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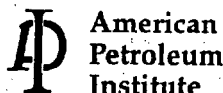
A RANGE-FINDING DEVELOPMENTAL INHALATION TOXICITY STUDY OF UNLEADED GASOLINE VAPOR CONDENSATE IN RATS AND MICE VIA WHOLE-BODY EXPOSURES

AN INHALATION DEVELOPMENTAL TOXICITY STUDY OF UNLEADED GASOLINE VAPOR CONDENSATE IN THE RAT VIA WHOLE-BODY EXPOSURE

Health and Environmental Sciences Department
Publication Numbers TR 412 and TR 414
April 1998



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Documents

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An Inhalation Developmental Toxicity Study of Unleaded Gasoline Vapor Condensate in the Rat via Whole-Body Exposure

Health and Environmental Sciences Department

API PUBLICATION NUMBER TR 414

PREPARED UNDER CONTRACT BY:

HUNTINGDON LIFE SCIENCES
METTLERS ROAD
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APRIL 1998

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THE FOLLOWING PEOPLE ARE RECOGNIZED FOR THEIR CONTRIBUTIONS OF TIME AND EXPERTISE DURING THIS STUDY AND IN THE PREPARATION OF THIS REPORT:

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PREFACE

This abridged publication consists of the full text and pertinent data from the study, *An Inhalation Developmental Toxicity Study of Unleaded Gasoline Vapor Condensate (API 94-02) in the Rat via Whole Body Exposure*. The study was conducted by Huntingdon Laboratories under contract to API. The complete appendices are too voluminous for distribution on a routine basis. The Table of Contents indicates omitted appendices in italics. The complete report is available through the API Information Specialist, 1220 L Street, Washington D.C., 20005.

ABSTRACT

This study was conducted for the American Petroleum Institute to assess the potential maternal toxicity and developmental toxicity of Unleaded Gasoline Vapor Condensate (API 94-02) administered via inhalation (whole-body exposure) to mated rats (24/group) 6 hours/day during the Day 6-19 gestation interval. Exposure levels were 0 (filtered air), 1000, 3000 and 9000 ppm. In this study, no maternal or developmental toxicity was seen in rats at an exposure level up to and including 9000 ppm.

Italicized sections in the Table of Contents have been omitted from this publication. These sections are on file at API and may be obtained by contacting the API Information Specialist, 1220 L St., Washington, D.C., 20005.

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LABORATORY TITLE PAGE

STUDY NO. 95-6083

API STUDY NO. 08200-0601-SH9343

AN INHALATION DEVELOPMENTAL TOXICITY STUDY
OF UNLEADED GASOLINE VAPOR CONDENSATE (API 94-02) IN THE RAT
VIA WHOLE-BODY EXPOSURE

LABORATORY

Huntingdon Life Sciences
Mettlers Road
P.O. Box 2360
East Millstone, N.J. 08875-2360

SPONSOR

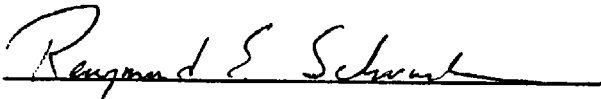
American Petroleum Institute
Health and Environmental Sciences Department
1220 L Street, N.W.
Washington, D.C. 20005

FINAL REPORT

date final report is produced

LABORATORY SIGNATURE PAGE

This report constitutes a true and faithful account of the procedures adopted and the results obtained in the performance of this study.



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Study Director

29 September 97
Date



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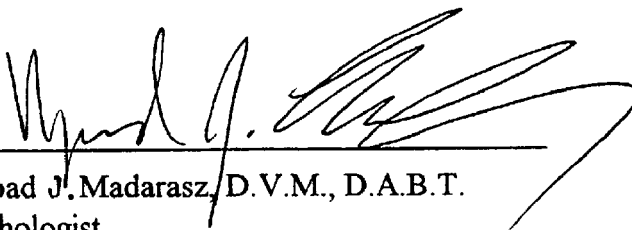
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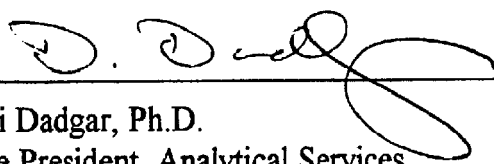
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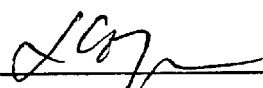
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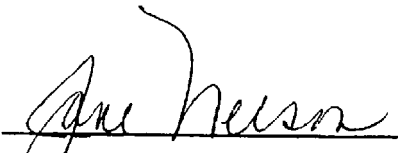

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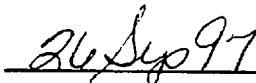
26 Sept. 1997
 Date

LABORATORY QUALITY ASSURANCE STATEMENT

Listed below are the dates that this study was inspected by the Quality Assurance Unit of Huntingdon Life Sciences, East Millstone, New Jersey, and the dates that findings were reported to the Study Director and Management.

Type of Inspection	Date(s) of Inspection(s)	Reported to Study Director	Reported to Management
GLP Protocol Review	25 Sep 95	25 Sep 95	27 Sep 95 and 4 Oct 95
Mating Observations	29 Sep 95	29 Sep 95	4 Oct 95 and 20 Oct 95
Exposure and Monitoring	3 Oct 95	5 Oct 95	10 Oct 95 and 20 Oct 95
Gestation Body Weights and Feeder Weights	5 Oct 95	5 Oct 95	16 Oct 95 and 20 Oct 95
Terminal Sacrifice	16 Oct 95	16 Oct 95	19 Oct 95 and 20 Oct 95
Final Analytical Report	7, 8 and 15 Mar 96	15 Mar 96	5 Apr 96
Final In-Life and Pathology Report	1 Apr 96 to 5 Apr 96	5 Apr 96	5 Apr 96
Report Revisions	22, 24 and 25 Apr 97	25 Apr 97	25 Apr 97
Report Revisions	1 Jul 97	1 Jul 97	1 Jul 97


 Jane Nelson
 Quality Assurance Senior Auditor


 Date

LABORATORY COMPLIANCE STATEMENT

This study was conducted in compliance with the United States Environmental Protection Agency's Good Laboratory Practice Standards 40 CFR Part 792.

Raymond E. Schroeder
Raymond E. Schroeder, M.S., D.A.B.T.
Study Director

17 Mar 98
Date

Lorraine E. Twerdok
Lorraine E. Twerdok, Ph.D.
Sponsor Representative

3/30/98
Date

GOLDMAN ASSOCIATES INTERNATIONAL, INC.

Quality Assurance Statement

Study No. 08200-0601-SH9343

In accordance with the Good Laboratory Practice regulations published at 40 CFR 792, this study was monitored on behalf of the Sponsor, the American Petroleum Institute.

An Inhalation Developmental Toxicity Study of Regular Unleaded Gasoline in the Rat via Whole Body Exposure.

was monitored on and reported to Sponsor on

September 7 and 17-19, 1995

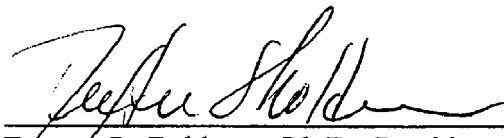
October 14, 1995

October 30-31, 1995

November 22, 1995

July 15-17, 1996

September 5, 1996



Dexter S. Goldman, Ph.D., President

Section 1

SUMMARY

This inhalation study was performed to provide information on the maternal and developmental toxicity of Unleaded Gasoline Vapor Condensate (API 94-02) in rats. Unleaded Gasoline Vapor Condensate (API 94-02) was administered as a vapor, via inhalation (whole-body) exposure, 6 hours/day to 72 mated rats (24/group) during Days 6-19 of gestation. Exposure levels were 1000, 3000 and 9000 ppm. Twenty-four mated rats which served as controls were chamber-housed and received filtered room air only, 6 hours/day over the same treatment intervals.

Study animals were observed twice daily for mortality/morbidity and for obvious pharmacologic and/or toxicological effects. In addition, each animal was removed from its cage and given a detailed physical examination on Day 0 of gestation, daily both pre- and post-exposure during the treatment period and at terminal sacrifice (Day 20 of gestation). Body weights were recorded on Days 0, 3, 6, 9, 12, 15, 18, and 20 of gestation. Food consumption was recorded on Days 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, and 18-20 of gestation.

Chamber exposure concentrations were evaluated daily using infrared spectrophotometry (hourly) and gas chromatography (single grab sample, daily). The latter were analyzed to monitor the ratio of the 11 major components of the test material in comparison to its largest component, isopentane.

At terminal sacrifice (Day 20 of gestation), animals were given a macroscopic postmortem examination. The gravid uterus with the ovaries attached was removed, weighed intact and evaluated for the number of fetuses and resorption sites. The number of *corpora lutea* on the ovaries was also recorded. Fetuses were removed from the uterus, weighed, sexed externally and evaluated for external irregularities. The intact fetuses were processed for either soft tissue or skeletal examinations. Approximately one-half of the fetuses in each litter were decapitated and

processed for soft tissue examinations using a microdissection procedure. The heads of these fetuses were fixed in Bouin's solution and evaluated using a razor blade sectioning procedure. The remaining fetuses in each litter were left intact, sacrificed with an overdose of inhaled carbon dioxide, eviscerated and processed for staining of the skeletal structures with Alizarin Red S. These fetuses were then evaluated for skeletal malformations and ossification variations.

The mean daily total hydrocarbon concentrations $\pm$ standard deviations from the infrared spectrophotometric evaluation over the entire exposure period for the 1000, 3000 and 9000 ppm groups were 1015 ± 35 , 2984 ± 147 and 8993 ± 278 ppm, respectively. Gas chromatograph analyses of syringe samples from the chambers demonstrated a similarity among all exposure groups, and a stability of the test material during each exposure day. In these data 11 major components of the test material were expressed as a ratio to isopentane, the largest component.

No mortality occurred in the control or treated animals. No maternal or developmental toxicity was seen at an exposure level up to and including 9000 ppm.

Section 2

INTRODUCTION

Presented in this report are the procedures used and the results obtained from an inhalation developmental toxicity study of Unleaded Gasoline Vapor Condensate (API 94-02) in rats via whole body exposure. This study was conducted at Huntingdon Life Sciences, Mettlers Road, P.O. Box 2360, East Millstone, New Jersey 08875-2360.

This inhalation study was performed to provide information on the maternal and developmental toxicity of Unleaded Gasoline Vapor Condensate (API 94-02) in rats when administered as a vapor, via whole-body exposure, 6 hours/day to 72 mated rats (24/group) on Days 6-19 of gestation. Exposure levels were 1000, 3000, and 9000 ppm. Twenty-four mated rats which served as controls were chamber-housed and received filtered room air only, 6 hours/day over the same interval.

Procedures used during the study are presented in the Materials and Methods/References section of the report. Maternal and developmental toxicity data for the rat are presented in Appendices A through L. Data regarding the inhalation exposures are presented in Appendix M. Results, as well as methods used, for the chamber analyses performed by the Testing Facility's Analytical Department are presented in Appendix N. Recent historical control data for this strain of rat in teratology/developmental toxicity studies conducted at this laboratory are presented in Appendix O. Feed and water analyses are presented in Appendix P. A copy of the study protocol and amendments is presented in Appendix Q.

Section 3

MATERIALS AND METHODS/REFERENCES

REGULATORY REFERENCES

Test Guideline

This study was designed to meet or exceed the requirements of the EPA (Environmental Protection Agency) TSCA (Toxic Substances Control Act) Test Guideline No. 798-4350: Inhalation Developmental Toxicity Study published in the Federal Register Vol. 50, No. 188 (September 27, 1985) pgs. 39426-39428 (and with revisions Federal Register Vol. 52, No. 97- May 20, 1987). Reference was also made to the EPA draft developmental toxicity study guidelines (870.300., July 1994) in determining the dosing schedule.

Good Laboratory Practices

This study was conducted in compliance with Part 792 of 40 CFR (EPA Good Laboratory Practices - TSCA).

Animal Welfare Act Compliance

This study complied with all appropriate parts of the Animal Welfare Act Regulations: 9 CFR Parts 1 and 2 Final Rules, Federal Register, Volume 54, No. 168, August 31, 1989, pp. 36112-36163 effective October 30, 1989 and 9 CFR Part 3 Animal Welfare Standards; Final Rule, Federal Register, Volume 56, No. 32, February 15, 1991, pp. 6426-6505 effective March 18, 1991.

Facilities Management/Animal Husbandry

Currently acceptable practices of good animal husbandry were followed, e.g., Guide for the Care and Use of Laboratory Animals; DHHS Publication No. (NIH) 86-23, Revised 1985. The laboratory of Huntingdon Life Sciences, East Millstone, New Jersey is fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

STUDY MANAGEMENT

Sponsor

American Petroleum Institute
Health and Environmental Sciences Department
1220 L Street, N.W.
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Sponsor Representative

Richard A. Rhoden, Ph.D.

Testing Facility

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Study Director

Raymond E. Schroeder, M.S., D.A.B.T.

EXPERIMENTAL DESIGN

Group	Exposure Level ^a (ppm)	Treatment Schedule ^b (GD)	Number of Animals				
			Mated	Sacrificed	Proportion of Gestation Day 20 Fetuses/Litters Evaluated for Malformations and/or Variations		
					External	Soft-Tissue	Skeletal
I	0	6-19	24	24	All	1/2	1/2
II	1000	6-19	24	24	All	1/2	1/2
III	3000	6-19	24	24	All	1/2	1/2
IV	9000 ^d	6-19	24	24	All	1/2	1/2

^aExposure levels were established on the basis of data from a range-finding study (Huntingdon Life Sciences Study No. 95-6082).

^bExposures were 6 hours/day. Groups II-IV received Unleaded Gasoline Vapor Condensate (API 94-02). Control animals received filtered room air only.

^cComplete macroscopic postmortem evaluations were performed on all animals.

^dThe Lower Explosive Limit (LEL) for Unleaded Gasoline Vapor Condensate (API 94-02) as reported in the American Petroleum Institute DTI Report 2509 was 1.25% (12,500 ppm). The high-exposure level of 9000 ppm represents approximately 75% of the LEL.

GD = gestation day

STUDY DATES

Study Initiation (Date Study Director signed the Protocol)

26 September 1995

Initiation of Mating (Experimental Start Date)

25 September 1995

Initiation of Exposure

2 October 1995

Termination of Exposure

31 October 1995

Terminal Sacrifice

16-20, 23-27, 30-31 October and 1 November 1995

Experimental Termination (Date of Last Data Collection)

7 December 1995

Study Termination

29 September 1997

TEST MATERIAL INFORMATION

TEST MATERIAL	LOT NO.	PURITY	DESCRIPTION	DATES RECEIVED	EXPIRATION DATE
Unleaded Gasoline Vapor Condensate (API 94-02)	API 94-02	Considered 100%	Clear liquid	First Shipment - 6 March 1995 Second Shipment - 25 October 1995	December 1999

Supplier

Chevron Research and Technology Company
100 Chevron Way
Richmond, California 94802-0627

Analysis

Documentation of the identity, strength, purity, composition; and synthesis, fabrication, and/or derivation of the test material were the responsibility of the Sponsor.

Stability

Documentation of the stability of the test material over the use interval of this study was the responsibility of the Sponsor.

Storage

Upon receipt, the test material was stored at ambient temperature in an outdoor solvent shed. During generations it was kept frozen at a temperature of -20°C. At other times the test material was stored indoors at room temperature.

Archival Sample

A sample of approximately 10 mL of test material is stored in the Archives of the Testing Facility.

Disposition

The unused portion of the test material, and any empty test material containers, will be returned to the Sponsor following submission of the final report.

TEST ANIMAL INFORMATION

Rats

Albino (Outbred) VAF/Plus[®]

Strain

Sprague-Dawley derived (CD[®]) [CrI: CD[®] BR]

Justification for Animal Selection

The rat is a rodent animal model commonly utilized in developmental toxicity studies as recommended in the EPA-TSCA guidelines. In addition, a tabulation of recent historical control data is available for this species in this laboratory for comparative evaluation with study data (Appendix U).

Number of Animals Purchased

140 females. All females were nulliparous and non-pregnant.

Number of Animals Placed On Test

96 females

Supplier - Males and Females

Charles River Laboratories
Portage, Michigan 49081

Date Received - Females

11 September 1995

Date Received - Males

1 May and 21 August 1995

Proven breeders were used solely for mating purposes (in-house breeding colony).

Age at Receipt - Females

71 days old

Age at Receipt - Males (Breeders)

49 days old from 1 May 1995 shipment and 56 days old from the 21 August 1995 shipment received.

Age at Initiation of Mating - Females

85 days old

Age at Initiation of Mating - Males (Breeders)

147 days old (1 May shipment); and 91 days old (21 August shipment)

Weight of Mated Females Used on Test (Gestation Day 0)

	<u>Mean</u> (grams)	<u>Range</u> (grams)
Rats:	262	219-318

Acclimation Period - Females

13 days

SELECTION

More females than required for the study were purchased and acclimated. Animals considered unsuitable for the study on the basis of pretest physical examinations were eliminated prior to initiation of mating.

MATING

Females selected for mating were placed with male rats nightly in a 1:1 ratio. Vaginal smears were taken early in the morning following intervals of nightly cohabitation and females were considered to have mated if sperm was noted microscopically in the vaginal rinse and/or a plug was observed in the vaginal opening. The day on which evidence of mating was observed was defined as Day 0 of gestation. The evenings for cohabitation of males with females were scheduled to provide females at Day 20 gestation sacrifice during the Monday-to-Friday work week. The number of females caged nightly with males was also controlled to limit the number of mated females available for sorting into groups on a particular day. In this study, the maximum number of females sorted into groups on a particular day was 12. When the number of females mated after an evening exceeded the number to be sorted among the groups that day, females were excluded from the sorting procedure using a random numbers table and the female's temporary cage card number.

Mating was conducted on 13 nights, 25-29 September and 2-6, 9-11 October 1995.

GROUP ASSIGNMENT

Females which mated were assigned to the groups daily in such a way as to provide an equal distribution of mated females among groups and equalize, as best possible, the Day 0 gestation mean body weights between groups.

ANIMAL IDENTIFICATION

Each female was assigned a temporary identification number upon receipt. Each mated female sorted into test groups was identified with a metal ear tag bearing its assigned animal number. This individual animal number plus the study number comprised a unique identification for each animal. Mated animals were eartagged on the day they were sorted into test groups which was Day 0 of gestation. Each non-exposure cage contained a card that was color coded for exposure level identification, and contained the study number and animal number.

ANIMAL HUSBANDRY - NON-EXPOSURE

Housing

Rats were housed individually, except during mating, in elevated, stainless steel suspended cages with wire mesh floors and fronts.

Food

Certified Rodent Diet, No. 5002; (Meal) (PMI Feeds, Inc., St Louis, MO) was available without restriction. Each animal's cage was designed to retain a glass feeder jar with a stainless steel lid. During the acclimation period, fresh feed was provided weekly to the rats. During gestation, fresh food was presented to the animals on Days 0, 6, 12 and 18. Note: On Day 18 some animals in each of the four groups were not presented with fresh feed. These animals were considered to have sufficient feed in their feeders to last to Day 20 gestation terminal sacrifice.

Analyses of Feed

Analyses of each feed lot used during this study were performed by the PMI Feeds, Inc. These data are maintained on file at the Testing Facility. Photocopies of analyses of feed lots used on study are included in Appendix P in the final report.

Water

Facility water was available without restriction (Supplier - Elizabethtown Water Company, Westfield, New Jersey, Raritan-East Millstone Plant) and provided to individual animal cages by an automated water delivery system.

Monthly Water Analyses

Monthly analyses of water supplied to this facility were provided by the Supplier. Photocopies of these analyses are included in Appendix P in the final report.

Biannual Water Analyses

Biannual chemical and microbiological analyses of water samples collected from representative rooms in the Testing Facility were conducted to assure that the water being provided met standards specified under the EPA National Primary Drinking Water Regulations (40 CFR Part 141). Photocopies of these analyses will be included in Appendix P in the final report.

Contaminants

There were no known contaminants in the feed or water which were considered capable of interfering with the results of this study.

Environmental Conditions

Twelve hour light/dark cycle via automatic timer. During acclimation, the light cycle in the animal room was approximately 0600 to 1800 hours. On Day 6 of gestation, animals were transferred to a different room for the remainder of the study. In this room the light cycle was also approximately 0600 to 1800 hours.

Temperature was monitored and recorded twice daily; relative humidity was monitored and recorded once daily.

	<u>Desired</u>	<u>Actual</u>
Temperature:	18 to 26°C	20 to 24°C
Relative Humidity:	40 to 70%	38 to 74%

ANIMAL HUSBANDRY DURING EXPOSURE

Housing

Animals were individually housed in wire mesh, stainless steel cages within a 1000 liter glass and stainless steel exposure chamber (see Appendix M for details).

Food and Water

None during exposure

Environmental Conditions

Chamber temperature and humidity were monitored and recorded every half hour during exposure and maintained, to the maximum extent possible, within the ranges presented below. See Appendix M for monitoring equipment details.

	<u>Desired</u>	<u>Actual</u>
Temperature:	20 to 24°C	20 to 25°C
Relative Humidity:	40 to 60%	34 to 70%

TEST MATERIAL ADMINISTRATION

Route of Administration

Inhalation, as a vapor, via whole-body exposure.

Justification for Route of Administration

The inhalation route is one of the potential routes of human exposure to the test material and is the route specified in the referenced guidelines.

Frequency and Duration of Exposure

Animals were exposed for six hours daily over the Day 6-19 gestation period.

Dates of Exposure

Day 6 of gestation. 2 - 18 October 1995

Day 19 of gestation. 15 - 31 October 1995

Prestudy Trials

Trials were performed to evaluate the optimal set of equipment and operating conditions to generate a stable atmosphere at the targeted exposure levels. See Appendix M, pages M-22 and M-23 for details of prestudy trials.

Chamber Operation

Chamber operation procedures as well as the chamber's airflow rate, time for air change and 99% equilibrium time (T_{99}) for each group are presented in Appendix M, page M-3.

TEST MATERIAL PREPARATION

The test material was used as received.

EXPOSURE PROCEDURES

Complete exposure procedures for all groups are presented in Appendix M, page M-6.

EXPOSURE CHAMBER SAMPLING

Total hydrocarbon levels were measured six times/exposure day for the 1000, 3000 and 9000 ppm groups and once daily for the controls using infrared spectrophotometry.

One sample/exposure for either the 1000, 3000 or 9000 ppm groups was analyzed rotating among the three exposure groups daily throughout the study, using a syringe grab sample and a gas chromatographic (GC) procedure to characterize airborne vapor components. The ratios of eleven major components to isopentane, the primary component of the test material, were analyzed. The twelve major components were: n-butane, isopentane, n-pentane, *trans*-2-pentene, 2-methyl-2-butene, 2,3-dimethylbutane, 2-methylpentane, 3-methylpentane, n-hexane, benzene, 2,2,4-trimethylpentane and toluene.

Details of the actual sampling procedures are presented in Appendix N.

Nominal Concentration

A nominal exposure concentration was calculated daily for the 1000, 3000 and 9000 ppm groups. The flow of air through the chamber was monitored using appropriate calibrated equipment. The test material consumed during the exposure was divided by the total volume of air passing through the chamber (volumetric flow rate multiplied by total exposure time) to give the nominal concentration.

Particle Size Distribution Analysis

Particle size distribution measurements were performed once during each exposure to characterize the aerodynamic particle size distribution of any aerosol present. This measurement determined whether any aerosol present was due to background aerosol vs. test material aerosol. Complete procedural information is presented in Appendix M.

EXPERIMENTAL EVALUATIONS

Observations

Viability Checks (In-Cage). Observations for mortality, general appearance and signs of severe toxic or pharmacologic effects were made twice daily (morning and afternoon).

Physical Examinations. Each rat was removed from its cage and given a detailed physical examination on Days 0, 6-19 and 20 of gestation. During the Day 6-19 period, animals were evaluated both pre- and post-exposure. The latter examination was performed approximately a half hour after exposures ceased when animals were removed from the chamber. Control animals were also removed from the chamber and examined at the same time as the test animals.

Body Weights

Each animal was weighed on Days 0, 3, 6, 9, 12, 15, 18 and 20 of gestation using a Mettler Balance, Model PE4000 (Mettler Instrument Corporation, Hightstown, New Jersey). Day 20 gestation body weights are presented as actual and corrected (the actual Day 20 gestation body weight minus the weight of the gravid uterus) values.

Food Consumption

Animals were presented with weighed feeders on Days 0, 3, 6, 9, 12, 15 and 18 of gestation. Feeders were removed on Days 3, 6, 9, 12, 15, 18 and 20 of gestation and weighed. All feeder weights were measured using a Mettler Balance, Model PE4000 (Mettler Instrument Corporation, Hightstown, New Jersey).

Calculations. To determine the amount of feed consumed, the weight of the feeder at the end of the measurement interval (feeder-out weight) was subtracted from the initial feeder weight (feeder-in weight). The resulting value represented the grams of feed consumed/interval. The following formula was used to calculate grams of feed consumed per kilogram of body weight per day (g/kg/day).

$$\text{g/kg/day} = \frac{\text{grams of feed consumed}}{\text{previous body weight (kilograms)}} \div \text{Number of days}$$

Measurement intervals (i.e., the number of days over which food consumption was measured) and the body weight used to calculate grams of feed consumed/kg body weight are as follows:

- Day 0-3 = 3-day interval using Day 0 body weight.
- Day 3-6 = 3-day interval using Day 3 body weight.
- Day 6-9 = 3-day interval using Day 6 body weight.
- Day 9-12 = 3-day interval using Day 9 body weight.
- Day 12-15 = 3-day interval using Day 12 body weight.
- Day 15-18 = 3-day interval using Day 15 body weight.
- Day 18-20 = 2-day interval using Day 18 body weight.

Body weights and feeder weights were recorded to the nearest tenth of a gram and are presented in this report as a rounded whole number; the reported g/kg/day of food consumption was calculated using the unrounded body weights and feeder weights.

MATERNAL POSTMORTEM EXAMINATIONS

Macroscopic Postmortem Examinations

Complete macroscopic postmortem examinations were performed on all test animals. This included examination of all surfaces, all orifices, the cranial cavity, carcass, the external surface of the spinal cord and sectioned surfaces of the brain, nasal cavity and paranasal sinuses, the thoracic, abdominal and pelvic cavities and their viscera and the cervical tissues and organs. The carcass of each female was discarded at completion of the macroscopic postmortem examination. Only gross lesions were saved in 10% neutral buffered formalin.

Animals Sacrificed

All animals were exsanguinated following anesthesia with inhaled carbon dioxide on Day 20 of gestation during the period of 16-31 October and 1 November 1995.

Reproductive System

The intact uterus (ovaries attached) was removed from the abdominal cavity and weighed. The ovaries were dissected free to be examined for the presence and number of *corpora lutea*. The uteri were dissected longitudinally along the antimesometrial border and the number and location of the following were recorded for each horn: live fetuses (movement in response to touch); dead fetuses (absence of movement in response to touch with no visible degeneration); late resorptions (recognizable dead fetus undergoing degeneration regardless of size); early resorptions (evidence of implantation but no recognizable fetus); and implantation sites (total of live, dead and resorbed fetuses).

When no uterine implants were grossly apparent, the uterus was stained with ammonium sulfide (Salewski, 1964). When no uterine foci were visualized poststaining, the female was considered not pregnant.

FETAL EVALUATIONS

External Evaluations

All fetuses were weighed, using a Mettler Balance, Model No. PE4000 (Mettler Instrument Corporation, Hightstown, New Jersey). In addition, all fetuses were sexed externally (general observation of ano-genital distance) and given a macroscopic external examination for malformations and variations that included observations for palatal defects.

Soft Tissue Evaluations

Approximately one-half of the fetuses in each litter (alternating fetuses within the litter) were evaluated for soft-tissue malformations/variations using a microdissection procedure similar to that described by Staples (1974). Evaluations were performed on the fresh fetal specimens shortly after removal from the uterus. Fetuses designated for soft tissue evaluation were decapitated (head placed in appropriately labelled tissue bags [i.e., teabags] and fixed in Bouin's solution for later evaluation). The decapitated fetal specimens were then secured beneath a dissecting microscope and dissected so as to permit evaluation of tissues in the thoracic, abdominal and pelvic cavities. At the completion of the fetal examination, the fetuses with viscera intact were placed in individual plastic cassettes and stored in a 10% neutral buffered formalin solution. Following a period of fixation, the fetal heads were sectioned using a razor blade. The serial, transverse sections generated during this procedure were evaluated for malformations of the palate, eyes and brain under a dissecting microscope. Following evaluation, head sections were placed in plastic cassettes for storage (one litter/jar) in a 70% ethanol solution.

Fetal Skeletal Evaluations

The remaining fetuses in each litter were killed via an overdose of inhaled carbon dioxide. The intact fetuses were eviscerated (internally sexed by inspection of the gonads) and processed for staining of the skeletal structures with Alizarin Red S using a staining procedure of Crary as modified by the Testing Facility as follows: 1. Specimens were not air dried but were placed in a 0.5% potassium hydroxide solution immediately following evisceration; 2. There was a provision for a separate Alizarin Red S staining step; 3. There was a provision for a separate destaining step using a 20% glycerin aqueous solution which contained a small amount of potassium

hydroxide (0.01%) to remove excess stain from the specimens; 4. Specimens were cleared in a process of moving through graded solutions of glycerin (50%, 80% and 100%); 5. Specimens were stored in 100% glycerin to which several crystals of thymol have been added. Fetal skeletal specimens were evaluated under a dissecting microscope for malformations and ossification variations.

Resorptions

Late resorptions were weighed, examined macroscopically for external malformations and discarded. Only late resorptions with obvious external malformations were saved (10% neutral buffered formalin). Early resorptions were discarded.

STATISTICAL ANALYSES

Continuous Data

The following parameters were analyzed statistically:

Mean body weights during gestation: Days 0, 3, 6, 9, 12, 15, 18 and 20

Mean body weight change during gestation: Days 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, 18-20 and cumulative to include Days 0-6 and 6-20. A cumulative weight gain for the Gestation Day 6-20 interval was also calculated for each animal using the corrected Day 20 gestation weights.

Mean food consumption values during gestation: Days 0-3, 3-6, 6-9, 9-12, 12-15, 15-18 and 18-20

Reproduction Data

Mean number of *corpora lutea*

Mean number of uterine implantation sites per female

Mean litter size (number of live fetuses per female)

Mean number of resorptions per female

Mean preimplantation loss ratio (*corpora lutea* minus implantations/*corpora lutea*)

Mean resorption/implant ratio

Mean number of male and female fetuses per female

Mean fetal weight (composite of both sexes and distinguished by sex).

Incidence Data

Females with resorptions

Pregnancy rates

Incidence of litters with resorptions

Incidence of fetuses with malformation/variations (external, soft tissue and skeletal)

Incidence of litters containing fetuses with malformations/variations (external, soft tissue and skeletal).

STATISTICAL ANALYSES/CONTINUOUS DATA

Interval Data - Multiple Group (Method A)

Statistical evaluation of equality of means was made by the appropriate one-way analysis of variance technique, followed by a multiple comparison procedure, if needed. First, Bartlett's test (Snedecor and Cochran, 1967) was performed to determine if groups had equal variance. If the variances were equal, parametric procedures were used; if not, nonparametric procedures were used. The parametric procedures were the standard one-way ANOVA (Snedecor and Cochran, 1967) using the F distribution to assess significance. If significant differences among the means were indicated, Dunnett's test (Dunnett, 1955; Dunnett, 1964) was used to determine which means were significantly different from the control. If a nonparametric procedure for testing equality of means was needed, the Kruskal-Wallis test (Hollander and Wolfe, 1973) was used, and if differences were indicated, a summed rank test (Dunn) (Hollander and Wolfe, 1973) was used to determine which treatments differed from control.

A statistical test for trend in the dose levels was also performed. When parametric procedures were appropriate (i.e., equal variance), standard regression techniques with a test for trend and lack-of-fit were used (Snedecor and Cochran, 1967). When nonparametric were appropriate, Jonckheere's test (Hollander and Wolfe, 1973) for monotonic trend was used.

All ratios (pre- and post-implantation loss indices) were transformed via Bartlett's transformation followed by the arc-sine transformation (Snedecor and Cochran, 1967) prior to analysis. Data are presented untransformed.

The test for equal variance (Bartlett's) was conducted at the 1% two-sided risk level. All other statistical tests were conducted at the 5% and 1%, two-sided risk levels.

Key to Statistical Symbols-Interval Data - Multiple Group (Method A)

STATISTICAL SYMBOL			STATISTICAL STATEMENT
No Sig	$p \leq 0.05$	$p \leq 0.01$	
<u>Parametric</u>			
A-			No statistical differences among the means (parametric ANOVA).
	A	A+	The means differ significantly (parametric ANOVA).
L-			The response is not linearly related to the dose levels.
	L	L+	The response is linearly related to the dose levels.
	Q	Q+	The response shows a lack-of-fit.
	*	**	Significantly different from control (Dunnett's).
NT			Not tested due to lack of variability.
<u>Nonparametric</u>			
K-			No statistical differences among the means (Kruskal-Wallis, nonparametric).
	K	K+	The means differ significantly (Kruskal-Wallis nonparametric).
J-			There is not an ordered response to dosage.
	J	J+	There is an ordered response to dosage.
	*	**	Significantly different from control (Dunn's Rank Sum).
NT			Not tested due to lack of variability.

Statistical symbols are presented on the mean and summary tables of the report.

STATISTICAL ANALYSES/INCIDENCE DATA

Incidence Data - Method B

Statistical analysis of incidence data was performed using contingency tables. First, a standard Chi-square analysis (Snedecor and Cochran, 1971) was performed to determine if the proportion of incidences differed between the groups tested. Next, each treatment group was compared to the control group using a 2x2 Fisher Exact Test (Bradley, J. V., 1968); the significance level was corrected via the Bonferroni inequality (Miller, R. G., Jr., 1966) to assure an overall test of the stated significance level. Thirdly, Armitage's test (Armitage, P., 1955) for linear trend in the dosage groups was performed. In keeping with standard statistical practice, if any one cell had

an expected value of less than 5, the Chi-square and Armitage's tests were not reported. When this occurred, only the Fisher Exact test (corrected via Bonferroni inequality) was performed and reported.

All tests were reported at the 5% and 1% level of significance.

Key to Statistical Symbols - Incidence Data - (Method B)

STATISTICAL SYMBOL			STATISTICAL STATEMENT
No Sig	$p \leq 0.05$	$p \leq 0.01$	
C-			No statistical differences among the groups (chi-square).
	C	C+	The groups differ significantly (chi-square).
	*	**	Significantly different from control (Fisher Exact Test).
	A	A+	The response is linearly related to the dose levels (Armitage Test).
	F	F+	The response shows a lack of fit.
NS			No statistical differences from control (Fisher Exact Test, when any one cell had an expected value less than 5).
NT			Not tested due to lack of variability.
(FE)			Indicates significance by the Fisher Exact Test when any one cell had an expected value less than 5. An asterisk (*) will appear next to the treated group which is significantly different from the control group.

Statistical symbols are presented on the mean and summary tables of the report.

PROTOCOL DEVIATIONS

The following protocol deviations occurred during the study but were not considered to have compromised the validity or integrity of the study:

1. Due to a technician error, no GC analyses were performed on 29 Oct. 95 for the mid-dose exposure (Group III). This deviates from Section 10.6 of the study protocol which states that "one fingerprint will be analyzed daily rotating among the three exposure groups throughout the exposure period." On the following day, a GC analysis was performed for the mid-dose exposure along with the scheduled analysis.
2. Because the number of components analyzed in the GC fingerprint was revised after the study had begun, one component, 2,2,4-trimethylpentane, was not included in these analysis until the fourth exposure day.

Section 4

RESULTS AND DISCUSSION/CONCLUSION

CHAMBER MONITORING (APPENDIX M)

Exposure Levels

The complete chamber monitoring results are presented in Appendix M. These results include total hydrocarbon exposure levels obtained from a MIRAN[®] infrared spectrophotometer and GC (gas chromatography) fingerprints obtained from syringe samples. Each day, one GC syringe sample was taken from the exposure chamber for either Group II (1000 ppm), III (3000 ppm) or IV (9000 ppm), to confirm composition and stability of the test material among exposure groups and over the course of the study.

Prestudy chamber trials were conducted to determine the optimum conditions for producing the target exposure levels. These trials also included distribution analyses (Appendix M, page M-23) which showed the test material was evenly distributed within each chamber.

The target, mean total hydrocarbon, and nominal concentrations for this study are summarized below (Table 1):

Table 1: Summary of Concentrations

	Target Concentration (ppm)	Miran Analytical Concentration (ppm)	Nominal Concentration (ppm)
		Mean $\pm$ S.D.	Mean $\pm$ S.D.
I	0	0	-
II	1000	1015 $\pm$ 35	1018 $\pm$ 117
III	3000	2984 $\pm$ 147	3227 $\pm$ 208
IV	9000	8993 $\pm$ 278	9033 $\pm$ 368

The achieved mean exposure concentration for each group was very close to the respective target and nominal concentrations. Chamber environmental conditions averaged 22°C and 54% relative humidity.

Syringe Samples

The results of the GC syringe sample fingerprints of the exposure atmospheres are summarized below (Table 2). The results, which present the ratio of eleven major components to isopentane, the largest component, showed similarity among all exposure groups as well as reasonable comparison to the neat liquid test material. In addition, the individual syringe sample data showed minimal variation, as reflected in the standard deviation, over the course of the study. A new shipment of test material (Lot No. API 94-02) was used for exposures of Groups II and IV for the last four exposure days (Group II Animal Nos. 2521 and 2522 and Group IV Animal Nos. 4521 and 4522 [Days 18-19]; Group II Animal No 2523 [Days 17-19]; Group II Animal No. 2524 and Group IV Animal Nos. 4523 and 4524 [Days 16-19]). This may account for any shift in the component ratios over this period.

Table 2: Summary of GC Syringe Sample Fingerprints of Exposure Atmosphere Results
Ratio of each of the following eleven major components to Isopentane:

	n-Butane	n-Pentane	trans-2-Pentene	2-Methyl-2-butene	2,3-Dimethyl-butane	2-Methyl-pentane	3-Methyl-pentane	n-Hexane	Benzene	2,2,4-Tri-methyl-pentane	Toluene
Neat Liquid											
Mean	0.534	0.240	0.0649	0.0944	0.0876	0.254	0.130	0.0817	0.0536	-	0.0737
Group II - 1000 ppm											
Mean	0.459	0.274	0.0813	0.115	0.0985	0.293	0.152	0.101	0.0844	0.043	0.1075
S.D.	0.1190	0.0140	0.00438	0.00868	0.0144	0.0448	0.0245	0.0199	0.0146	0.00962	0.023
Group III - 3000 ppm											
Mean	0.639	0.257	0.0768	0.108	0.0837	0.246	0.127	0.0835	0.0682	0.0334	0.0851
S.D.	0.0433	0.00359	0.00101	0.00163	0.00236	0.00788	0.00440	0.00358	0.00258	0.00163	0.00372
Group IV - 9000 ppm											
Mean	0.593	0.259	0.0775	0.109	0.0865	0.255	0.132	0.0856	0.0713	0.0345	0.0873
S.D.	0.0787	0.00585	0.00195	0.00313	0.00520	0.0159	0.00894	0.00624	0.00529	0.00324	0.00934

Particle Sizing

Particle size distribution measurements of the background aerosol from all the exposure groups are summarized on the next page (Table 3):

Table 3: Summary of Particle Size Distribution Measurements

Group	Mass Median Aerodynamic Diameter (μm)	Geometric Standard Deviation	Total Mass Concentration (mg/m^3)
I	2.8	2.1	3.08×10^{-3}
II	2.5	2.1	4.18×10^{-3}
III	2.2	2.0	2.95×10^{-3}
IV	2.0	1.9	2.77×10^{-3}

The similarity of concentration and particle size of the background aerosol among all of these groups indicated that there was no measurable test material aerosol present.

MATERNAL DATA

Mortality (Appendix A)

No mortality occurred among the control or treated groups as all animals survived to scheduled sacrifice.

Pregnancy Rates (Appendix G)

Pregnancy rates in the treated groups were comparable to control data and no adverse effect of treatment with Unleaded Gasoline Vapor Condensate (API 94-02) was indicated from these data.

Pregnancy rates are summarized below (Table 4):

Table 4: Pregnancy Rates

Group (ppm)	Pregnancy Rate	
	No. pregnant ^a	Percent
I (0)	24	100
II (1000)	22	91.7
III (3000)	22	91.7
IV (9000)	21	87.5

No statistically significant differences from control (see Appendix G, page G-1).

^aEach group contained 24 mated females.

Gestation Body Weight and Weight Gain Data (Figure 1 and Appendices B, C and D)

Mean maternal body weights and weight gain data during gestation were not adversely affected by treatment. Mean weight gain over the entire treatment period (Days 6-20 of gestation) for the treated groups was slightly higher than control data: these values for the control, low-, mid- and high-dose groups were 112, 120, 121 and 118 grams, respectively. Mean weight gain for the treated groups over the Day 6-20 gestation interval using the corrected Day 20 gestation weights, were comparable to control data; these values for the control, low-, mid- and high-dose groups were 31.2, 30.5, 31.6 and 28.1 grams, respectively.

Food Consumption Data - Gestation Period (Figure 2 and Appendix E)

Mean food consumption data during the pre-treatment (Days 0-3 and 3-6) and treatment period (Days 6-9, 9-12, 12-15, 15-18 and 18-20) for the Unleaded Gasoline Vapor Condensate-treated groups were comparable to control data and no adverse effect of treatment was indicated.

Physical Observation Data (Pre- and post-exposure data - Appendix F)

No adverse effect of treatment from exposure to Unleaded Gasoline Vapor Condensate (API 94-02) was indicated from the detailed physical examinations performed both pre- and post-exposure during the treatment period. The types of observations seen among the treated groups occurred at low incidence or with comparable frequency to the control group and were not considered to be related to treatment.

Corpora lutea and Uterine Implantation Data (Appendices G and H)

No adverse effect of treatment with Unleaded Gasoline Vapor Condensate (API 94-02) was evident from uterine implantation data. No aborted pregnancies or premature deliveries occurred among the control or treated groups. The number of litters containing viable fetuses recovered at Day 20 gestation maternal sacrifice for the control, low-, mid- and high-dose groups was 24, 22, 22 and 21, respectively.

The mean numbers of *corpora lutea*, uterine implantation sites, live fetuses and resorptions per pregnant female for the treated groups were comparable to control data. Likewise, the mean pre- and post-implantation loss indices for the treated groups were comparable to control data. No dead fetuses were recovered from the control or treated groups.

Macroscopic Postmortem Evaluations (Appendix I)

All animals were examined postmortem for the presence of macroscopic abnormalities. Those observed occurred sporadically and were considered incidental and not related to the test material.

FETAL DATA

Fetal Body Weight Data (Appendices G and J)

No adverse effect of treatment was indicated from fetal weight data. Mean fetal weights, distinguished by sex and as a composite for both sexes, for the Unleaded Gasoline Vapor Condensate-treated groups were comparable to control data. Fetal weights for the treated and control groups were within the range of recent historical control data for this laboratory (Appendix O, page O-4).

Fetal Sex Distribution Data (Appendices G and J)

No adverse effect of treatment was indicated from fetal sex distribution data. The mean number of male and female fetuses per pregnant female and the ratio of total male to female fetuses for the Unleaded Gasoline Vapor Condensate-treated groups were comparable to control data.

Fetal External Examination Data (Appendix J)

No external malformations or variations were seen among fetuses recovered from the control or Unleaded Gasoline Vapor Condensate-treated groups. The numbers of fetuses and litters evaluated were as follows: control group - 327 fetuses from 24 litters; low-dose group - 334 fetuses from 22 litters; mid-dose group - 336 fetuses from 22 litters; and high-dose group - 325 fetuses from 21 litters.