$	8 moles
c.	KCl	33 moles
d.	glucose-6-phosphate	5 moles
e.	NADP	4 moles
f.	sodium phosphate	100 moles pH 7.4

The S-9 mix will be freshly prepared on the day of the experiment, passed through 0.45 membrane filters and kept on ice; the unused portion will be discarded at the end of the day. Stock solutions of reagents b, c and f are stored in the refrigerator and stocks of reagents d and e are held frozen.

F. Preparation of Chemical Solutions

All test and control compounds will be weighed, and their solutions prepared in a chemical fume hood. The chemicals will be dissolved in a suitable solvent indicated by the sponsor or as determined by solubility tests.

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G. Test of Toxicity

The greatest number of induced mutations per plate and therefore the most sensitive test for mutagenesis should result from treatment of the indicator organisms with the highest concentration of chemical that causes minimal killing of the organisms. The exact kinetics of toxicity and mutation induction will vary with different compounds; however, in general the optimal concentration should allow survival of about 50 percent of the cells. The method described below will be used in our laboratory to indicate the levels at which compounds should be tested for mutagenesis.

The concentration of the chemical found satisfactory for testing based on the solubility study will serve as the initial dose for the toxicity evaluation. A minimum of five tenfold serial dilutions of the agent will be prepared in the appropriate solvent to contain the test dose in 0.1 ml volumes.

To each of 2 ml of complete top agar, 0.1 ml of an overnight broth culture of S. typhimurium strain TA100 and 0.1 ml of the appropriate dilution of the test compound or diluent will be added. The contents of the tube will be thoroughly mixed, poured and uniformly distributed over VBE minimal agar (see appendix). The plates will be allowed to harden approximately one hour, inverted and put into a dark 37 ± 0.5°C incubator. After two days, the background lawn of growth and revertant colonies in both test and control plates will be scored. Observations of chemical precipitation will be similarly recorded. The concentration at which inhibition of the bacteria as indicated by a reduction in the bacterial lawn and/or a decrease in spontaneous revertants occurs, will furnish the data for further studies. If toxicity is not evident at any of the doses, higher concentrations of the compound will be examined. If chemical precipitation interferes with the observations of growth, the highest level suitable for use in future studies will not exceed the limits of solubility.

H. Test of Mutagenicity

Exposure of auxotrophic bacteria will be done directly by the plate incorporation assay. For the induction of reverse mutations, each chemical will be tested against the five bacterial strains with and without the metabolic activation systems. Each concentration of the compound and controls will be plated in triplicate.

To ensure uniformity in all experiments, media will be prepared in bulk volumes. Twenty-ml aliquots will be aseptically dispensed, using a Petrimat, into 100x15 mm disposable petri

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dishes and stored at room temperature in the same location until required. All other media and reagents will be prepared in a similar manner.

Data derived from the toxicity studies will be utilized to establish test levels of each compound for further investigation. A minimum of five concentrations of the test compound will be prepared to contain the test dose in 0.1 ml volumes for evaluation with and without metabolic activation. The highest dose tested will be that level of the chemical exhibiting inhibition of the bacterial growth in toxicity studies, unless this is not possible due to limited solubility of the investigated agent.

To each of 2 ml of complete top agar, 0.1 ml of an overnight broth culture of each tester strain, 0.1 ml of the test compound or diluent, and 0.5 ml of the S-9 mix for the activated test will be added. The contents of the tube will be mixed thoroughly and poured onto VBE minimal agar plates. Plates will be gently rotated and tilted to assure uniform distribution of the top agar, allowed to harden on an even surface for one hour, inverted and put in a dark 37 $\pm$ 0.5°C incubator. After two days, the colonies in both test plates and controls will be counted. For the experiment to be considered valid, at least three of the doses tested should be below the level at which toxicity is evident, and the responses of the positive controls should fall within the expected ranges.

I. Controls

In addition to internal controls described in previous sections, each test will include media and solvent negative controls, and will incorporate appropriate known mutagenic chemicals as positive controls. Final selection of known mutagens for each test system may be made in conjunction with the sponsor; however, compounds may be made in conjunction with the sponsor; however, compounds recommended to confirm mutagenesis for the tester strains are:

Bacterial Strains	Chemical	Enzyme Activation
S. typhimurium		
TA1535, TA100	MNNG[A]	-
TA1537	9-aminoacridine	-
TA1538, TA98	2-nitrofluorene	-
All strains	2-aminoanthracene	+

A = methylnitronitrosoguanidine

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I. Results

S. typhimurium revertants to histidine prototrophy on test and control plates will be counted with an electronic colony counter. Results of toxicity determinations will be confirmed and the number of revertants on treated and control plates will be compared. While the results of each experiment will be considered independently, the spontaneous reversion rate and the sensitivity and reproducibility of the bacterial strains and metabolic activation systems will be evaluated both by reference to previous work with the method and by the concurrent use of positive and negative controls.

A chemical will be considered negative if the maximum noninhibitory level exhibits less than a twofold increase in the number of induced revertants when compared to the solvent control. A non-linear dose response over three concentrations of the chemical will indicate mutagenic potential, but a dose-related response provides more persuasive evidence of mutagenesis.

K. Report

The report will include the following:

1. Experimental design and methods.

2. Results

   a. Toxicity evaluation.

   b. Comparison of the number of revertants in test and control groups.

3. Conclusions.

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APPENDIX

Media for *Salmonella typhimurium* Strains

Vogel-Bonner Agar Minimum E

50x Stock Solution

$MgSO_4$ 7 H_2O	10.0 grams
Citric acid H_2O	100.0 grams
K_2HPO_4 anhydrous	500.0 grams
$NaNH_4HPO_4$ anhydrous	175.0 grams
Distilled water	to 1.0 liter pH 7.0

Dispensed in 100 ml volumes; autoclave 15 minutes.

50x Stock Solution	30.0 ml
Glucose	30.0 grams
Agar	22.5 grams
Distilled water	to 1500 ml

Autoclave 20 minutes at 121°C.

Top Agar

NaCl	0.5 grams
Agar (Difco)	0.6 grams
Distilled water	100.0 ml

Autoclave 15 minutes at 121°C. Aseptically add 10 ml of 0.5mM histidine - 0.5mM biotin to 100 ml of top agar.

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TEST FOR MUTAGENESIS OF MAMMALIAN CELLS
MOUSE LYMPHOMA ASSAY

I. BACKGROUND

The objective of mutagenicity testing is to assess the potential of a given chemical or mixture of chemicals to modify or interact with human genetic material and cause a heritable change. Many in vitro test systems have been developed for the detection of potential mutagens over the last several years. To date microbial assays primarily dependent upon detection of reverse mutations have been most widely used due to their low cost, efficiency, rapidity, and sensitivity. Since the ultimate objective of mutagenicity testing is to define the potential risk to humans, there is an obvious need for in vitro test systems using mammalian cells. To serve this need we utilize the L5178Y mouse lymphoma cell mutational assay developed by Clive and associates (1,2). This test system permits quantitation of forward mutational events at the autosomal thymidine kinase (TK) specific locus.

II. PURPOSE

The objective of this study is to evaluate the potential of compounds to cause forward mutational events at the TK locus in L5178Y mouse lymphoma cells.

III. EXPERIMENTAL DESIGN

A. Chemicals

1. Supply and Storage of Test Chemicals

Chemicals to be tested will be supplied by the sponsor. Approximately five grams of a sample from a single manufactured lot of each chemical will be required. Chemicals will be stored at Hazleton Laboratories America, Inc. (HLA) under conditions compatible with safety and stability until required for use. After completion of the tests, and the sponsor's acceptance of the final report, the excess samples will be returned to the sponsor or discarded.

2. Handling of Toxic Materials

Preparation of test chemicals for assay will be performed using precautions for handling toxic substances which conform to the safety standards of the Toxicologic Subcommittee for Carcinogen Standards, U.S. Department of Health, Education and Welfare.

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B. Characteristics of Cell Lines

The L5178Y mouse lymphoma cell line is presumed to be diploid in nature and three TK phenotypes have been recognized: TK+/+, TK+/-, and TK-/-. The TK+/+ and TK+/- cells are sensitive to 5-bromodeoxyuridine (BUdR) and trifluorothymidine (TFT) and resistant to a solution of thymidine, hypoxanthine, methotrexate and glycine (THMG). The TK-/- phenotype exhibits reverse sensitivity and resistance patterns (2). The heterozygous TK+/- phenotype will be used as the target cell in this study.

C. Maintenance of Cell Lines

Frozen stocks of L5178Y mouse lymphoma cells grown from the TK+/- -3.7.2C heterozygote subline are held in a liquid nitrogen storage tank. Suspension cultures of the TK+/- cells will be maintained in Fischer's medium $F_{10}P$ (see appendix) and will be cleansed of pre-existing spontaneous TK-/- mutants by treatment with THMG (1) prior to use in experiments.

D. Metabolic Activation

1. Preparation of Liver Homogenates (S-9)

Enzymes will be induced in male Sprague Dawley rats by a single intraperitoneal injection of polychlorinated biphenyl mixture (200 mg Monsanto Aroclor 1254/ml corn oil) at a dosage of 500 mg/kg five days before sacrifice. The animals will be deprived of food and water prior to sacrifice.

The rats will be sacrificed by decapitation, the livers removed, placed in sterile ice-cold beakers, and washed in an equal volume of 0.15M KCl. All livers will be minced with sterile scissors in three volumes of the sterile KCl rinsing solutions and homogenized. The homogenates will be centrifuged at 9000xg in a refrigerated centrifuge and the supernate (S-9 fraction) retained. The S-9 fraction will be distributed in aliquots, quickly frozen on dry ice, and stored in a freezer. As needed, sufficient S-9 fraction will be thawed and kept on ice until incorporated into the S-9 mix. The unused portion will be discarded at the end of the day. Optimum induced S-9 concentrations will be verified using 2-acetylaminofluorene (2-AAF) as a test chemical.

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D. Preparation of S-9 Mix

The S-9 mix contains per 100 ml:

Fischer's Medium	75 ml
S-9	25 ml
NADP	600 mg
isocitric acid	1125 mg

The S-9 mix will be prepared just prior to use in the experiment and kept on ice until used. The unused portion will be discarded at the end of the day.

E. Preparation of Chemical Solutions

All test and control compounds will be weighed, and their solutions prepared in a chemical fume hood. The chemicals will be dissolved in a suitable solvent indicated by the sponsor or as determined by solubility tests.

F. Toxicity Tests

The solubility and toxicity of a compound dictate the doses used in the mutagenicity test. The toxicity of a compound is determined by testing a wide range of chemical concentrations for relative inhibition of the growth of TK+/- cells in suspension in $F_{10}P$ following a four hour exposure to the chemical. The highest concentration of the chemical tested will depend upon its solubility. The test compound is prepared at 100x concentration in the solvent in a series of dilutions (e.g., 1,000, 500, 100, 50, 10, 1, and 0.1 g/ml final concentration). A solvent control and a positive control are included for comparison. One-tenth ml of each chemical concentration and the solvent are added to sterile, screw-cap, 50 ml centrifuge tubes containing 6.0×10^6 TK+/- cells in ten ml of $F_{10}P$. The cells are exposed to the chemical for four hours, washed, resuspended in 20 ml $F_{10}P$, and incubated for two days. The exposure and incubation conditions used are identical to those used in the mutagenicity assay. After the first and second day of incubation, the cell counts and viability in each culture will be determined by counting a sample of the cells using a hemocytometer in the presence of trypan blue. Following the first day of incubation the viable cell count will be adjusted to 3.0×10^5 cells/ml in all cultures which have grown significantly above this level. Based on the cell counts the relative toxicity of the chemical doses as compared to the solvent control will be determined. From this information eight concentrations of the test compound will be formulated for use in the mutagenicity test. The highest dose tested will usually be selected to give approximatey 50 to 90% inhibition of suspension cell growth.

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However, the upper dose may be limited by [illegible] of the compound. Seven succeeding dilutions will be tested with the lower doses in a range of non-toxicity relative to the solvent control.

G. Mutagenicity Test - Forward Mutational Assay

Eight concentrations of the test compound will be prepared to contain the test dose in 0.1 ml volumes. The highest dose tested and the succeeding dilutions will be determined from the toxicity evaluation. The assay includes a solvent control tested with and without metabolic activation: a positive control (ethyl methane-sulfonate) that does not require activation to induce mutations; a promutagen (2-AAF) which depends on S-9 activation for its mutagenic properties; and eight concentrations of the unknown chemical tested with and without S-9.

Six million precleansed TK+/- cells in six ml of $F_{10}p$ are added to each of 20 sterile, screw-cap, 50 ml centrifuge tubes. An additional four ml of $F_{10}p$ are added to ten of the tubes, and four ml of the S-9 mix are added to the remaining ten tubes. Immediately thereafter, 0.1 ml of 100x concentrations of the test chemical dilutions and the positive controls, and 0.1 ml of the solvent are added to the appropriate tubes. Each tube will be mixed, gasses with a mixture of CO_2 and air, and incubated at 37 $\pm$ 0.5°C on a revolving roller drum for four hours. Following this incubation the tubes will be centrifuged and the treatment solutions decanted. The cells will be washed twice with $F_{10}p$ after the second wash. The tube cultures will then be gassed and reincubated as described above for a two day expression time. Growth of the cells will be monitored at one and two days post-exposure and the cultures readjusted to 3.0×10^5 cells/ml if necessary.

At the end of the expression period, a sample from each of the cultures will be centrifuged, and the cells resuspended at 1.0×10^6 viable cells/ml in $F_{10}p$. The concentrated cells will be serially diluted and appropriate dilutions will be plated in triplicate in cloning medium (see appendix) with and without TFT. The plates will be incubated for 12 $\pm$ 2 days. The mutant colonies (TK-/-) will be counted on the selective TFT containing plates and the survivors (TK+/- and TK-/-) will be counted on the nonselective medium plates.

H. Results

The daily cell counts, the total number of colony forming cells and the number of TFT resistant colony forming cells will be determined for the test and control cultures. The number of mutants in the test and control groups will be compared and

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their mutation ranges are determined. [illegible] which experiment will be considered unacceptable, the spontaneous mutation frequency and the responses of the positive controls should be within acceptable ranges as determined by a development historical data base. A chemical will be considered positive if a dose-related response is obtained in which the mutation frequencies at two or more test concentrations are at least three-fold higher than the mutation frequency of the solvent control.

I. Report

The report will include the following:

1. Experimental design and methods

2. Results

   a. Daily cell counts

   b. Number of surviving colony forming cells in test and control groups

   c. Number of mutants in test and control groups

   d. Mutation frequencies in test and control groups

3. Conclusions

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Media

Fischer's Medium

- Fischer's medium for leukemic cells of mice (10x) (Gibco)	50 ml
- Sterile, deionized, glass distilled H_2O	450 ml
- Media pH adjusted visually with approximately 7.5% NaH_2CO_3 (Gibco)	5 ml

E10R

- Fischer's Medium	500 ml
- Penicillin-Streptomycin (50,000 units and g/ml)	1 ml
- Sodium Pyruvate (2.2%)	2.3 ml
- L-Glutamine (200mM)	5 ml
- Pluronics (10% Solution)	5 ml
- Horse Serum (Heat Inactivated 30 min., 56°C)	50 ml

Non-Selective Cloning Medium

- Fischer's Medium	350 ml
- Penicillin-Streptomycin (50,000 units and g/ml)	1 ml
- Sodium Pyruvate (2.2%)	5 ml
- L-Glutamine (200mM)	5 ml
- Horse Serum (Heat Inactivated 30 min., 56°C)	100 ml
- 4% Molten Noble Agar	40 ml

Selective Cloning Medium

As above with the addition of 2 ug/ml trifluorothymidine (TFT).

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Triage of 8(e) Submissions

Date sent to triage: 2/5/96

NON-CAP (CAP)

Submission number: 12533A

TSCA Inventory: (Y) N D

Study type (circle appropriate):

Group 1 - Dick Clements (1 copy total)

ECO AQUATO

Group 2 - Ernie Falke (1 copy total)

ATOX SBTOX SEN w/NEUR

Group 3 - Elizabeth Margosches (1 copy each)

STOX CTOX EPI RTOX (GTOX)

STOX/ONCO CTOX/ONCO IMMUNO CYTO NEUR

Other (FATE, EXPO, MET, etc.):

Notes:

THIS IS THE ORIGINAL 8(e) SUBMISSION; PLEASE REFILE AFTER TRIAGE DATABASE ENTRY

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entire document: (0) 1 2 **pages** 1 **pages** 1,2, tabs

Notes:

Contractor reviewer : LPS **Date:** 5/11/95

CECATS\TRIAGE TRACKING DBASE ENTRY FORM

CECATS DATA:
Submission # 8EHQ- 0992 -12533 SEQ. A

TYPE: INT SUPP FLWP

SUBMITTER NAME: Phillips Petroleum Company

INFORMATION REQUESTED: FLWP DATE:
0501 NO INFO REQUESTED
0502 INFO REQUESTED (TECH)
0503 INFO REQUESTED (VOL ACTIONS)
0504 INFO REQUESTED (REPORTING RATIONALE)

DISPOSITION:
0639 REFER TO CHEMICAL SCREENING
0678 CAP NOTICE

VOLUNTARY ACTIONS:
0401 NO ACTION REPORTED
0402 STUDIES PLANNED/UNDERWAY
0403 NOTIFICATION OF WORKERS/USERS
0404 LABEL/MSDS CHANGES
0405 PROCESS/HANDLING CHANGES
0406 APP/USE DISCONTINUED
0407 PRODUCTION DISCONTINUED
0408 CONFIDENTIAL

SUB. DATE: 08/24/92 OTS DATE: 09/02/92 CSRAD DATE: 03/20/95

CHEMICAL NAME:

CAS#
64741 - 79-3

INFORMATION TYPE:		P F C	INFORMATION TYPE:		P F C	INFORMATION TYPE:		P F C
0201	ONCO (HUMAN)	01 02 04	0216	EPI/CLIN	01 02 04	0241	IMMUNO (ANIMAL)	01 02 04
0202	ONCO (ANIMAL)	01 02 04	0217	HUMAN EXPOS (PROD CONTAM)	01 02 04	0242	IMMUNO (HUMAN)	01 02 04
0203	CELL TRANS (IN VITRO)	01 02 04	0218	HUMAN EXPOS (ACCIDENTAL)	01 02 04	0243	CHEM/PHYS PROP	01 02 04
0204	MUTA (IN VITRO)	01 02 04	0219	HUMAN EXPOS (MONITORING)	01 02 04	0244	CLASTO (IN VITRO)	01 02 04
0205	MUTA (IN VIVO)	01 02 04	0220	ECO/AQUA TOX	01 02 04	0245	CLASTO (ANIMAL)	01 02 04
0206	REPRO/TERATO (HUMAN)	01 02 04	0221	ENV. OCCC/REL/FATE	01 02 04	0246	CLASTO (HUMAN)	01 02 04
0207	REPRO/TERATO (ANIMAL)	01 02 04	0222	EMER INCI OF ENV CONTAM	01 02 04	0247	DNA DAM/REPAIR	01 02 04
0208	NEURO (HUMAN)	01 02 04	0223	RESPONSE REQEST DELAY	01 02 04	0248	PROD/USE/PROC	01 02 04
0209	NEURO (ANIMAL)	01 02 04	0224	PROD/COMP/CHEM ID	01 02 04	0251	MSDS	01 02 04
0210	ACUTE TOX. (HUMAN)	01 02 04	0225	REPORTING RATIONALE	01 02 04	0299	OTHER	01 02 04
0211	CHR. TOX. (HUMAN)	01 02 04	0226	CONFIDENTIAL	01 02 04			
0212	ACUTE TOX. (ANIMAL)	01 02 04	0227	ALLERG (HUMAN)	01 02 04			
0213	SUB ACUTE TOX (ANIMAL)	01 02 04	0228	ALLERG (ANIMAL)	01 02 04			
0214	SUB CHRONIC TOX (ANIMAL)	01 02 04	0239	METAB/PHARMACO (ANIMAL)	01 02 04			
0215	CHRONIC TOX (ANIMAL)	01 02 04	0240	METAB/PHARMACO (HUMAN)	01 02 04			

TRIAGE DATA:	NON-CBI INVENTORY	ONGOING REVIEW	SPECIES	TOXICOLOGICAL CONCERN:	USE:	PRODUCTION:
	YES	YES (DROP/REFER)	RAT In Vitro	LOW		
CAS SR	NO	NO (CONTINUE)		MED		
	IN TRANSIT	REFER		HIGH		

COMMENTS

7) ✓ 8EHQ-92-12533: Rank - medium.

Chemical: petroleum coking sample 4-1-140 (CAS# 64741-79-3).

In vivo and in vitro mutagenicity studies, Hazelton Laboratories, Inc., Vienna VA, dated February 27, 1980: Negative for gene mutations in Salmonella typhimurium in strains TA98, TA100, TA1535, TA1537 and TA1538 both without and with metabolic activation.

Negative for gene mutations in L5178Y $TK^{+/-}$ mouse lymphoma gene mutation assay in vitro both without and with metabolic activation.

Positive for chromosome mutations (aberrations) in male rats exposed in vivo by inhalation.