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In accordance with API's policy of providing the federal government with copies of research designed to determine whether any chemical substance or mixture manufactured, processed or distributed by API member companies may cause a risk of injury to health or the environment, we are enclosing a copy of the following final reports:

(Identification no: Not Assigned)

Title: *A Range-Finding Developmental Inhalation Toxicity Study of Unleaded Gasoline Vapor Condensate in Rats and Mice via Whole-Body Exposure*

(Identification no: Not Assigned)

Title: *An Inhalation Developmental Toxicity Study of Unleaded Gasoline Vapor Condensate in Rats via Whole-Body Exposure*

Please note that this information is provided in accordance with the full disclosure policy of API and does not constitute a formal submission as required by a test rule. These documents do not contain confidential information. If you have any questions, please communicate with me.

Sincerely,

Carol J. Henry, Ph.D.

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# GOLDMAN ASSOCIATES INTERNATIONAL, INC.

## Quality Assurance Statement

Study Title: A Range-Finding Developmental Inhalation Toxicity Study of Regular Unleaded Gasoline Vapor Condensate (API 94-02) in Rats and Mice Via Whole-Body Exposure - Study No.: 95-6082 and API Study No.: 08200-0601-SH9343

Test Article: Unleaded Gasoline Vapor Condensate (API 94-02)

Quality assurance inspections/audits on this study were conducted by Goldman Associates International, Inc. on the schedule shown below.

Date of Inspection	Type of Inspection	Date Issued
June 6, 1995	Protocol	July 14, 1995
June 30, 1995	Phase audit-test substance generation, analytical procedures, and animal exposure	July 14, 1995
July 12, 1995	Phase audit-animal observations, body weights, feeder weights, animal loading into chambers, chamber start-up, analytical sampling, animal sacrifice, necropsy and fetal evaluations	August 5, 1995
July 22, 1995	Protocol Amendment 1	August 5, 1995
October 31 - November 2, 1995	Draft Report	December 22, 1995
April 28-29, 1997	Reformatted Report audit	May 9, 1997
July 14, 1997	Second Reformatted Report audit	September 3, 1997

*Linda J. Calisti* 9/3/97  
Linda J. Calisti, E.S. Date  
Goldman Associates International, Inc.

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

Health and Environmental Sciences Department  
Publication Number TR 412  
October 1997

**A Range-Finding Developmental  
Inhalation Toxicity Study of Unleaded  
Gasoline Vapor Condensate in Rats and  
Mice via Whole-Body Exposures**

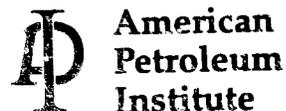
**Health and Environmental Sciences Department**

API PUBLICATION NUMBER TR 412

PREPARED UNDER CONTRACT BY:

HUNTINGDON LIFE SCIENCES  
METTLERS ROAD  
PO Box 2360  
EAST MILLSTONE, NJ 08875-2360

OCTOBER 1997



## ABSTRACT

This range-finding study was conducted for American Petroleum Institute seeking concentration levels appropriate to assess the potential maternal toxicity and developmental toxicity of Unleaded Gasoline Vapor Condensate (API 94-02) administered by inhalation (whole-body exposure) to mated rats during the Day 6-19 gestation interval and to mated mice during the Day 6-17 gestation interval. Exposure levels were 0 (filtered air), 300, 1000, 3000 and 9000 ppm. These data will be used to identify the appropriate rodent species and exposure levels to be used in a definitive inhalation developmental toxicity study. In this study, no maternal or developmental toxicity in either rats or mice was seen up to an exposure level of 3000 ppm. At the highest exposure level evaluated (9000 ppm), maternal toxicity was seen only in the rat (reduced body weights, weight gain and food consumption during the treatment period). No evidence of developmental toxicity was seen in either species at the 9000 ppm exposure level.

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STUDY NO. 95-6082

API STUDY NO. 08200-0601-SH9343

A RANGE-FINDING DEVELOPMENTAL INHALATION TOXICITY STUDY  
OF UNLEADED GASOLINE VAPOR CONDENSATE (API 94-02) IN RATS AND MICE  
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.

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

29 September 97  
Date

Carol S. Auletta  
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Senior Director, Toxicology

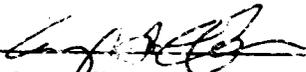
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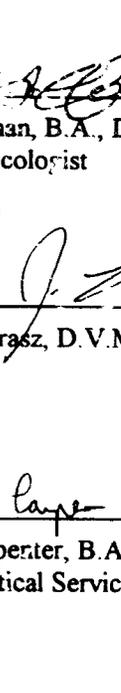
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Vice President, Research and Pathology

29 Sep 97  
Date

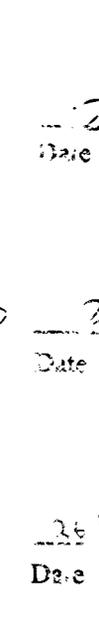
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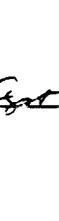
LABORATORY INDIVIDUAL SIGNATURE PAGE

  
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Pathologist  
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Howard L. Carpenter, B.A.  
Scientist, Analytical Services  
Date 26 Sept 97

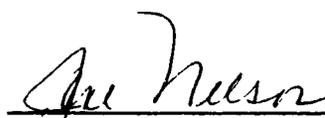
  
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Dari Dadgar, Ph.D.  
Vice President, Analytical Services  
Date 25 Sept 1997

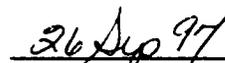
  
\_\_\_\_\_  
Laura V. Cojocaru, B.S. Chem. Eng.  
Manager, Analytical Services  
Date 16 Sept 1997

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	8 Jun 95	09 Jun 95	19 Jun 95 and 30 Jun 95
Mating Observations	27 Jun 95	27 Jun 95	3 Jul 95 and 14 Jul 95
Exposure and Monitoring	26 Jun 95	27 Jun 95	30 Jul 95 and 23 Aug 95
Gestation Body Weights and Feeder Weights	7 Jul 95	7 Jul 95	14 Jul 95 and 23 Aug 95
Terminal Sacrifice	10 Jul 95	10 Jul 95	14 Jul 95 and 23 Aug 95
Final Analytical Report	16 Oct 95 to 19 Oct 95	19 Oct 95	20 Oct 95
Final In-Life and Pathology Report	16 Oct 95 to 20 Oct 95	20 Oct 95	20 Oct 95
Report Revisions	2 Apr 97	2 Apr 97	2 Apr 97
Report Revisions	26 Jun 97 to 1 Jul 97	7 Jul 97	7 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

25 Sept 87  
Date

## Section I

### SUMMARY

This inhalation study was performed to provide range-finding information on the maternal and developmental toxicity of Unleaded Gasoline Vapor Condensate (API 94-02) in rats and mice. Data from this study will be used to identify the appropriate rodent species and exposure levels to use in a definitive inhalation developmental toxicity study. Unleaded Gasoline Vapor Condensate (API 94-02) was administered as a vapor, via inhalation (whole-body) exposure, 6 hours/day to 40 mated rats (10/group) during Days 6-19 of gestation and to 40 mated mice (10/group) during Days 6-17 of gestation. Exposure levels were 300, 1000, 3000, and 9000 ppm. Ten mated rats and ten mated mice, 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. Body weights were recorded on Days 0, 3, 6, 9, 12, 15, 18, and 20 of gestation in the rat and Days 0, 3, 6, 9, 12, 15, and 18 of gestation in the mouse. Food consumption was recorded on Days 0-3, 3-6, 6-9, 9-12, 12-15, 15-18, and 18-20 of gestation for the rats and Days 0-3, 3-6, 6-8, 8-10, 10-12, 12-14, 14-16, and 16-18 of gestation for the mice.

Chamber exposure concentrations were evaluated daily using infrared spectrophotometry (hourly), gas chromatography (single grab sample, daily) and analysis of charcoal tubes (four samples/day). The latter were analyzed to monitor the ratio of the ten major components of the test material.

At terminal sacrifice (Day 18 of gestation for the mouse and Day 20 of gestation for the rat), 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 fetuses were then sacrificed with an overdose of inhaled carbon dioxide and discarded.

The mean daily total hydrocarbon concentrations  $\pm$  standard deviations from the infrared spectrophotometric evaluation over the entire exposure period for the 300, 1000, 3000 and 9000 ppm groups were  $314 \pm 31$ ,  $1034 \pm 63$ ,  $3073 \pm 146$  and  $9025 \pm 417$  ppm, respectively. Gas chromatograph analyses of syringe and charcoal tube samples of the exposure atmospheres, demonstrated a similarity among all exposure groups and a stability of the test material during each exposure day. In these data, nine 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 for either species.

In the mouse, no maternal or developmental toxicity was seen at any exposure level of Unleaded Gasoline Vapor Condensate (API 94-02) evaluated.

In the rat, no maternal or developmental toxicity was seen at exposure levels up to and including 3000 ppm. At the 9000 ppm exposure level, the following statistically significant changes were seen in comparison to control data and were considered indicative of maternal toxicity: reduced body weights on Days 12, 15 and 20 of gestation; reduced weight gain over the Day 6-20 gestation interval using the actual Day 20 gestation weight; and reduced food consumption over the Day 6-9 gestation interval. No developmental toxicity was seen in the rat at the 9000 ppm exposure level.

## Section 2

### INTRODUCTION

The procedures and results obtained from a range-finding inhalation toxicity study of Unleaded Gasoline Vapor Condensate (API 94-02) in rats and mice via whole body exposure are presented in this report. 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 range-finding information on the maternal and developmental toxicity of Unleaded Gasoline Vapor Condensate (API 94-02) in rats and mice. Data from this study will be used to identify the appropriate rodent species and exposure levels to use in a definitive inhalation developmental toxicity study. Unleaded Gasoline Vapor Condensate (API 94-02) was administered as a vapor, via inhalation (whole-body) exposure, 6 hours/day to 40 mated rats (10/group) during Days 6-19 of gestation and to 40 mated mice (10/group) during Days 6-17 of gestation. Exposure levels were 300, 1000, 3000, and 9000 ppm. Ten mated rats and ten mated mice which served as controls, were chamber-housed and received filtered room air only, 6 hours/day over the same treatment intervals.

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 J and similar data for the mouse are presented in Appendices K through T. Data regarding the inhalation exposures are presented in Appendix U. Results, as well as methods used, for the chamber analyses performed by the Testing Facility's Metabolism and Analytical Department are presented in Appendix V. Historical control data for both rats and mice are presented in Appendix W. A copy of the study protocol and protocol amendments are presented in Appendix X.

### Section 3

## MATERIALS AND METHODS/REFERENCES

### REGULATORY REFERENCES

#### 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.

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.  
Washington, D.C. 20005

#### Sponsor Representative

Richard A. Rhoden, Ph.D.

**Testing Facility**

Huntingdon Life Sciences  
Mettlers Road  
P.O. Box 2360  
East Millstone, New Jersey 08875-2360

**Study Director**

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

**EXPERIMENTAL DESIGN**

Group	Test Material	Exposure Level (ppm)	Treatment Schedule <sup>a</sup>		Number of Animals				Proportion of Fetuses/Litter Evaluated for External Malformations and/or Variations
			Rats (GD)	Mice (GD)	Mated		Sacrificed <sup>b</sup>		
					Rats	Mice	Rats (GD 20)	Mice (GD 18)	
I	Control	0	6-19	6-17	10	10	10	10	All
II	Unleaded Gasoline Vapor Condensate (API 94-02)	300	6-19	6-17	10	10	10	10	All
III	Unleaded Gasoline Vapor Condensate (API 94-02)	1000	6-19	6-17	10	10	10	10	All
IV	Unleaded Gasoline Vapor Condensate (API 94-02)	3000	6-19	6-17	10	10	10	10	All
V	Unleaded Gasoline Vapor Condensate (API 94-02)	9000 <sup>c</sup>	6-19	6-17	10	10	10	10	All

<sup>a</sup>Exposures were 6 hours/day.

<sup>b</sup>Complete gross postmortem evaluations were performed on all animals.

GD = gestation day

<sup>c</sup>The 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.

## STUDY DATES

### Study Initiation (Date Study Director signed the Protocol)

14 June 1995

### Initiation of Mating (Experimental Start Date)

Rats: 19 June 1995

Mice: 21 June 1995

### Initiation of Exposure

Rats: 26 June 1995

Mice: 28 June 1995

### Termination of Exposure

Rats: 19 July 1995

Mice: 13 July 1995

### Terminal Sacrifice

Rats: 10-20 July 1995

Mice: 10-14 July 1995

### Experimental Termination (Date of Last Data Collection)

Rats: 20 July 1995

Mice: 14 July 1995

### Study Termination

Date Final Report is signed by the Study Director.

## TEST MATERIAL INFORMATION

TEST MATERIAL	LOT NO	PURITY	DESCRIPTION	DATE RECEIVED	EXPIRATION DATE
Unleaded Gasoline Vapor Condensate (API 94-02)	API 94-02	Considered 100%	Clear liquid	6 March 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 was the responsibility of the Sponsor.

### Stability

Documentation of the stability of the test material was the responsibility of the Sponsor.

### Storage

Upon receipt, the test material was stored at ambient temperature in an outdoor solvent shed. When transferred indoors for use, the test material was stored at room temperature except during atmosphere generation when it was kept frozen at a temperature of -20°C.

### 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, are being retained at the Testing Facility until completion of the definitive study.

## TEST ANIMAL INFORMATION

### Test Animals

Rats: Albino (Outbred) VAF/Plus®

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

Mice: Albino

Strain: CD-1™ [CrI: CD-1®(ICR) BR]

### Justification for Animal Selection

The rat and mouse are rodent animal models commonly used in developmental toxicity studies as recommended in the EPA-TSCA guidelines. In addition, historical control data are available for these species in this laboratory for comparative evaluation.

### Number of Animals Purchased

Rats: 75 females

Mice: 90 females

All purchased animals were nulliparous and non-pregnant.

### Number of Animals Placed On Test

Rats: 50 females

Mice: 50 females

### Supplier - Males and Females

Charles River Laboratories  
Portage, Michigan 49081

### Date Received - Females

Rats and Mice: 22 May 1995

**Date Received - Males**

Rats and Mice: 1 May 1995

For rats, proven breeders were used solely for mating purposes (in-house breeding colony).

For mice, young males were received and used strictly for mating this study.

**Age at Receipt - Females**

Rats and Mice: 57 days old

**Age at Receipt - Males**

Rats: 50 days old

Mice: 43 days old

**Age at Initiation of Mating - Females**

Rats: 85 days old

Mice: 87 days old

**Age at Initiation of Mating - Males**

Rats: 99 days old

Mice: 92 days old

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

	<u>Mean</u> (grams)	<u>Range</u> (grams)
Rats:	260	219-293
Mice:	29	25-34

**Acclimation Period - Females**

Rats: 28 days

Mice: 30 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

### Rats

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.

Mating was conducted on nine nights, 19-23, 26-29 June 1995.

### Mice

Females selected for mating were placed with male mice nightly in a 1:1 ratio. In the morning, following the nightly interval of cohousing, females were evaluated for the presence of a vaginal copulatory plug. 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 18 gestation sacrifice during the Monday-to-Friday work week.

Mating was conducted on five nights, 21-25 June 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 animal sorted into test groups was identified with a metal ear tag (rats) or a tail tattoo (mice) bearing its assigned animal number. This individual animal number plus the study number comprised a unique identification for each animal. Mated animals were either eartagged or tail tattooed on the day they were sorted into test groups, which was Day 0 of gestation. Each non-exposure cage was provided with 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.

Two mice of the same sex were housed together in stainless steel, suspended cages with wire mesh floors and fronts for the first week of acclimation. Thereafter, except during mating, animals were housed individually.

### Food

Certified Rodent Diet, No. 5002; (Meal) (PMI Feeds, Inc., St Louis, MO) was available without restriction. Each animal's cage was fitted to retain a glass feeder jar with a stainless steel lid. During the acclimation period, fresh feed was provided weekly to the rats while the mice received fresh feed every three to four days. During gestation, fresh food was presented to the rats on Days 0, 6 and 12. The mice were provided with fresh food on Days 0, 3, 6, 10 and 14 of gestation. (Note: For the mice, one animal each from the control, 300, 1000 and 3000 ppm groups and two animals from the 9000 ppm groups were not provided with fresh feeders on Day 3 of gestation due to an oversight. This oversight was not considered to have compromised the validity or integrity of the study as the feeders contained sufficient feed for the animals to Day 6.)

### 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.

### Water

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

### Monthly Water Analyses

Monthly analyses of water supplied to this facility were provided by the Supplier. These data are maintained on file at the Testing Facility.

### 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). These data are maintained on file at the Testing Facility.

### 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 0700 to 1900 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 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%	42 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 U for details).

### Food and Water

None

### 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 U for monitoring equipment details.

	<u>Desired</u>	<u>Actual</u>
Temperature:	20 to 24°C	21 to 26°C
Relative Humidity:	40 to 60%	42 to 68%

## 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.

#### Frequency and Duration Of Exposure

Rats were exposed for 6 hours daily over the Day 6-19 gestation period. Mice were exposed for 6 hours daily over the Day 6-17 gestation period.

#### Dates of Exposure

Day 6 of gestation. Rats: 26 June - 6 July 1995

Mice: 28 June - 2 July 1995

Day 19 of gestation. Rats: 9-19 July 1995

Day 17 of gestation. Mice: 9-13 July 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 U, pages 23 and 24 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 U.

#### TEST MATERIAL PREPARATION

The test material was used as received.

#### EXPOSURE PROCEDURES

Complete exposure procedures for all groups, both species, are presented in Appendix U.

## EXPOSURE CHAMBER SAMPLING

Total hydrocarbon levels were measured six times/exposure for the 300, 1000, 3000 and 9000 ppm groups and once daily for the controls using infrared spectrophotometry.

One sample/exposure for the 300, 1000, 3000 and 9000 ppm groups was analyzed using a syringe grab sample and a gas chromatographic (GC) procedure to characterize airborne vapor components. The ten major components listed below were identified for analysis: isopentane, n-Butane; N-Pentane; *trans*-2 Pentene; 2-Methyl-2-butene; 2,3 Dimethylbutane; 2-Methylpentane; 3-Methylpentane; n-Hexane; Toluene.

Four samples/exposure for the 300, 1000, 3000 and 9000 ppm groups were collected in charcoal tubes and analyzed to monitor the ratio of the major components. The ten major components listed above were identified for analysis. Details of the actual sampling procedures are presented in Appendix U.

### Nominal Concentration

A nominal exposure concentration was calculated daily for the 300, 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 using the following calculation:

$$\text{Nominal concentration (ppm)} = \frac{\Delta \text{wt (g)}}{\text{Vol (L)}} \times \frac{24.457 \text{ L/mole}}{\text{MW (g/mole)}} \times 10^6 \mu\text{l/L.}$$

### 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 U.

## 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) on both species.

Physical Examinations. Each rat was removed from its cage and given a detailed physical examination on Days 0, 6-19 and 20 of gestation. In the mouse, these detailed examinations were performed on Days 0, 6-17 and 18 of gestation. During the exposure period, animals were evaluated both pre-exposure 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 rat 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.

Each mouse was weighed on Days 0, 3, 6, 9, 12, 15 and 18 of gestation using a Sartorius Electric Top Loading Balance, Model Number U3600 (Sartorius Corporation, Edgewood, New York). Day 18 gestation body weights are presented as actual and corrected (the actual Day 18 gestation body weight minus the weight of the gravid uterus) values.

### Food Consumption

Rats 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).

Mice were presented with weighed feeders on Days 0, 3, 6, 8, 10, 12, 14 and 16 of gestation. Feeders were removed on Days 3, 6, 8, 10, 12, 14, 16 and 18 of gestation and weighed. All feeder weights were measured using a Sartorius Electric Top Loading Balance, Model U3600 (Sartorius Corporation, Edgewood, New York).

**Calculations.** The resulting weight of the feeder at the end of the measurement interval was subtracted from the initial feeder weight. The resulting value represented the grams of feed consumed/interval. The following formula was used to calculate grams of feed 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 food consumption are as follows:

**Rats**

- 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.

**Mice**

- Day 0-3 = 3-day interval using Day 0 body weight.
- Day 3-6 = 3-day interval using Day 3 body weight.
- Day 6-8 = 2-day interval using Day 6 body weight.
- Day 8-10 = 2-day interval using Day 6 body weight.
- Day 10-12 = 2-day interval using Day 9 body weight.
- Day 12-14 = 2-day interval using Day 12 body weight.
- Day 14-16 = 2-day interval using Day 12 body weight.
- Day 16-18 = 2-day interval using Day 15 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. Rats were sacrificed on Day 20 of gestation during the period of 10-20 July 1995 and mice were sacrificed on Day 18 of gestation during the period of 10-14 July 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 rat fetuses were weighed, using a Mettler Balance, Model No. PE4000 (Mettler Instrument Corporation, Hightstown, New Jersey) and all mouse fetuses were weighed using an AND Electronic Balance Model FX3200 (A&D Engineering, Inc., Milpitas, California). All fetuses were sexed externally (ano-genital distance) and given a macroscopic external examination for malformations and variations that included observations for palatal defects.

Following these examinations, all fetuses were euthanized via an overdose of carbon dioxide and discarded. Only fetuses with external malformations were saved in 10% neutral buffered formalin at the discretion of the Study Director.

### Resorptions

Late resorptions were weighed, examined macroscopically for external malformations and discarded.

## STATISTICAL ANALYSES

### Continuous Data

The following parameters were analyzed statistically:

#### Rats

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.

#### Mice

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

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

Mean food consumption values during gestation: Days 0-3, 3-6, 6-8, 8-10, 10-12, 12-14, 14-16, and 16-18.

### Reproduction Data (both species)

Mean number of *corpora lutea*

Mean number of uterine implantation sites per female

Mean number of live and dead fetuses per female

Mean number of resorptions per female

Mean pre-implantation loss ratio (*corpora lutea* - 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

Females with viable fetuses

Females that deliver prematurely

Incidence of fetuses with external malformations

Incidence of litters containing fetuses with external malformations

## 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. In the parametric case (i.e., equal variance), standard regression techniques with a test for trend and lack-of-fit were used (Snedecor and Cochran, 1967). In the nonparametric case, Jonckheere's test (Hollander and Wolfe, 1973) for monotonic trend was used.

All ratios 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≤0.05	p≤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≤0.05	p≤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 DEVIATION

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

The percent isopentane was assayed by the analytical chemistry department. This value was used to calculate chamber concentrations by gas chromatography for comparison to the MIRAN<sup>®</sup> values. This procedure was for comparison only. It was not required by protocol and was not reported.

## Section 4

### RESULTS AND DISCUSSION/CONCLUSION

#### CHAMBER MONITORING (APPENDIX U)

##### Exposure Levels

The complete chamber monitoring results are presented in Appendix U. These results include total hydrocarbon exposure levels obtained from a MIRAN<sup>®</sup> infrared spectrophotometer and GC (gas chromatography) fingerprints obtained from both syringe and charcoal tube samples. The GC syringe samples allowed for the most accurate analysis of the components in the test material because capture and retention of the test material mixture's most volatile components on charcoal tubes were subject to inaccuracies caused by vapor leakage. Because of the time necessary for elution of the GC samples, one GC syringe sample was taken each day to confirm composition and stability of the test material among exposure groups and over the course of the study. The multiple daily charcoal tube samples confirmed stability of the test material composition during the 6 hour exposure day.

Prestudy chamber trials were conducted to determine the optimum conditions for producing the target exposure levels. These trials also included distribution analyses (page U-24) 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

Group	Target Concentration (ppm)	Miran Analytical Concentration (ppm)	Nominal Concentration (ppm)
		Mean ± S.D.	Mean ± S.D.
I	0	0	-
II	300	314 ± 31	331 ± 50
III	1000	1034 ± 63	1121 ± 75
IV	3000	3073 ± 146	3354 ± 215
V	9000	9025 ± 417	9218 ± 452

The achieved mean exposure concentration for each group was very close to the respective target and nominal concentrations. Chamber environmental conditions averaged 23°C and 55% relative humidity.

#### Syringe Samples

The results of the GC syringe sample fingerprints of the exposure atmospheres are summarized in Table 2 on the following page. The results, which present the ratio of nine major components to isopentane, the largest component, showed similarity among all exposure groups. The only exception was for toluene, for which the ratio generally decreased with increasing exposure level. In addition, the individual syringe sample data show minimal variation, as reflected in the standard deviation, over the course of the study.

Table 2: Summary of GC Sample Fingerprints of Exposure Atmosphere Results:  
Ratio of each of the following nine major components to isopentane

	N-Butane Ratio	N-Pentane Ratio	Trans-2- Pentene Ratio	2-Methyl -2-Butene Ratio	2,3-Dimethyl Butane Ratio	2-Methyl Pentane Ratio	3-Methyl Pentane Ratio	N-Hexane Ratio	Toluene Ratio
<b>GROUP II - 300 ppm</b>									
MEAN	0.604	0.255	0.674	0.106	0.089	0.243	0.128	0.078	0.102
S.D.	0.069	0.005	0.001	0.002	0.005	0.014	0.008	0.005	0.054
<b>GROUP III - 1000 ppm</b>									
MEAN	0.692	0.248	0.073	0.104	0.083	0.230	0.119	0.073	0.072
S.D.	0.011	0.013	0.002	0.002	0.008	0.005	0.003	0.003	0.005
<b>GROUP IV - 3000 ppm</b>									
MEAN	0.621	0.258	0.077	0.107	0.086	0.246	0.125	0.081	0.075
S.D.	0.111	0.007	0.002	0.004	0.008	0.023	0.012	0.009	0.015
<b>GROUP V - 9000 ppm</b>									
MEAN	0.717	0.252	0.077	0.105	0.079	0.225	0.113	0.072	0.056
S.D.	0.028	0.002	0.001	0.001	0.002	0.005	0.003	0.002	0.006

#### Charcoal Tube Samples

The results of the charcoal tube sample fingerprints are summarized in Table 3 on the following page. These results show that the composition of the test material was stable from sample to sample during the exposure day. Therefore, the test animals were exposed to a test material of uniform composition over the course of the study.

Table 3: Charcoal Tube Samples - Summary of Daily Mean Ratios

	N-Butane Ratio	N-Pentane Ratio	Trans-2- Pentene Ratio	2-Methyl -2-Butene Ratio	2,3-Dimethyl Butane Ratio	2-Methyl Pentane Ratio	3-Methyl Pentane Ratio	N-Hexane Ratio	Toluene Ratio
<b>Group II - 300 ppm</b>									
Sample No.									
1	0.566	0.267	0.080	0.114	0.095	0.270	0.141	0.093	0.097
2	0.568	0.266	0.080	0.113	0.093	0.267	0.139	0.093	0.095
3	0.567	0.267	0.080	0.113	0.094	0.268	0.139	0.091	0.095
4	0.562	0.267	0.080	0.114	0.092	0.270	0.140	0.093	0.097
MEAN	0.566	0.267	0.080	0.114	0.093	0.269	0.140	0.092	0.096
SD	0.003	0.000	0.000	0.000	0.001	0.001	0.001	0.001	0.001
<b>Group III - 1000 ppm</b>									
Sample No.									
1	0.661	0.259	0.078	0.110	0.087	0.249	0.129	0.083	0.084
2	0.657	0.260	0.079	0.111	0.086	0.251	0.119	0.088	0.087
3	0.657	0.261	0.079	0.111	0.087	0.251	0.128	0.088	0.088
4	0.654	0.261	0.079	0.111	0.087	0.252	0.134	0.086	0.086
MEAN	0.657	0.260	0.079	0.111	0.086	0.251	0.127	0.086	0.086
SD	0.003	0.001	0.000	0.000	0.002	0.001	0.008	0.002	0.002
<b>Group IV - 3000 ppm</b>									
Sample No.									
1	0.581	0.267	0.080	0.115	0.093	0.269	0.140	0.095	0.097
2	0.586	0.266	0.081	0.114	0.092	0.266	0.138	0.092	0.093
3	0.586	0.267	0.081	0.114	0.093	0.267	0.139	0.092	0.099
4	0.587	0.266	0.081	0.114	0.091	0.266	0.138	0.091	0.094
MEAN	0.585	0.266	0.080	0.114	0.092	0.267	0.139	0.092	0.096
SD	0.003	0.000	0.000	0.001	0.001	0.001	0.001	0.002	0.002
<b>Group V - 9000 ppm</b>									
Sample No.									
1	0.668	0.261	0.079	0.111	0.086	0.251	0.130	0.086	0.087
2	0.665	0.261	0.079	0.111	0.089	0.252	0.131	0.086	0.087
3	0.667	0.261	0.079	0.111	0.087	0.251	0.130	0.085	0.087
4	0.671	0.260	0.079	0.111	0.086	0.249	0.129	0.085	0.085
MEAN	0.668	0.261	0.079	0.111	0.087	0.251	0.130	0.086	0.087
SD	0.003	0.000	0.000	0.000	0.001	0.001	0.001	0.001	0.001

Note: Sample numbers: Four samples were taken, approximately 60 minutes apart, during each exposure.

### Particle Sizing

Particle size distribution measurements of the background aerosol from all the exposure groups are summarized below (Table 4):

Table 4: Summary of Particle Size Distribution Measurements

Group	Mass Median Aerodynamic Diameter ( $\mu\text{m}$ )	Geometric Standard Deviation	Total Mass Concentration ( $\text{mg}/\text{m}^3$ )
I	1.7	1.7	$5.96 \times 10^{-3}$
II	1.6	1.9	$5.67 \times 10^{-3}$
III	1.6	1.9	$5.58 \times 10^{-3}$
IV	1.8	1.8	$5.95 \times 10^{-3}$
V	1.4	1.9	$5.27 \times 10^{-3}$

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 - Rats; Appendix K - Mice)

No adverse effect of treatment from exposure to Unleaded Gasoline Vapor Condensate (API 94-02) was indicated from mortality data as all treated animals (rats and mice) survived to scheduled sacrifice.

#### Pregnancy Rates (Appendix G - Rats; Appendix Q - Mice)

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

Pregnancy rates are summarized below (Table 5):

Table 5: Pregnancy Rates

Group (ppm)	Pregnancy Rate	
	No. pregnant <sup>a</sup>	%
Rats		
I (0)	10	100
II (300)	10	100
III (1000)	10	100
IV (3000)	10	100
V (9000)	9	90
Mice		
I (0)	8	80
II (300)	8	80
III (1000)	10	100
IV (3000)	9	90
V (9000)	10	100

<sup>a</sup>Each group contained 10 mated females.

Gestation Body Weight and Weight Gain Data (Figure 1 and Appendices B, C and D - Rats; Figure 3 and Appendices L, M and N - Mice)

**Rats.** Mean maternal body weights and weight gain data during gestation for the groups treated at the 300, 1000, and 3000 ppm levels were comparable to control and no adverse effect of treatment was indicated.

At the 9000 ppm exposure level, mean maternal body weights were lower than control throughout the treatment period and these differences were statistically significant on Gestation Days 12, 15 and 20. A statistically significant reduction in weight gain over the Day 6-20 gestation interval using the actual Day 20 gestation weights, was also seen in the 9000 ppm group in comparison to the control data. The reduction in gestation body weights and weight gain data during the treatment period at the 9000 ppm level was assumed to be an adverse response to treatment, since the definitive study does not seem to bear out the earlier finding.

0045

Mice. Mean body weights and weight gain data during gestation in the Unleaded Gasoline Vapor Condensate-treated groups were comparable to control data and no adverse effect of treatment was indicated from these data.

Food Consumption Data - Gestation Period (Figure 2 and Appendix E - Rats; Figure 4 and Appendix O - Mice)

Rats. Mean food consumption data during the treatment period (Days 6-9, 9-12, 12-15, 15-18 and 18-20) for animals treated with Unleaded Gasoline Vapor Condensate (API 94-02) at exposure levels of 300, 1000 and 3000 ppm were comparable to control and no adverse effect of treatment was indicated from these data. At the 9000 ppm exposure level, a statistically significant reduction in food consumption was seen over the Day 6-9 gestation interval. For the remainder of the treatment period mean food consumption for this group was comparable to control data.

Mice. Mean maternal food consumption data during gestation in the Unleaded Gasoline Vapor Condensate-treated groups were comparable to control data and no adverse effect of treatment was indicated from these data.

Physical Observation Data (Appendix F - Rats; Appendix P - Mice)

No adverse effect of treatment from exposure to Unleaded Gasoline Vapor Condensate (API 94-02) was indicated in either the rats or mice from the detailed physical examinations.

**Corpora lutea and Uterine Implantation Data (Appendix G - Rats and Appendix O - Mice):**

**Rats.** No adverse effect of treatment with Unleaded Gasoline Vapor Condensate (API 94-02) was evident from uterine implantation data. No females delivered prematurely. The number of *in utero* litters recovered at Day 20 gestation maternal sacrifice from the control, 300, 1000, 3000 and 9000 ppm groups was ten, ten, ten, ten and nine, respectively. The mean numbers of *corpora lutea*, uterine implantation sites, live fetuses and resorptions per 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.

**Mice.** One control female (Animal No. 1518) delivered prematurely on Day 18 of gestation. This female was noted with 13 live pups and one partially cannibalized pup. At sacrifice, 14 uterine implantation scars were seen. No premature deliveries were seen among the treated females.

No adverse effect of treatment with Unleaded Gasoline Vapor Condensate (API 94-02) was evident from uterine implantation data. The number of *in utero* litters recovered at Day 18 gestation maternal sacrifice from the control, 300, 1000, 3000 and 9000 ppm groups was seven, eight, ten, nine and ten, respectively. The mean number of *corpora lutea*, uterine implants, and live fetuses per female and the mean pre-implantation loss indices for the treated groups were comparable to control data. One dead fetus was recovered in the control and 1000 ppm treated group. No dead fetuses were seen in the remaining groups. Resorption data (mean number of resorption per female, the resorption/implant ratio and incidence of females with resorptions) were greater in the unleaded gasoline-treated groups in comparison to control data. This was not considered to represent a treatment-related response but was attributed to unusually low resorption data in the control group. The mean number of resorptions per female, the mean resorption/implant ratio and the incidence of females with resorptions in the control group

were outside the range of historical control data for this laboratory (0.6-1.7, 0.048-0.149, and 46.4%-69.2%, respectively, See Appendix W, pages W-10 - W-12). These same data for the treated groups were generally within the range of these historical data. Differences in resorption data for the treated groups were not statistically significant in comparison to control data and not dose-responsive. Therefore, this increase in resorption data in the treated groups was not considered treatment-related.

Macroscopic Postmortem Evaluations (Appendix I - Rats; Appendix S - Mice):

All animals in both species 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 (Appendix G - Rats and Appendix Q - Mice)

No adverse effect of treatment was indicated from fetal weight data in either rats or mice. For both species, mean fetal weights distinguished by sex and for both sexes combined in the treated groups were considered comparable to control data and generally within the range of historical control data for this laboratory (combined ranges were 3.28-3.76 and 1.30-1.34, respectively, See Appendix W, pages W-4 and W-10).

Fetal Sex Distribution Data (Appendix G - Rats and Appendix Q - Mice)

No adverse effect of treatment was indicated from fetal sex distribution data. For both species, the mean number of male and female fetuses per female was comparable to control data. Likewise, in both species the ratio of total male to female fetuses for the treated groups was considered similar to control data.

**Fetal External Examination Data (Appendix J - Rats and Appendix T - Mice)**

**Rats.** No external malformations were seen among fetuses recovered from the treated groups. The numbers of fetuses/litters evaluated in each of the treated groups were as follows: 300 ppm group - 148 fetuses from ten litters; 1000 ppm group - 163 fetuses from ten litters; 3000 ppm group - 152 fetuses from ten litters; and 9000 ppm group - 130 fetuses from nine litters.

In the control group, one fetus from the litter of Female No. 1501 had a filamentous tail. No other malformations were seen in the remaining 157 fetuses from a total of ten litters evaluated in the control group.

**Mice.** The incidences of fetuses with external malformations in the control, 300, 1000, 3000 and 9000 ppm groups were 0% (89 fetuses evaluated), 0% (96 fetuses evaluated), 0.8% (1/131 fetuses affected), 0% (100 fetuses evaluated) and 0.8% (1/120 fetuses affected), respectively. The litter incidences for these same groups were 0% (seven litters evaluated), 0% (eight litters evaluated), 10% (1/10 litters affected), 0% (nine litters evaluated) and 10% (1/10 litters affected), respectively.

Hindlimb flexure was seen in one fetus from the 1000 ppm group (Female No. 3511) and unilateral open eye was seen in one fetus at the 9000 ppm group (Female No. 5515). The low incidence of these dissimilar malformations within the treated groups was not considered indicative of a treatment-related response.

Cleft palate was seen in one of 13 intact pups recovered from the prematurely delivered litter of control Female No. 1518.

## CONCLUSION

In this range-finding inhalation developmental toxicity study with Unleaded Gasoline Vapor Condensate (API 94-02), no developmental toxicity was seen in either rats or mice at exposure levels up to and including 9000 ppm (75% of the lower explosive limit). No evidence of maternal toxicity was observed in the mouse at any dose level. In the rat, evidence of maternal toxicity (reduced body weights, weight gain and food consumption) was seen at 9000 ppm; no maternal effects occurred at 3000 ppm.

## Section 5

### REFERENCES

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Snedecor, G.W. and Cochran, W.G., 1971. *Statistical Methods*, 7th edition. Iowa State University Press, Ames, Iowa.

## Section 6

### LOCATION OF SPECIMENS, RAW DATA AND FINAL REPORT

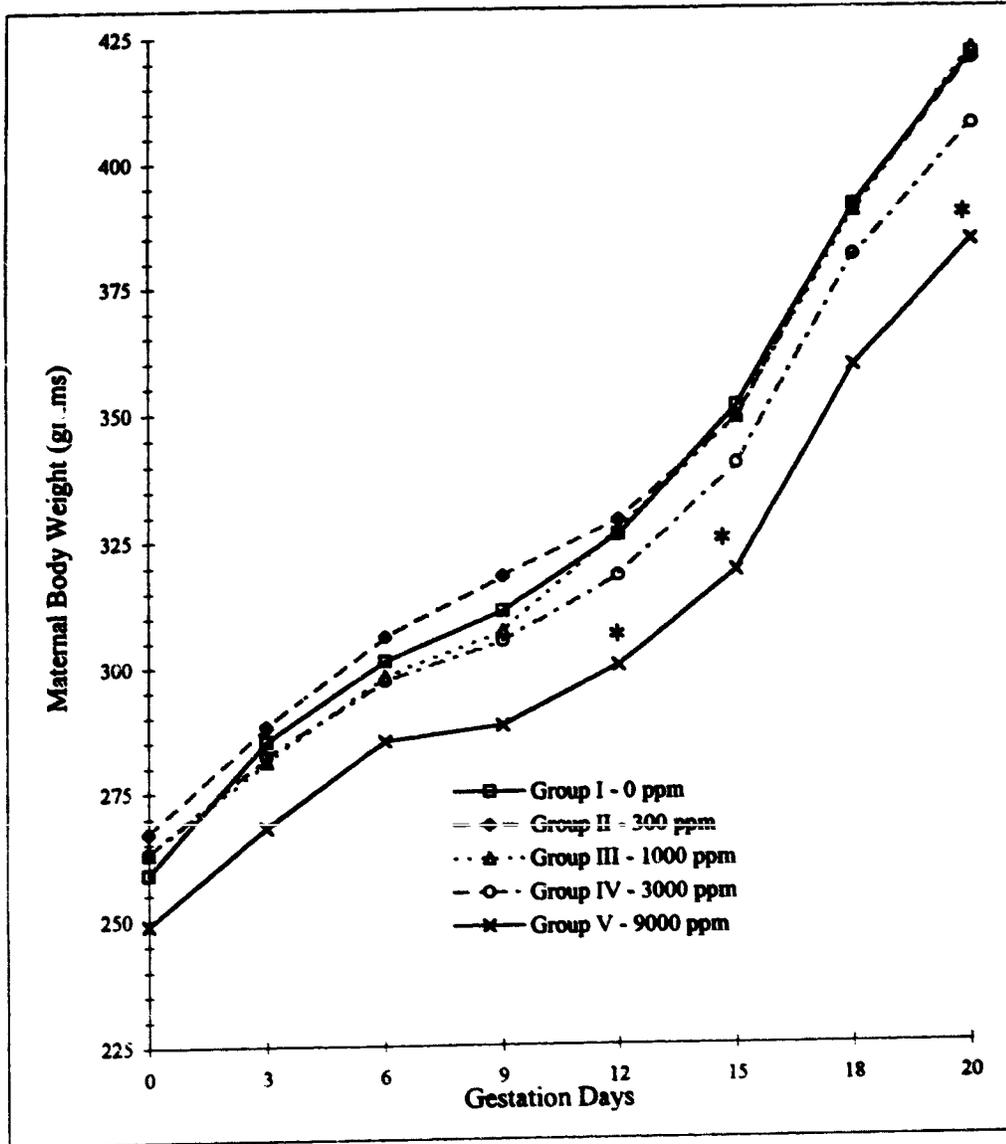
All data documenting experimental details, study procedures and observations were recorded and maintained as raw data.

All raw data, preserved specimens, and retained samples, as well as the original study protocol and the original final report are to be maintained in the Archives of the Testing Facility upon completion of the study.

Section 7

FIGURES

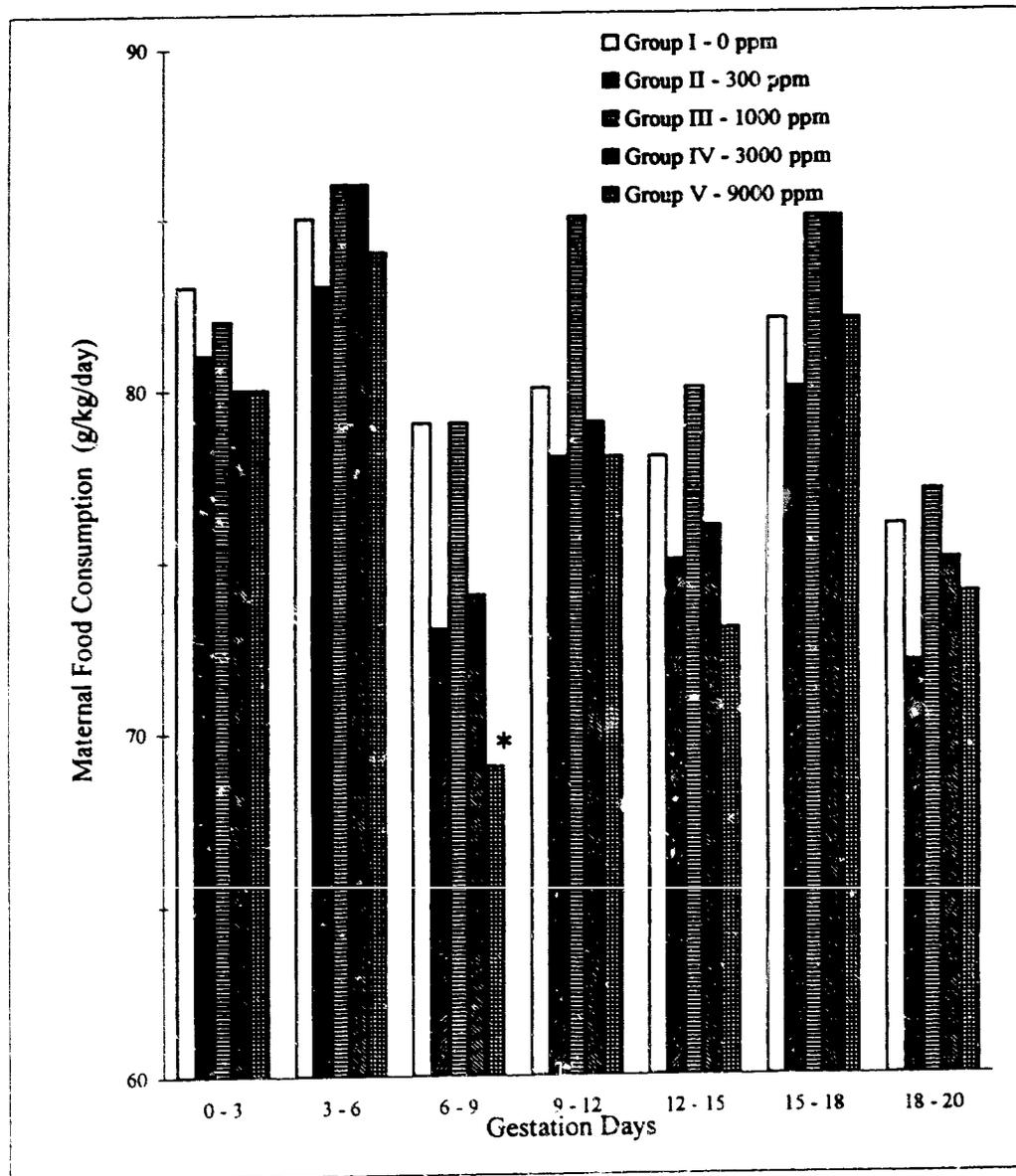
Figure 1: Mean Maternal Body Weights During Gestation - Rats



\*Significantly different from control mean,  $p < 0.05$  (Dunnett's).

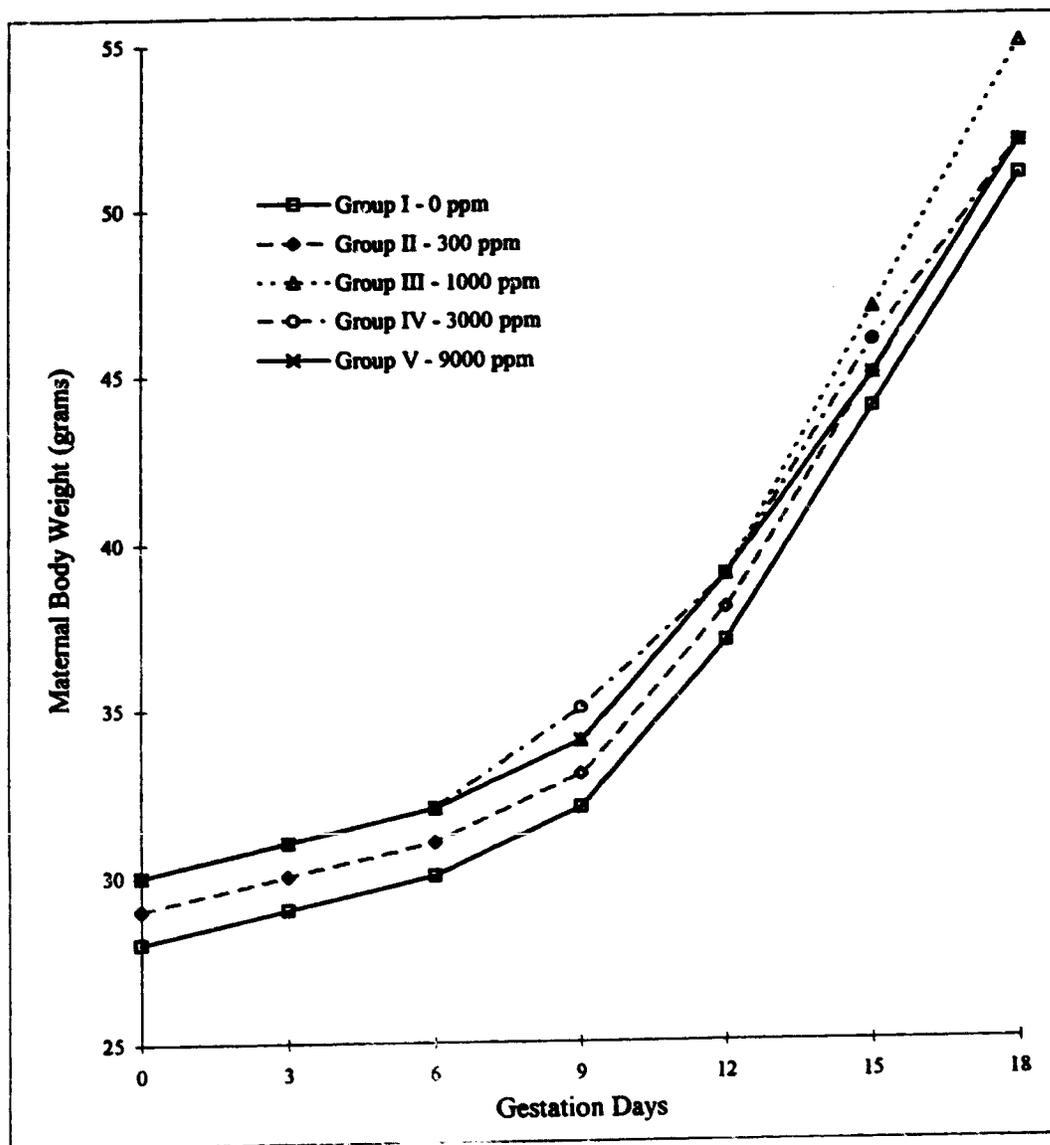
0054

Figure 2: Mean Maternal Food Consumption During Gestation - Rats



\*Significantly different from control mean  $p \leq 0.05$  (Dunnnett's).

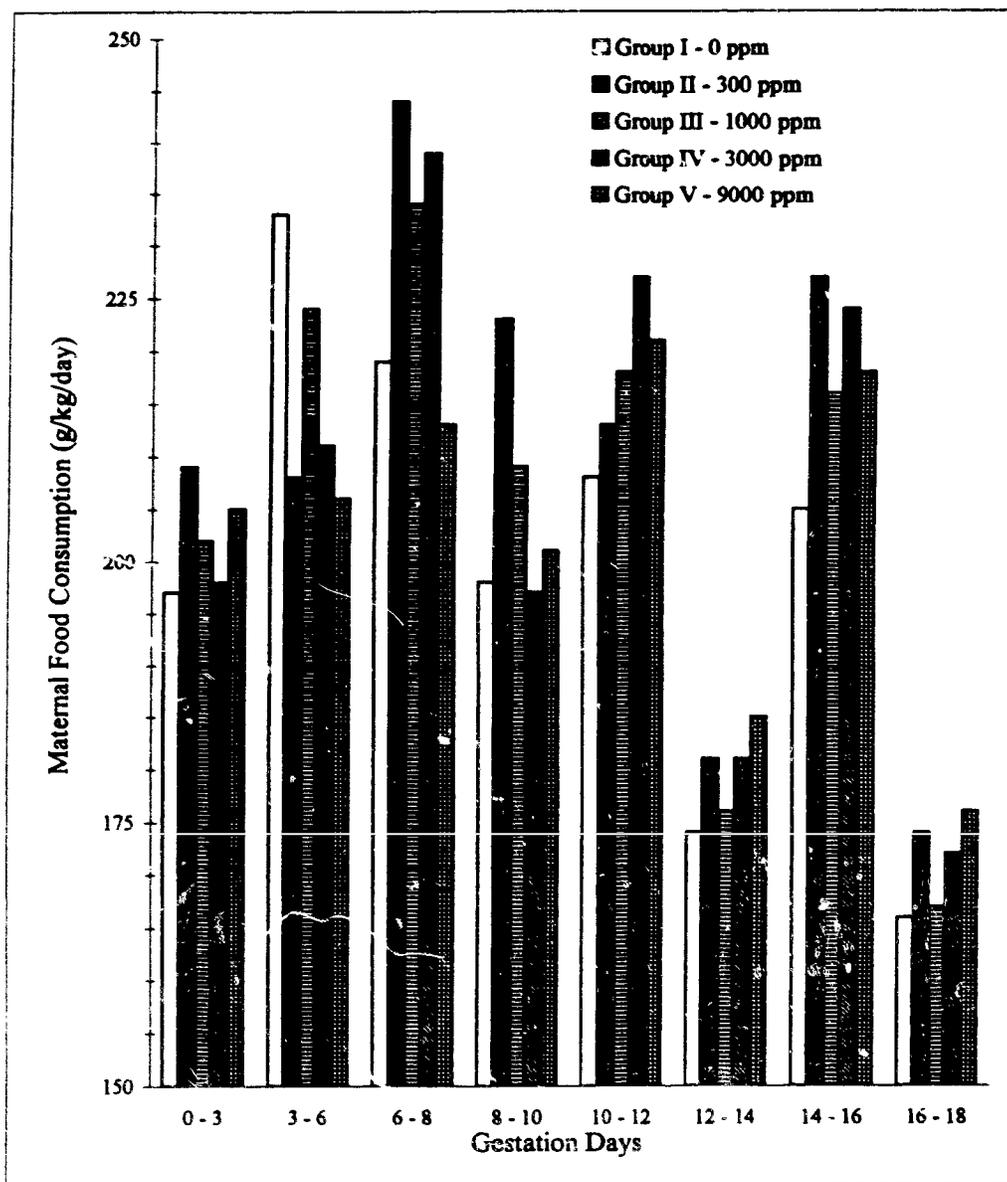
Figure 3: Mean Maternal Body Weights During Gestation - Mice



No statistically significant differences.

0-0-5-5

Figure 4: Mean Maternal Food Consumption During Gestation - Mice



No statistically significant differences.

APPENDIX A  
ANIMAL TERMINATION HISTORY  
RATS

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Appendix A

ANIMAL TERMINATION HISTORY  
RATS

ANIMAL NUMBER	SEX	TYPE OF DEATH	DATE OF DEATH	DAYS ON STUDY	NUMBER OF DAYS EXPOSED
GROUP I - 0 PPM					
1501 P	F	SCHEDULED SACRIFICE	10-Jul-95	20	14
1502 P	F	SCHEDULED SACRIFICE	12-Jul-95	20	14
1503 P	F	SCHEDULED SACRIFICE	12-Jul-95	20	14
1504 P	F	SCHEDULED SACRIFICE	12-Jul-95	20	14
1505 P	F	SCHEDULED SACRIFICE	12-Jul-95	20	14
1506 P	F	SCHEDULED SACRIFICE	14-Jul-95	20	14
1507 P	F	SCHEDULED SACRIFICE	14-Jul-95	20	14
1508 P	F	SCHEDULED SACRIFICE	19-Jul-95	20	14
1509 P	F	SCHEDULED SACRIFICE	20-Jul-95	20	14
1510 P	F	SCHEDULED SACRIFICE	20-Jul-95	20	14
GROUP II - 300 PPM					
2501 P	F	SCHEDULED SACRIFICE	10-Jul-95	20	14
2502 P	F	SCHEDULED SACRIFICE	11-Jul-95	20	14
2503 P	F	SCHEDULED SACRIFICE	12-Jul-95	20	14
2504 P	F	SCHEDULED SACRIFICE	12-Jul-95	20	14
2505 P	F	SCHEDULED SACRIFICE	12-Jul-95	20	14
2506 P	F	SCHEDULED SACRIFICE	14-Jul-95	20	14
2507 P	F	SCHEDULED SACRIFICE	17-Jul-95	20	14
2508 P	F	SCHEDULED SACRIFICE	19-Jul-95	20	14
2509 P	F	SCHEDULED SACRIFICE	20-Jul-95	20	14
2510 P	F	SCHEDULED SACRIFICE	20-Jul-95	20	14
GROUP III - 1000 PPM					
3501 P	F	SCHEDULED SACRIFICE	10-Jul-95	20	14
3502 P	F	SCHEDULED SACRIFICE	11-Jul-95	20	14
3503 P	F	SCHEDULED SACRIFICE	12-Jul-95	20	14
3504 P	F	SCHEDULED SACRIFICE	12-Jul-95	20	14
3505 P	F	SCHEDULED SACRIFICE	12-Jul-95	20	14
3506 P	F	SCHEDULED SACRIFICE	14-Jul-95	20	14
3507 P	F	SCHEDULED SACRIFICE	18-Jul-95	20	14
3508 P	F	SCHEDULED SACRIFICE	19-Jul-95	20	14
3509 P	F	SCHEDULED SACRIFICE	20-Jul-95	20	14
3510 P	F	SCHEDULED SACRIFICE	20-Jul-95	20	14

P=PREGNANT.

**APPENDIX X**  
**PROTOCOL AND PROTOCOL AMENDMENTS**

**PHARMACO LSR  
TOXICOLOGY SERVICES WORLDWIDE**

**A RANGE-FINDING DEVELOPMENTAL INHALATION TOXICITY  
STUDY OF REGULAR UNLEADED GASOLINE IN RATS AND  
MICE VIA WHOLE-BODY EXPOSURE**

**QUOTE NO.: Q8935, Option A**

**STUDY NO.: 95-6082**

**ISSUE NO.: 3**

**SPONSOR STUDY NO.: 08200-0601-SH9343**

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

**ATTENTION: Richard A. Rhoden, Ph.D.**

**DATE: 23 May 1995**

## 1 INTRODUCTION:

- 1.1 STUDY NO.: 95-6082
- 1.2 SPONSOR STUDY NO: 08200-0601-SH9343
- 1.3 ISSUE NO.: 3
- 1.4 STUDY TITLE: A Range-Finding Developmental  
Inhalation Toxicity Study of  
Regula: Unleaded Gasoline in Rats  
and Mice via Whole-Body  
Exposure
- 1.5 TEST MATERIAL: Regular Unleaded Gasoline  
(130F fraction)
- 1.6 SPONSOR: American Petroleum Institute  
Health and Environmental Sciences  
Department  
1220 L Street, N.W.  
Washington, D.C. 20005
- 1.7 SPONSOR REPRESENTATIVE: Richard A. Rhoden, Ph.D.  
Phone No.: 202-682-8480  
Fax No.: 202-682-8270
- 1.8 TESTING FACILITY: Pharmaco LSR  
Toxicology Services Worldwide  
Mettlers Road  
P.O. Box 2360  
East Millstone, NJ 08875-2360
- 1.9 PURPOSE:

This study is designed to assess the potential maternal toxicity and developmental toxicity of a test material administered via inhalation (whole-body exposure) to pregnant rats during the Day 6-19 gestation interval and to pregnant mice during the Day 6-17 gestation interval to determine dose levels for a more definitive developmental study using one of these species.

## 2 REGULATORY REFERENCES:

### 2.1 GOOD LABORATORY PRACTICES:

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

### 2.2 FACILITIES MANAGEMENT/ANIMAL HUSBANDRY:

Currently acceptable practices of good animal husbandry will be followed, e.g., Guide for the Care and Use of Laboratory Animals; DHHS Publication No. (NIH) 86-23, Revised 1985. Pharmacology LSR, Toxicology Services Worldwide, is fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

### 2.3 ANIMAL WELFARE ACT COMPLIANCE:

This study will comply with all appropriate parts of the Animal Welfare Act regulations: 9 CFR Parts 1 and 2 Final Rules, Federal Register, Volume 54, No. 158, 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. The Sponsor should make particular note of the following:

1. The Sponsor's signature on this protocol documents for the study described, there are no generally accepted non-animal alternatives and the study does not unnecessarily duplicate previous experiments.
2. All procedures used in this study have been designed to avoid discomfort, distress and pain to the animals. All methods are described in this study protocol or in written laboratory standard operating procedures.
3. Any aspects of this study which cause more than momentary or slight pain or distress to the animals will be performed with appropriate sedatives, analgesics or anesthetics unless the withholding of these agents is justified for scientific reasons, in writing by the Sponsor and the Study Director, in which case the procedure will continue for the minimum time necessary.
4. Animals that experience severe or chronic pain or distress that cannot be relieved will be painlessly euthanized as deemed appropriate by the Testing Facility's veterinary staff and the Study Director. The Sponsor will be advised by the Study Director of all circumstances which could lead to this action in as timely a manner as possible.

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**2.3 ANIMAL WELFARE ACT COMPLIANCE:**

5. Methods of euthanasia used during this study are in conformance with the above referenced regulations.

**3 QUALITY ASSURANCE MONITORING:**

The Pharmaco LSR, Toxicology Services Worldwide Quality Assurance Unit will monitor the facilities, equipment, personnel, methods, practices, records and controls used in this study to assure that they are in conformance with this protocol, company SOP's, and the appropriate Good Laboratory Practice regulations.

**4 ALTERATION OF DESIGN:**

Alterations of this protocol may be made as the study progresses. No changes in the protocol will be made without the consent of the Sponsor. In the event that the Sponsor authorizes a protocol change verbally, such changes will be honored by the Testing Facility and will be followed by a written verification. All protocol modifications will be signed by the Study Director and a Sponsor representative. Any modifications potentially affecting animal welfare will also be signed by two members of the Institutional Animal Care and Use Committee prior to the modification's implementation.

**5 STUDY PERSONNEL:**

Study Director:	Raymond E. Schroeder, M.S., D.A.B.T.
Inhalation Toxicologist:	Paul E. Newton, Ph.D., D.A.B.T.
Study Pathologist:	Arpad Madarasz, D.V.M.

Additional personnel will be documented in the project file and presented in the final report.

**6 PROPOSED STUDY DATES:**

*See p. 33*

Study initiation date:

Date Study Director signs protocol - See Section 16.2.

Receipt of test animals:

•

Initiation of mating:

•

Initiation of exposure:

•

Termination of exposure:

•

Necropsy:

•

Submission of draft final report:

•

Experimental termination:  
(Date of last data collection)

•

Study completion date:

Date final report is signed by Study Director.

**7 EXPERIMENTAL DESIGN:**

Group	Exposure Level (ppm)	Treatment Schedule*	Number of Mated Animals				Proportion of Fetuses/Litter Evaluated for External Malformations and/or Variations
			Rats	Mice	Sacrificed		
					Rats (GD 20)	Mice (GD 18)	
I (Chamber exposed, air control)	0	Rats - GD 6-19 Mice - GD 6-17	10	10	A.S.	A.S.	All
II	300	Rats - GD 6-19 Mice - GD 6-17	10	10	A.S.	A.S.	All
III	1000	Rats - GD 6-19 Mice - GD 6-17	10	10	A.S.	A.S.	All
IV	3000	Rats - GD 6-19 Mice - GD 6-17	10	10	A.S.	A.S.	All
V	9000	Rats - GD 6-19 Mice - GD 6-17	10	10	A.S.	A.S.	All

\* Exposures will be 6 hours/day.

Key: A.S. = All Survivors; complete gross postmortem evaluations will be performed on animals found dead, euthanized in a moribund condition or killed at termination;  
GD - gestation day.

## 8 TEST MATERIAL:

8.1 TEST MATERIAL: Regular unleaded gasoline (130F fraction)

Description, lot number, storage, expiration date and handling procedures, as well as other pertinent information will be documented in the study data. In case of emergency contact Dr. Dick Rhoden, Sponsor Representative (Phone no. 202-682-8480).

### 8.2 IDENTIFICATION OF TEST MATERIAL:

Unless otherwise noted, the identity, strength, purity, composition, stability, and method of synthesis, fabrication and/or derivation of each batch of the test material will be documented by the Sponsor before its use in the study. This documentation will be maintained by the Sponsor at the address indicated in Section 1.6 of this protocol.

### 8.3 ARCHIVAL SAMPLES:

A sample from each lot of test material used on study will be taken and stored in the Archives of the Testing Facility. If multiple studies are conducted with the same material, a common archival sample may be taken and appropriately labeled.

8.4 UNUSED TEST MATERIAL: *See p. 23. fsh/si*

The unused portion of the test material as well as any empty test material containers will be returned to the Sponsor following completion of the study. Materials will be shipped to:

Name:  
Address:  
Phone:

In the event the Sponsor wishes the Testing Facility to arrange for disposal, a cost for this service will be provided.

**9 TEST ANIMALS:**

Albino Rats (Outbred) VAF/Plus<sup>®</sup>  
Albino Mice

**9.1 STRAIN:**

**9.1.1 Rats:**

Sprague Dawley - derived (CD<sup>®</sup>)  
[Cri: CD<sup>®</sup> BR]

**9.1.2 Mice:**

CD-1<sup>®</sup> [Cri: CD-1<sup>®</sup> (ICR) BR]

**9.2 SUPPLIER:**

Charles River Laboratories  
Documentation of the specific breeding facility will be maintained in the  
study file.

**9.3 JUSTIFICATION FOR TEST SYSTEM SELECTION:**

The rat and mouse are rodent animal models commonly utilized in develop-  
mental toxicity studies as recommended in the EPA TSCA guidelines. In  
addition, historical control data are available for these species in this  
laboratory for comparative evaluation.

**9.4 ANIMAL REQUIREMENTS/SPECIFICATIONS:**

**9.4.1 Number:**

**9.4.1.1 Rats:**

	<u>Females</u>
Purchased - approximately	75
Placed on test	50

Males: In-house breeding colony used solely for mating.

**9.4.1.2 Mice:**

	<u>Females</u>
Purchased - approximately	90
Placed on test	50

Males: In-house breeding colony used solely for mating.

9.4.2 Age:

9.4.2.1 Rats: *Cytec p. 23* *PSI/MS*

Females will be approximately seven weeks at receipt; approximately nine weeks at initiation of mating.

Note: Females will be nulliparous and non-pregnant.

9.4.2.2 Mice: *Cytec p. 24* *PSI/MS*

Females will be approximately seven weeks at receipt; approximately nine weeks at initiation of mating.

Note: Females will be nulliparous and non-pregnant.

9.5 ACCLIMATION PERIOD: *Cytec p. 24* *PSI/MS*

Approximately two weeks; all animals will be checked for viability twice daily. Prior to assignment to study all animals will be examined by a staff veterinarian to ascertain suitability for study.

9.6 ANIMAL HUSBANDRY/NON-EXPOSURE:

9.6.1 Housing:

Animals will be housed individually in suspended stainless steel wire mesh cages except as follows:

Mating: one male and one female co-housed nightly.

9.6.2 Food:

Certified Rodent Diet, No. 5002; (Meal) (PMI Feeds, Inc., St. Louis, MO) *ad libitum*.

9.6.3 Water:

Elizabethtown Water Company, Westfield, NJ; *ad libitum*, via automated watering system.

9.6.4 Feed Analysis:

Analytical certification of batches of feed provided by the manufacturer will be maintained on file at the Testing Facility. There are

**9.6.4 Feed Analysis:**

no known contaminants in the feed which are expected to interfere with the results of this study.

**9.6.5 Water Analysis:**

Monthly water analysis, provided by Elizabethtown Water Company, will be maintained on file at the Testing Facility. Biannual chemical and microbiological analyses of water samples collected from representative rooms in this facility are conducted to assure that water meets standards specified under the EPA National Primary Drinking Water Regulations (40 CFR Part 141). Results are maintained on file. There are no known contaminants in the water which are expected to interfere with the results of this study.

**9.6.6 Veterinary Care:**

Animals are monitored by the technical staff for any conditions requiring possible veterinary care. If any such conditions are identified, a staff veterinarian will be notified for an examination and evaluation. Any medical veterinary intervention will be made only with approval of the staff veterinarian and the Study Director. The Sponsor will be consulted whenever possible. However, in emergency situations, decisions will be made as needed and the Sponsor will be advised as soon as possible.

**9.6.7 Environmental Conditions:**

**9.6.7.1 Light/Dark Cycle:**

Twelve hour light/dark cycle (light period will be approximately 6:00 to 18:00 hrs each day) provided via automatic timer.

**9.6.7.2 Temperature:**

Monitored and recorded twice daily. The desired temperature range is 64-79°F (18-26°C). Temperature will be maintained in this range to the maximum extent possible.

**9.6.7.3 Humidity:**

Monitored and recorded once daily. The desired humidity range is 40-70%. Humidity will be maintained in this range to the maximum extent possible.

#### 9.7 ANIMAL HUSBANDRY/EXPOSURE:

##### 9.7.1 Housing:

- 9.7.1.1 Rats: Individually in wire mesh cages.
- 9.7.1.2 Mice: Individually in wire mesh cages.
- 9.7.2 Food: None.
- 9.7.3 Water: None.

#### 9.8 SELECTION FOR STUDY:

More animals than required for the study will be purchased and equilibrated. Animals considered suitable for study on the basis of pretest physical examinations will be incorporated into the mating phase of the study. Disposition of all animals not utilized in the study will be maintained in the study file.

#### 9.9 ANIMAL IDENTIFICATION:

Each female will be assigned a temporary identification number upon receipt. Mated animals assigned to the study will be provided with a metal eartag (rats) or tail tattooed (mice) with a number assigned by the Testing Facility. This number plus the study number will comprise the unique identification for each animal. Each non-exposure cage will be provided with a card which will be color-coded for exposure level identification and will contain the study number and animal number.

#### 9.10 MATING PROCEDURE:

##### 9.10.1 Rats:

Females selected for mating will be placed with the male rats nightly in a 1:1 ratio. Vaginal smears will be taken early in the morning following nightly interval of co-housing and females will be considered to have mated if sperm is observed microscopically in the vaginal smear and/or a vaginal plug is observed. The day on which evidence of mating is observed will be defined as Day 0 of gestation.

**9.10.2 Mice:**

Females selected for mating will be placed with the male mice nightly in a 1:1 ratio. In the morning following nightly interval of co-housing, females will be evaluated for the presence of a vaginal copulatory plug. The day on which a vaginal plug is observed will be defined as Day 0 of gestation.

**10 TEST MATERIAL ADMINISTRATION:**

**10.1 ROUTE OF ADMINISTRATION:**

Inhalation via whole-body exposures.

**10.2 JUSTIFICATION FOR ROUTE OF ADMINISTRATION:**

The inhalation route is one of the potential routes of human exposure to this test material.

**10.3 FREQUENCY OF ADMINISTRATION:**

Once daily, 6 hours/day.

**10.4 DURATION OF ADMINISTRATION:**

Mated rats will be exposed for 6 hours/day over the Day 6-19 gestation period and mated mice will be exposed over the Day 6-17 gestation period.

**10.5 ADMINISTRATION OF TEST MATERIAL:**

The test material will be administered as a vapor in the breathing air of the animals. The test atmosphere will be generated by an appropriate procedure determined during pre-study trials. The trials will be performed to evaluate the optimal set of conditions and equipment to generate a stable atmosphere at the target exposure levels. The method will be described in the raw data of the study and in the report.

The whole-body exposure chambers will each have a volume of approximately 1000 liters. Each chamber will be operated at a minimum flow rate of 200 liters per minute. The final airflow will be set to provide at least one air change in 5.0 minutes (12 air changes/hour) and a  $T_{90}$  equilibrium time of at most 23 minutes. This chamber size and air flow rate is considered adequate to maintain the oxygen level above 19% and the animal loading factor below 5%. At the end of the exposure, all animals will remain in the chamber for a minimum of 30 minutes. During

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**10.5 ADMINISTRATION OF TEST MATERIAL:**

this time the chamber will be operated at approximately the same flow rate using clean air only.

**10.6 EXPOSURE CONCENTRATION DETERMINATION:**

A nominal exposure concentration will be calculated if possible. The flow of air through the chamber will be monitored using appropriate calibrated equipment. The test material consumed during the exposure will be divided by the total volume of air passing through the chamber (volumetric flow rate times total exposure time) to give the nominal concentration.

The exposure level will be measured six times/day (Groups II-V) by using an infrared spectrophotometer (Miran) and at least one time/day (Group I). During intervals when the Miran is not being used to analyze chamber concentrations in the different groups it will monitor concentration levels in the high-exposure chamber (Group V). This real time monitoring will allow accurate and precise control of the concentrations in each chamber each day. In addition, one sample/day (Groups II-V) will be analyzed using a syringe grab sample and a gas chromatographic (GC) procedure to characterize airborne vapor components. Finally, four samples/day (Groups II-V) will be collected on charcoal tubes. These samples will be analyzed for a consistent ratio of the major components.

Prior to initiation of animal exposures additional samples will be taken to determine the distribution of the test material in the exposure chamber. If more than the nominal amount of trialing is required because of test material generation or monitoring problems (2 weeks or 150 technician hours), the Sponsor will be consulted prior to additional trialing (additional cost).

**10.7 PARTICLE SIZE DISTRIBUTION ANALYSIS:**

During each exposure, particle size determinations will be performed using a cascade impactor or other appropriate device, to characterize the aerodynamic particle size distribution of any aerosol present.

**10.8 CHAMBER ENVIRONMENT:**

Temperature, humidity and air flow rate will be recorded every 20 minutes during exposure. Chamber temperature and relative humidity will be maintained, to the extent possible, between 20 to 24°C and 40 to 60%, respectively.

## 10.9 SUMMARY OF CHAMBER ACTIVITY:

The minimum frequency of chamber activity is summarized below.

<u>Activity</u>	<u>Frequency/chamber/day</u>
Measured Test Material Conc: n Infrared spectrophotometry GC procedures	6X 5X (1 syringe sample and 4 charcoal tube samples)
Particle Size	1X
Temperature	13X
Relative Humidity	13X
Airflow Rate	13X
Nominal Test Material Concentration (excluding the air control chamber)	1X
Rotation Pattern of Exposure Cages	1X
Loading/Unloading Verification	1X

## 11 EXPERIMENTAL EVALUATION:

### 11.1 OBSERVATIONS:

#### 11.1.1 Viability Checks (In-Cage):

Both species: Observations for mortality, general appearance and signs of severe toxic or pharmacologic effects will be made at least twice daily. Animals in extremely poor health or in a possible moribund condition will be identified for further monitoring and possible euthanasia.

#### 11.1.2 Physical Examinations:

Each animal will be removed from its cage and examined pretest and on Days 0, 6-17, 18 (mice) and Days 0, 6-19 and 20 (rats) of gestation. Examinations will include observations of general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia as well as evaluations of respiration. During the exposure period animals will be evaluated pre- and post-exposure.

## 11.2 BODY WEIGHTS:

### 11.2.1 Rats:

Body weights will be recorded on Days 0, 3, 6, 9, 12, 15, 18 and 20 of gestation.

### 11.2.2 Mice:

Body weights will be recorded on Days 0, 3, 6, 9, 12, 15 and 18 of gestation.

## 11.3 FOOD CONSUMPTION:

### 11.3.1 Rats:

Food consumption will be recorded for each female during the following intervals of gestation: Days 0-3, 3-6, 6-9, 9-12, 12-15, 15-18 and 18-20.

### 11.3.2 Mice:

Food consumption will be recorded for each female during the following intervals of gestation: Days 0-3, 3-6, 6-8, 8-10, 10-12, 12-14, 14-16 and 16-18.

## 12 POSTMORTEM:

### 12.1 MACROSCOPIC POSTMORTEM EXAMINATIONS:

Complete macroscopic postmortem examinations will be performed on all mated animals, including those dying spontaneously or killed in a moribund condition and on females sacrificed after aborting or premature delivery of a litter. Only gross lesions will be saved in 10% neutral buffered formalin. Dams showing signs of abortion or premature delivery (expulsion of concepti) will be killed (exsanguination following an anesthetic dose of inhaled carbon dioxide) on the day such evidence is observed. Reproductive tracts will be examined and fetuses obtained 17 days (mice) or 19 days (rats) or later will be given an external examination, sacrificed with an overdose of carbon dioxide and discarded.

All females surviving on Day 18 (mice) or Day 20 (rats) of gestation will be sacrificed by exsanguination following anesthesia with inhaled carbon dioxide and the following examinations performed:

### 12.1.1 Reproductive System:

The intact uteri (ovaries attached) will be examined *in situ* externally for signs of hemorrhage. The uterus with ovaries attached will then be removed and weighed. The ovaries will then be dissected free and the uterus dissected longitudinally along the antimesometrial border and the number and location of the following will be recorded for each uterine 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)
- implantation sites (total of live, dead and resorbed fetuses).

Uteri without grossly visible implantations will be stained according to the procedure of Salewski<sup>a</sup>. If stained foci are present, the female will be considered pregnant for purposes of calculating pregnancy rates. The number of foci will not be used in the calculation of uterine implantation data.

### 12.1.2 Ovaries:

Corpora lutea of pregnancy will be counted.

## 12.2 FETAL EVALUATIONS:

### 12.2.1 External Evaluations:

All fetuses will be weighed, sexed and given a gross external examination for malformations/variations to include observation of the palate. All fetuses will then be euthanatized via an overdose of carbon dioxide and discarded. Fetuses with external malformations will be saved in 10% neutral buffered formalin at the discretion of the Study Director.

---

<sup>a</sup> Salewski, E. (1964). Farbemethode zum makroskopischen nachweis von implantationsstellen am uterus der ratte. Archiv. Path. Exp. Pharmacol., 247:367.

**12.2.2 Resorptions:**

Late resorptions will be weighed, examined for external malformations and if unremarkable discarded. Resorptions with external findings will be saved at the discretion of the Study Director.

**13 PRESERVATION OF RECORDS AND SPECIMENS:**

All data documenting experimental details and study procedures and observations will be recorded and maintained as raw data.

At the completion of the study, all reports, raw data, preserved specimens and retained samples will be maintained in the Testing Facility's Archives for a period of one year after submission of the signed final report.

The Sponsor will then be contacted in order to determine the final disposition of these materials. The Sponsor will be responsible for all costs associated with the storage of these materials beyond one year from the issuance of the final report and for any costs associated with the shipment of these materials to the Sponsor or to any other facility designated by the Sponsor.

**14 STATISTICAL EVALUATIONS:**

The following items will be analyzed statistically in the final report:

**14.1 CONTINUOUS DATA:**

- Mean body weights (all recorded intervals)
- Mean body weight change (between all recorded interval and cumulative to include Days 0-6, 6-18 in the mouse and Days 0-6 and 6-20 in the rat), a cumulative weight gain for the Gestation Day 6-18 (mouse) or 6-20 (rat) intervals will also be calculated using the corrected Day 18 (mouse) or 20 (rat) gestation weights.
- Mean food consumption values (all recorded intervals)
- Mean number of corpora lutea
- Uterine implantation data (number of live, dead and resorbed fetuses)
- Ratio of resorptions to implantations
- Mean pre-implantation loss ratio
- Mean number of male and female fetuses
- Mean fetal weight (composite for both sexes and distinguished by sex)

#### 14.2 CONTINUOUS DATA - MULTIPLE GROUP ANALYSIS:

Employed when more than one treated group is compared to control.

Statistical evaluation of equality of means will be made by the appropriate one way analysis of variance technique, followed by a multiple comparison procedure if needed. Bartlett's test will be performed to determine if groups have equal variance. If the variances are equal, parametric procedures will be used; if not, nonparametric procedures will be used. The parametric procedures will be the standard one way ANOVA using the F distribution to assess significance. If significant differences among the means are indicated, Dunnett's test will be used to determine which means are significantly different from the control. If a nonparametric procedure for testing equality of means is needed, the Kruskal-Wallis test will be used, and if differences are indicated, a summed rank test (Dunn) will be used to determine which treatments differ from control.

A statistical test for trend in the dose levels will also be performed. In the parametric case (i.e., equal variance), standard regression techniques with a test for trend and lack of fit will be used. In the non-parametric case, Jonckheere's test for monotonic trend will be used.

All ratios will be transformed via Bartlett's transformation followed by the arc-sine transformation prior to analysis. Data will be presented untransformed.

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

**14.2 CONTINUOUS DATA - MULTIPLE GROUP ANALYSIS:**

References for these techniques are Snedecor, G.W., Cochran, W.G., *Statistical Methods*, 6<sup>th</sup> edition, Iowa State Univ. Press (1967); Hollander and Wolfe, *Nonparametric Statistical Methods*, John Wiley and Sons, New York (1973); Dunnett, C.W., *J. Am. Sta. Assn.* 50: 1096-1121 (1955) and *Biometrics* 20: 482 (1964).

Bartlett's Test	pp. 296-298	Snedecor & Cochran
ANOVA	pp. 277-279	Snedecor & Cochran
Dunnett's Test	pp. 1096-1121	Dunnett
	pp. 482-491	Biometrics
Kruskal-Wallis	pp. 114-116	Hollander & Wolfe
Summed Rank Test (Dunn)	p. 131	Hollander & Wolfe
Regression Analysis - Trend	pp. 135-153	Snedecor & Cochran
Lack of fit	pp. 456-459	Snedecor & Cochran
Arc Sine Transformation	pp. 327-329	Snedecor & Cochran
Jonckheere's Statistic	pp. 120-123	Hollander & Wolfe

**14.3 INCIDENCE DATA:**

Mortality rate  
Pregnancy rates  
Incidence of fetuses with malformations/variations (external).  
Incidence of litters containing fetuses with malformations/variations (external)  
Incidence of litters (females) with resorptions

**14.4 INCIDENCE DATA ANALYSIS:**

Statistical analysis of incidence data will be performed using contingency tables. First, a standard chi-square analysis will be performed to determine if the proportion of incidences differed between the groups tested. Next, each treatment group will be compared to the control group using a 2x2 Fisher Exact Test; the significance level will be corrected via the Bonferroni inequality to assure an overall test of the stated significance level. Thirdly, Armitage's test for linear trend in the dosage groups will be performed. In keeping with standard statistical practice, if any one cell has an expected value of less than 5, the chi-square and Armitage's tests will not be reported. When this occurs, only the Fisher Exact test (corrected via Bonferroni inequality) will be performed and reported.

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

#### 14.4 INCIDENCE DATA ANALYSIS:

References for the techniques are Snedecor, G.W., and Cochran, W.G., *Statistical methods*, 6<sup>th</sup> ed., Iowa State University Press, Ames, Iowa (1971); Bradley, J.V., *Distribution Free Statistical Tests*, Prentice-Hall, Englewood Cliffs, New Jersey (1968); Miller, R.G., Jr., *Simultaneous Statistical Inference*, McGraw-Hill Book Co., New York (1966); Armitage, P., "Tests for Linear Trends in Proportions and Frequencies", *Biometrics*, (Sept. 1955).

Chi-square	pp. 250-253	Snedecor & Cochran
Fisher Exact Test	pp. 195-203	Bradley
Bonferroni inequality	p. 15	Miller
Armitage's Test	pp. 375-386	Armitage

#### 15 REPORT:

One copy of a draft report will be submitted within four months following termination of the study. Within 25 days of receipt of the Sponsor's comments to the draft report, appropriate changes will be made and two copies of a signed, final report will be issued. All data and documents shall be submitted in both hard copy and magnetic form: one (1) camera-ready copy of the final report on a 5 1/4 inch floppy disk in a mutually agreeable format shall also be provided. Additional copies of the reports if requested, will be provided at additional cost. The final report will include:

- Abstract
- Introduction
- Experimental Design
- Materials and Methods
- Discussion of Study Results
- Conclusion and No-observed-effect level (NOEL) Statement
- Tables of daily mean exposure concentrations
- Mortality data
- Pregnancy data
- Mean body weight and weight gain data during gestation
- Mean food consumption data
- Mean uterine weight data and corrected Day 18 (mouse) or Day 20 (rat) gestation weights
- Mean number of corpora lutea, implantation sites, resorptions and fetuses
- Mean pre-implantation loss ratio
- Incidence of litters (females) with resorptions
- Mean ratio of resorptions to implants
- Mean fetal weight (composite of both sexes and distinguished by sex)
- Mean number of male and female fetuses per dam
- Incidence of fetuses with external malformations/variations

---

**15 REPORT:**

Incidence of litters containing fetuses with external malformations/vari-  
ations  
Summary statement on physical in-life observation  
Summary of maternal gross postmortem examination data

Appended data will include but not be limited to the following:

Individual maternal body weight data and weight gain during gestation  
Individual food consumption data  
Individual female uterine weight data and corrected terminal gestation  
weight data  
Individual female uterine implantation data and corpora lutea data  
Individual fetal weights  
Maternal gross postmortem observations  
Fetal external malformations/variation observations - individual fetal data  
Historical control data  
Statistical procedures  
Personnel involved in the study  
Quality Assurance statement  
Compliance statement

**16 SIGNATURES:**

**16.1 INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC):**

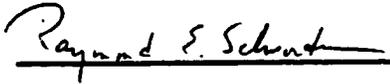
The IACUC Protocol Review Subcommittee has reviewed this protocol and found it to be in compliance with all appropriate regulations.

BY:  DATE: 12 June 1995

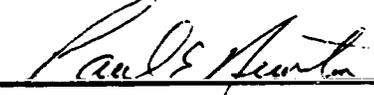
BY:  DATE: 13 June 95

FOR: Pharmaco LSR  
Toxicology Services Worldwide  
Institutional Animal Care and Use Committee

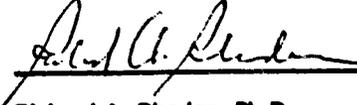
**16.2 PROTOCOL REVIEWED AND ACCEPTED:**

BY:  DATE: 14 June 95

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

BY:  DATE: 14 June 95

TITLE: Paul E. Newton, Ph.D., D.A.B.T.  
Inhalation Toxicologist  
FOR: Pharmaco LSR  
Toxicology Services Worldwide

BY:  DATE: 5/30/95

TITLE: Richard A. Rhoden, Ph.D.  
Sponsor's Representative  
FOR: American Petroleum Institute

PHARMACO LSR 10. STUD NO.: 95-6082  
SPONSOR STUDY NO. 08200-0601-SH9343

AMENDMENT NO.: 1

STUDY TITLE: A Range-finding Developmental Inhalation Toxicity Study of Regular Unleaded Gasoline in Rats and Mice via Whole-Body Exposure

TEST MATERIAL: Regular Unleaded Gasoline (130F fraction)

Changes

1. Page 5, 6. PROPOSED STUDY DATES: add the following dates:

Receipt of test animals:	22 May 1995
Initiation of mating:	19 June 1995 (rats) 21 June 1995 (mice)
Initiation of exposure:	26 June 1995 (rats) 28 June 1995 (mice)
Termination of exposure:	19 July 1995
Necropsy:	10-20 July 1995
Submission of draft final report:	20 October 1995
Experimental termination: (Date of last data collection)	20 July 1995

2. Page 7, 8. TEST MATERIAL:

8.4 UNUSED TEST MATERIAL:

Change the following: "The unused portion of the test material as well as any empty test material containers will be returned to the Sponsor following completion of the study".

To: "The unused portion of the test material as well as any empty test material containers will be retained at the Testing Facility until completion of the definitive study". Also delete reference to the Name, Address and Phone Number where materials were to be shipped.

3. Page 9, 9. TEST ANIMALS:

9.4.2.1 Rats:

Change the approximate age at receipt from approximately "seven weeks" to "eight weeks" and also change the approximate age at initiation of mating from approximately "nine weeks" to "12 weeks".

PHARMACO LSR INC. STUDY NO.: 95-6082  
SPONSOR STUDY NO. 08200-0601-SH9343

AMENDMENT NO.: 1

STUDY TITLE: A Range-finding Developmental Inhalation Toxicity Study of Regular Unleaded Gasoline in Rats and Mice via Whole-Body Exposure

TEST MATERIAL: Regular Unleaded Gasoline (130F fraction)

Changes (cont.):

3. Page 5, 6. TEST ANIMALS (cont.):

9.4.2.2 Mice:  
Change the approximate age at receipt from approximately "seven weeks" to "eight weeks" and also change the approximate age at initiation of mating from approximately "nine weeks" to "12 weeks".

4. Page 8, 9.4.5 ACCLIMATION PERIOD:

Change acclimation period from "approximately two weeks" to "approximately four weeks".

5. Page 10, 9.6.7. Environmental Conditions:

9.6.7.1 Light/Dark Cycle:  
Change to: "Following from "twelve hour light/dark cycle (light period will be approximately 6:00 to 18:00 hrs was provided via automatic timer".

Reason for changes

- The protocol was deficient in study schedule dates.
- Unused test material will be retained for use in definitive study.
- Animals were slightly older at receipt. The initiation of mating was delayed as additional time was required to develop the appropriate generation procedure and to provide adequate chamber exposure trial data. Therefore, animals were older at initiation of mating.
- The acclimation period was extended (see No. 3 above).
- Animals received a 12 hour light/dark photoperiod; however, during acclimation the light cycle was 7:00 to 19:00 hours and once mating the light cycle was 6:00 to 18:00 hours.

-24-  
D. J. [unclear]  
6/25/51

PROTOCOL AMENDMENT

Page 2 of 3

PHARMACO LSR INC. STUDY NO.: 95-6082  
SPONSOR STUDY NO. 08200-0601-SH9343

AMENDMENT NO.: 1

**STUDY TITLE:** A Range-finding Developmental Inhalation Toxicity Study of Regular Unleaded Gasoline in Rats and Mice via Whole-Body Exposure

**TEST MATERIAL:** Regular Unleaded Gasoline (130F fraction)

Changes (cont.):

---

3. Page 9, 9. TEST ANIMALS (cont.):

9.4.2.2 Mice:

Change the approximate age at receipt from approximately "seven weeks" to "eight weeks" and also change the approximate age at initiation of mating from approximately "nine weeks" to "12 weeks".

4. Page 9, 9.5 ACCLIMATION PERIOD:

Change acclimation period from "approximately two weeks" to "approximately four weeks".

5. Page 10, 9.6.7. Environmental Conditions:

9.6.7.1 Light/Dark Cycle:

Change the following from: "Twelve hour light/dark cycle (light period will be approximately 6:00 to 18:00 hrs each day) provided via automatic timer".

To:

"Twelve hour light/dark cycle provided via automatic timer".

Reasons for Changes

---

1. The protocol was deficient in study schedule dates.
2. Unused test material will be retained for use in definitive study.
3. Animals were slightly older at receipt. The initiation of mating was delayed as additional time was required to develop the appropriate generation procedure and to provide adequate chamber exposure trial data. Therefore, animals were older at initiation of mating.
4. The acclimation period was extended (see No. 3 above).
5. Animals received a 12 hour light/dark photoperiod; however, during acclimation the light cycle was 7:00 to 19:00 hours and once mated the light cycle was 6:00 to 18:00 hours.

-25-

*OK*

PROTOCOL AMENDMENT

PHARMACO LSR INC. STUDY NO.: 95-6082  
SPONSOR STUDY NO. 08200-0601-SH9343

AMENDMENT NO.: 1

STUDY TITLE: A Range-finding Developmental Inhalation Toxicity Study of  
Regular Unleaded Gasoline in Rats and Mice via Whole-Body  
Exposure

TEST MATERIAL: Regular Unleaded Gasoline (130F fraction)

Additional Cost Required:      Yes   X   No

Amendment approved by:

*Raymond E. Schroeder*  
Raymond E. Schroeder, M.S., D.A.B.T.  
Title: Study Director  
For: Pharmaco LSR Inc.  
Toxicology Services Worldwide

28 July 95  
Date

*Paul E. Newton*  
Paul E. Newton, Ph.D., D.A.B.T.  
Title: Inhalation Toxicologist  
For: Pharmaco LSR Inc.  
Toxicology Services Worldwide

28 July 95  
Date

*Richard A. Rhoden*  
Richard A. Rhoden, Ph.D.  
Title: Sponsor's Representative  
For: American Petroleum Institute

7/27/95  
Date

# GOLDMAN ASSOCIATES INTERNATIONAL, INC.

FYI -1097 - 001299

## Quality Assurance Statement

Study Title: An Inhalation Developmental Toxicity Study of Unleaded Gasoline Vapor Condensate (API 94-02) in the Rat Via Whole Body Exposure - Study No.: 95-6083 and API Study No.: 08200-0601-SH9343

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Test Article: Unleaded Gasoline Vapor Condensate (API 94-02)

Quality assurance inspections/audits on this study were conducted by Goldman Associates International, Inc. on the schedule shown below.

Date of Inspection	Type of Inspection	Date Issued
September 17-19, 1995	Protocol	October 9, 1995
October 30-31, 1995	Phase audit-test substance generation, analytical samplings and procedures, animal exposure, necropsy, and fetal external evaluations	December 22, 1995
July 15-17, 1996	Draft Report audit	September 3, 1996
April 28-29, 1997	Reformatted Report audit	May 9, 1997
July 14, 1997	Second Reformatted Report audit	September 3, 1997

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*Linda J. Calisti* 9/3/97  
Linda J. Calisti, B.S. Date  
Goldman Associates International, Inc.

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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  
Publication Number TR 414  
October 1997

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

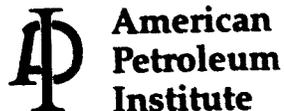
**Health and Environmental Sciences Department**

**API PUBLICATION NUMBER TR 414**

**PREPARED UNDER CONTRACT BY:**

**HUNTINGDON LIFE SCIENCES  
METTLERS ROAD  
PO Box 2360  
EAST MILLSTONE, NJ 08875-2360**

**OCTOBER 1997**



## 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.

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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.

Raymond E. Schroeder

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

29 September 97

Date

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Carol S. Auletta, B.A., D.A.B.T.  
Senior Director, Toxicology

29 Sept 97

Date

Ward R. Richter

Ward R. Richter, D.V.M., M.S.,  
Diplomate, A.C.V.P.  
Vice President, Research and Pathology

29 Sep 97

Date

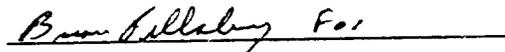
LABORATORY INDIVIDUAL SIGNATURE PAGE

  
Gary M. Hoffman, B.A., D.A.B.T.  
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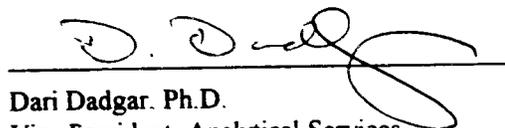
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Laura V. Cojocaru, B.S. Chem. Eng.  
Manager, Analytical Services

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	1 <sup>st</sup> 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

29 September 97  
Date

## 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  $1015 \pm 35$ ,  $2984 \pm 147$  and  $8993 \pm 278$  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.  
Washington, D.C. 20005

### Sponsor Representative

Richard A. Rhoden, Ph.D.

### Testing Facility

Huntingdon Life Sciences  
Mettlers Road  
P.O. Box 2360  
East Millstone, New Jersey 08875-2360

### Study Director

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

## EXPERIMENTAL DESIGN

Group	Exposure Level <sup>a</sup> (ppm)	Treatment Schedule <sup>b</sup> (GD)	Number of Animals				
			Mated	Sacrificed GD 20 <sup>c</sup>	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 <sup>d</sup>	6-19	24	24	All	1/2	1/2

<sup>a</sup>Exposure levels were established on the basis of data from a range-finding study (Huntingdon Life Sciences Study No. 95-6082).

<sup>b</sup>Exposures were 6 hours/day. Groups II-IV received Unleaded Gasoline Vapor Condensate (API 94-02). Control animals received filtered room air only.

<sup>c</sup>Complete macroscopic postmortem evaluations were performed on all animals.

<sup>d</sup>The 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**

Date Final Report is signed by the Study Director.

## 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<sup>®</sup>

### Strain

Sprague-Dawley derived (CD<sup>®</sup>) [Cri: CD<sup>®</sup> 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<sub>99</sub>) 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 M.

### 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).

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

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 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)**

<b>STATISTICAL SYMBOL</b>			<b>STATISTICAL STATEMENT</b>
<b>No Sig</b>	<b>p≤0.05</b>	<b>p≤0.01</b>	
<b><u>Parametric</u></b>			
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.
<b><u>Nonparametric</u></b>			
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

07 3 4 6

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
<u>No Sig</u>	<u>p≤0.05</u>	<u>p≤0.01</u>	
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<sup>®</sup> 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 ± S.D.	Mean ± S.D.
I	0	0	-
II	1000	1015 ± 35	1018 ± 117
III	3000	2984 ± 147	3227 ± 208
IV	9000	8993 ± 278	9033 ± 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):

0-3-5-0

Table 3: Summary of Particle Size Distribution Measurements

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

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 <sup>a</sup>	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).

<sup>a</sup>Each 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.

#### Fetal Soft Tissue Examination Data (Appendix K)

**Soft Tissue Malformations.** The incidences of fetuses with soft tissue malformations for the control, low-, mid- and high-dose groups were 0% (168 fetuses), 0.6% (1/172), 0% (174 fetuses) and 0% (169 fetuses), respectively. The incidences of litters containing fetuses with soft tissue malformations for these same groups were 0% (24 litters), 4.5% (1/22), 0% (22 litters) and 0% (21 litters), respectively. In the absence of soft tissue malformations among the mid- and high-dose fetuses, the single malformation seen in the low-dose group was not considered indicative of a treatment-related response.

Unilateral microphthalmia (left eye) was seen in one fetus at the low-dose level. This malformation has been seen at low incidence in this laboratory as indicated from recent historical control data (Appendix O, page O-8). In the absence of similar malformations among fetuses at the higher dose levels, the low incidence in occurrence of this finding in the low-dose group was not considered indicative of a treatment-related response.

**Soft Tissue Variations.** Soft tissue variations are findings that involve subtle changes in size, shape or appearance of the visceral organs/tissues. These types of observations are considered to represent transient developmental stages. Such subtle changes are not considered indicative of malformation but an increase in incidence of fetuses with certain soft tissue variations when seen to occur in a dose-related pattern may be indicative of a response to treatment (i.e., delayed maturation).

The incidences of fetuses with soft tissue variations for the control, low-, mid- and high-dose groups were 0% (168 fetuses), 0.6% (1/172), 0.6% (1/174) and 0% (169 fetuses), respectively. The incidences of litters containing fetuses with soft tissue variations for these same groups were 0% (24 litters), 4.5% (1/22), 4.5% (1/22) and 0% (21 litters), respectively. These incidences for the Unleaded Gasoline Vapor Condensate-treated groups, on both a per fetus and per litter basis, did not differ statistically from control data and were not considered indicative of a treatment-related response.

The only soft tissue variation seen during this study was tortuous ureters. This was seen in one fetus each from the low- and mid-dose groups. This soft tissue variation is seen commonly in this laboratory in the Day 20 gestation fetal rat as indicated from recent historical control data (Appendix O, page O-9 to O-11). The incidence of this finding in the low- and mid-dose groups was within the range of these historical control data and no adverse effect of treatment was indicated from its low incidence of occurrence in this study.

#### Fetal Skeletal Examination Data (Appendix L)

Skeletal Malformations. The incidences of fetuses with skeletal malformations for the control, low-, mid- and high-dose groups were 1.3% (2/160), 0% (162 fetuses), 0.6% (1/162) and 1.3% (2/156), respectively. The incidences of litters containing fetuses with skeletal malformations for these same groups were 8.3% (2/24), 0% (22 litters), 4.5% (1/22) and 4.8% (1/21), respectively.

Fourteenth rib or rib pair and 27 presacral vertebrae (seven cervical, 14 thoracic and six lumbar vertebrae) were seen in one fetus each from the control and mid-dose groups and two fetuses from one litter in the high-dose group. These incidences, on both a per fetus and per litter basis, for the mid- and high-dose groups were considered similar to control data and no adverse effect of treatment was indicated. This finding (i.e., 27 presacral vertebrae rather than the normal 26 vertebrae) is seen at low incidence historically in this laboratory (Appendix O, page O-12).

The only other skeletal malformation seen during the study was the presence of five lumbar vertebrae. This was seen in one control fetus.

Ossification Variations. Ossification variations may represent delays in the ossification process (retarded ossification) or slight ossification irregularities which may or may not be present in the adult specimen. Ossification variations are not considered representative of malformation. Increases in the incidences of fetuses and/or litters containing fetuses with particular ossification variations in relation to the concurrent control data and/or recent historical control data (Appendix O, pages O-13 to O-14) may be indicative of retarded ossification.

The incidences of fetuses with one or more ossification variations for the control, low-, mid- and high-dose groups were 71.9% (115/160), 72.8% (118/162), 66.7% (108/162) and 69.9% (109/156), respectively. The incidences of litters containing fetuses with ossification variations for these same groups were 95.8% (23/24), 100.0% (22/22), 95.5% (21/22) and 100.0% (21/21), respectively. These incidences, on both a per fetus and per litter basis, for the treated groups were comparable to control data.

The only ossification variation noted with increased incidence in the treated groups was rudimentary rib(s). This finding is seen frequently in the Day 20 gestation rat fetus in this laboratory as indicated from the tabulation of recent historical control (presented in Appendix O, page O-14 and summarized below Table 5).

Table 5: Study Incidence of the Ossification Variation "Rib(s) - 1st Lumbar Rudimentary" in Comparison to Recent Laboratory Historical Control Data

	Group (ppm)				Laboratory's Recent Historical Control Data <sup>a</sup>
	I (0)	II (1000)	III (3000)	IV (9000)	
Number of Fetuses (Litters) Examined:	160(24)	162(22)	162(22)	156(21)	
Rib(s) - 1st Lumbar Rudimentary:					
Fetal Incidence (%)	17(10.6)	27(16.7)	33(20.4)	33(21.2)	Mean: 5.4% Max: 18.5%
Litter Incidence (%) <sup>b</sup>	9(37.5)	15(68.2)	13(59.1)	16(76.2)	Mean: 21.1% Max: 63.6

<sup>a</sup>Represents data for 18 developmental toxicity studies conducted over the 1989-1994 period.

<sup>b</sup>Total number of litters containing at least one fetus with a rudimentary rib, either unilateral or bilateral.

While the incidence data for this finding in the concurrent control group were within this historical control range, the incidences for the treated groups were at or just outside these data. These incidence data were also compared to the recently published data on the CD rat from MARTA (Middle Atlantic Reproduction and Teratology Association) and MTA (Midwest Teratology Association). The incidence of rudimentary rib in these historical data (MARTA/MTA, 1995) are summarized below (Table 6) in comparison to the incidence of rudimentary rib seen among the control and treated groups in this study. The finding has been distinguished as unilateral or bilateral to better compare with the MARTA/MTA data. The incidences of rudimentary rib on both a per fetus and per litter basis in the present study were generally within the range of these historical control data.

Table 6: Incidence of the Ossification Variation "Rib(s) - 1st Lumbar Rudimentary" in Comparison to MARTA/MTA data base

	Group (ppm)				MARTA/MTA Historical Control Data
	I (0)	II (1000)	III (3000)	IV (9000)	
Number of Fetuses (Litters) Examined:	160(24)	162(22)	162(22)	156(21)	
Rib(s) - 1st Lumbar Rudimentary <sup>a</sup> :					
Fetal Incidence (%):					
Unilateral:	14(8.8)	21(13.0)	18(11.1)	19(12.2)	Av.: 0.5%; Max: 7.3%
Bilateral:	3(1.9)	6(3.7)	15(9.3)	14(9.0)	Av.: 0.7%; Max: 15.8%
Litter Incidence (%):					
Unilateral:	9(37.5)	13(59.1)	10(45.5)	16(76.2)	Av.: 4.1%; Max: 55.0%
Bilateral:	2(8.3)	5(22.7)	9(40.9)	7(33.3)	Av.: 3.9%; Max: 55.0%

<sup>a</sup>Referred to in MARTA/MTA data as "Rib Supernumerary". This is considered to represent the same observation as rudimentary rib noted in this report.

When reviewed in conjunction with all the ossification data for this study, the slight increase in rudimentary rib seen among the treated groups was not considered biologically significant. The increase in incidence on both a per fetus and per litter basis was not dose-responsive and the incidence data for other ossification variations seen during this study in the treated groups were similar to or slightly lower than the concurrent control data. Additionally, there were several other ossification variations seen during this study (e.g., incompletely ossified vertebral transverse processes [cervical and sacral], unossified vertebral processes [sacral and caudal] and unossified sternbrae [2nd and 6th] that occurred with notably lower frequency in fetuses from the treated groups than in control fetuses. Thus, the slight increase in incidence of rudimentary rib(s) in this study in the treated groups was not considered toxicologically significant because:

- other ossification variations seen in this study occurred with similar or increased frequency in the control group;
- it was not dose-responsive;
- it is not unusual to see this type of variation in ossification among groups in these types of studies; and
- there were no statistically significant differences between the control and treated groups in overall incidence of fetuses or litters containing fetuses with ossification variations.

#### CONCLUSION

Thus, in this inhalation developmental toxicity study with Unleaded Gasoline Vapor Condensate (API 94-02), no maternal or developmental toxicity was seen in rats at an exposure level up to and including 9000 ppm.

## Section 5

### REFERENCES

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## Section 6

### LOCATION OF SPECIMENS, RAW DATA AND FINAL REPORT

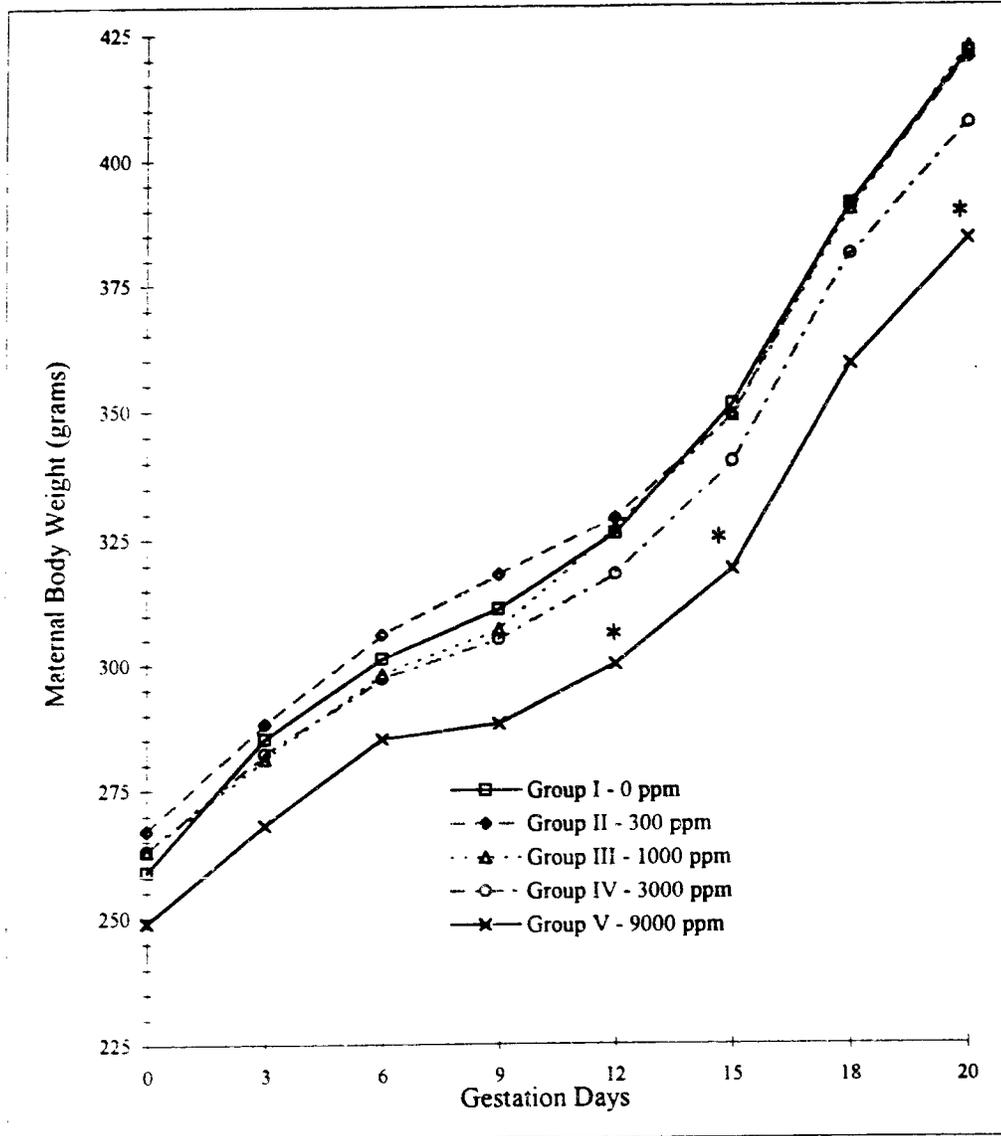
All data documenting experimental details, study procedures and observations were recorded and maintained as raw data.

All raw data, preserved specimens, and retained samples, as well as the original study protocol and the original final report are to be maintained in the Archives of the Testing Facility upon completion of the study.

Section 7

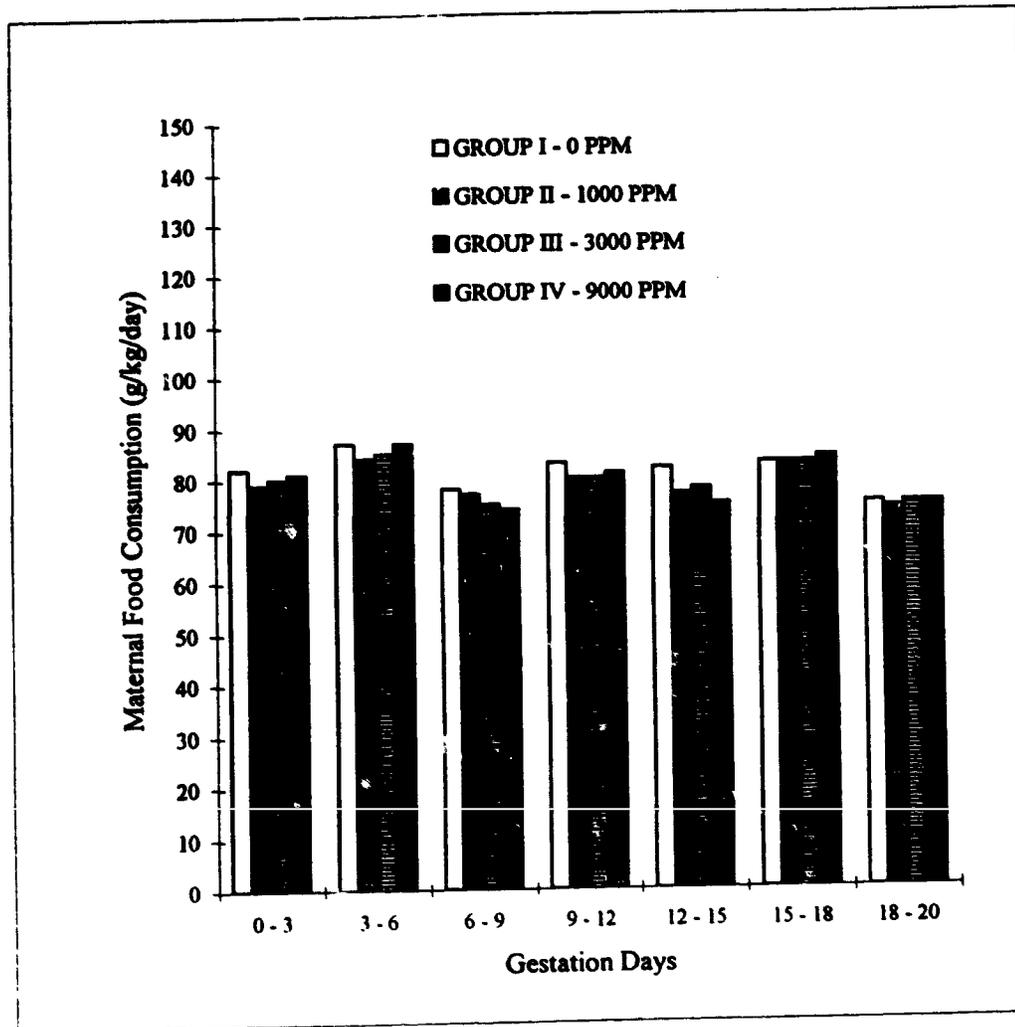
FIGURES

Figure 1: Mean Maternal Body Weights During Gestation - Rats



\*Significantly different from control mean;  $p \leq 0.05$  (Dunnett's).

Figure 2: Mean Maternal Food Consumption During Gestation



No statistically significant differences.

APPENDIX A  
ANIMAL TERMINATION HISTORY

APPENDIX Q  
PROTOCOL AND PROTOCOL AMENDMENTS

# PHARMACO::LSR

PHARMACO LSR  
TOXICOLOGY SERVICES WORLDWIDE

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

STUDY NO.: 95-6083

ISSUE NO.: 4

SPONSOR STUDY NO.: 08200-0601-SH9343

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

ATTENTION: Richard A. Rhoden, Ph.D.

DATE: 21 September 1995

Q-1

**1 INTRODUCTION:**

- 1.1 STUDY NO.:** 95-6083
- 1.2 ISSUE NO.:** 4
- 1.3 STUDY TITLE:** An Inhalation Developmental Toxicity Study of Unleaded Gasoline in the Rat via Whole-body Exposure
- 1.4 TEST MATERIAL:** Unleaded Gasoline (130F fraction)
- 1.5 SPONSOR:** American Petroleum Institute  
Health and Environmental Sciences  
Department  
1220 L Street, N.W.  
Washington, D.C. 20005
- 1.6 SPONSOR REPRESENTATIVE:** Richard A. Rhoden, Ph.D.  
Phone No.: 202-682-8480  
Fax No.: 202-682-8270
- 1.7 TESTING FACILITY:** Pharmaco LSR  
Toxicology Services Worldwide  
Mettlers Road  
P.O. Box 2360  
East Millstone, NJ 08875-2360
- 1.8 PURPOSE:**

This study is designed to assess the potential maternal toxicity and developmental toxicity of a test material administered via inhalation (whole-body exposure) to pregnant rats during the Day 6-19 gestation interval.

## 2 REGULATORY REFERENCES:

### 2.1 TEST GUIDELINE:

This study is 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 (870.3700, July 1994) in determining the dosing schedule.

### 2.2 GOOD LABORATORY PRACTICES:

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

### 2.3 FACILITIES MANAGEMENT/ANIMAL HUSBANDRY:

Currently acceptable practices of good animal husbandry will be followed, e.g., Guide for the Care and Use of Laboratory Animals; DHHS Publication No. (NIH) 86-23, Revised 1985. Pharmaco LSR, Toxicology Services Worldwide, is fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

### 2.4 ANIMAL WELFARE ACT COMPLIANCE:

This study will comply 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. The Sponsor should make particular note of the following:

1. The Sponsor's signature on this protocol documents for the study described, there are no generally accepted non-animal alternatives and the study does not unnecessarily duplicate previous experiments.
2. All procedures used in this study have been designed to avoid discomfort, distress and pain to the animals. All methods are described in this study protocol or in written laboratory standard operating procedures.

**2.4 ANIMAL WELFARE ACT COMPLIANCE:**

3. Any aspects of this study which cause more than momentary or slight pain or distress to the animals will be performed with appropriate sedatives, analgesics or anesthetics unless the withholding of these agents is justified for scientific reasons, in writing by the Sponsor and the Study Director, in which case the procedure will continue for the minimum time necessary.
4. Animals that experience severe or chronic pain or distress that cannot be relieved will be painlessly euthanized as deemed appropriate by the Testing Facility's veterinary staff and the Study Director. The Sponsor will be advised by the Study Director of all circumstances which could lead to this action in as timely a manner as possible.
5. Methods of euthanasia used during this study are in conformance with the above referenced regulations.

**3 QUALITY ASSURANCE MONITORING:**

The Pharmaco LSR, Toxicology Services Worldwide Quality Assurance Unit will monitor the facilities, equipment, personnel, methods, practices, records and controls used in this study to assure that they are in conformance with this protocol, company SOP's, and the appropriate Good Laboratory Practice regulations.

#### 4 ALTERATION OF DESIGN:

Alterations of this protocol may be made as the study progresses. No changes in the protocol will be made without the consent of the Sponsor. In the event that the Sponsor authorizes a protocol change verbally, such changes will be honored by the Testing Facility and will be followed by a written verification. All protocol modifications will be signed by the Study Director and a Sponsor representative. Any modifications potentially affecting animal welfare will also be signed by two members of the Institutional Animal Care and Use Committee prior to the modification's implementation.

#### 5 STUDY PERSONNEL:

Study Director:	Raymond E. Schroeder, M.S., D.A.B.T.
Inhalation Toxicology	Paul E. Newton, Ph.D., D.A.B.T.
Study Pathologist:	Arpad Madarasz, D.V.M.

Additional personnel will be documented in the project file and presented in the final report.

#### 6 PROPOSED STUDY DATES:

Study initiation date:	Date Study Director Signs protocol - See Section 16.2.
Receipt of test animals:	11 September 1995
Initiation of mating:	25 September 1995
Initiation of exposure:	2 October 1995
Termination of exposure:	1 November 1995
Necropsy:	16 October - 2 November 1995
Submission of draft final report:	1 April 1996
Experimental termination: (date of last data collection)	2 February 1996
Study completion date:	Date final report is signed by Study Director.

**7 EXPERIMENTAL DESIGN:**

Group	Dose Level ppm	Treatment Schedule*	Mated	Number of Animals			
				Sacrificed	Proportion of Gestation Day 20 Fetuses/Litter Evaluated for Malformations/Variations		
					Gestation Day 20	External	Soft Tissue
I (chamber-exposed, air control)	0	Gestation Days 6-19	24	A.S.	All	%	%
II	1000	Gestation Days 6-19	24	A.S.	All	%	%
III	3000	Gestation Days 6-19	24	A.S.	All	%	%
IV	9000	Gestation Days 6-19	24	A.S.	All	%	%

\* Six hours/day  
 Key: A.S. - all survivors

## 8 TEST MATERIAL:

### 8.1 TEST MATERIAL: Unleaded Gasoline (130F fraction)

Description, lot number, storage, expiration date and handling procedures, as well as other pertinent information will be documented in the study data.

### 8.2 IDENTIFICATION OF TEST MATERIAL:

Unless otherwise noted, the identity, strength, purity, composition, stability, and method of synthesis, fabrication and/or derivation of each batch of the test material will be documented by the Sponsor before its use in the study. This documentation will be maintained by the Sponsor at the address indicated in Section 1.5 of this protocol.

### 8.3 ARCHIVAL SAMPLES:

An archival sample from each lot of test material will be taken and stored in the Archives of the Testing Facility. If multiple studies are conducted with the same material, a common archival sample may be taken and appropriately labeled.

### 8.4 UNUSED TEST MATERIAL:

The unused portion of the test material as well as any empty test material containers will be returned to the Sponsor following completion of the study. Materials will be shipped to:

Name: Experimental Pathology Laboratories, Inc.  
Address: 22866 Shaw Road, Sterling Virginia  
Phone: Work - 703-471-4131/Fax - 703-471-8447

## 9 TEST ANIMALS:

Albino Rats (Outbred) VAF/Plus<sup>®</sup>

### 9.1 STRAIN:

Sprague Dawley - derived (CD<sup>®</sup>)  
[Cr: CD<sup>®</sup> BR]

### 9.2 SUPPLIER:

Charles River Laboratories  
Portage, Michigan

**9.3 JUSTIFICATION FOR TEST SYSTEM SELECTION:**

The rat is a rodent animal model commonly utilized in developmental toxicity studies as recommended in the referenced guidelines. In addition, a historical control data base for this laboratory is available for comparative evaluation.

**9.4 ANIMAL REQUIREMENTS/SPECIFICATIONS:**

**9.4.1 Number:**

	<u>Females</u>
Purchased - approximately	140
Placed on test	96

Males: In-house colony used solely for mating.

**9.4.2 Age:**

Approximately 10 weeks at receipt; 12 weeks at initiation of mating.  
Note: Females will be nulliparous and non-pregnant.

**9.5 ACCLIMATION PERIOD:**

Approximately two weeks; all animals will be checked for viability twice daily. Prior to assignment to study all animals will be examined to ascertain suitability for use on study.

**9.6 ANIMAL HUSBANDRY/NON-EXPOSURE:**

**9.6.1 Housing:**

Animals will be housed individually in suspended stainless steel wire mesh cages except as follows:

Mating: one male and one female co-housed nightly.

**9.6.2 Food:**

Certified Rodent Diet, No. 5002; (meal) (PMI Feeds, Inc., St. Louis, MO) *ad libitum*.

**9.6.3 Water:**

Elizabethtown Water Company, Westfield, NJ; *ad libitum*, via automated watering system.

**9.6.4 Feed Analysis:**

Analytical certification of batches of feed provided by the manufacturer will be maintained on file at the Testing Facility. Photocopies of these certification records for diets used on study will be appended to the final report. There are no known contaminants in the feed which are expected to interfere with the results of this study.

**9.6.5 Water Analysis:**

Monthly water analyses, provided by Elizabethtown Water Company, will be maintained on file at the Testing Facility. Biannual chemical and microbiological analyses of water samples collected from representative rooms in this facility are conducted to assure that water meets standards specified under the EPA National Primary Drinking Water Regulations (40 CFR Part 141). Results are maintained on file. Photocopies of these analytical data for the water supplied to the laboratory over the study interval will be appended to the final report. There are no known contaminants in the water which are expected to interfere with the results of this study.

**9.6.6 Veterinary Care:**

Animals are monitored by the technical staff for any conditions requiring possible veterinary care. If any such conditions are identified, a staff veterinarian will be notified for an examination and evaluation. Any medical veterinary intervention will be made only with approval of the staff veterinarian and the Study Director. The Sponsor will be consulted whenever possible. However, in emergency situations, decisions will be made as needed and the Sponsor will be advised as soon as possible.

**9.6.7 Environmental Conditions:**

**9.6.7.1 Light/Dark Cycle:**

Twelve hour light/dark cycle provided by automatic timer. The actual times for turning the animal room lights on in the morning and off in the afternoon will be similar between the rooms for acclimation/mating and exposures.

**9.6.7.2 Temperature:**

Monitored and recorded twice daily. The desired temperature range is 64-79°F (18-26°C). Temperature will be maintained in this range to the maximum extent possible.

**9.6.7.3 Humidity:**

Monitored and recorded once daily. The desired humidity range is 40-70%. Humidity will be maintained in this range to the maximum extent possible.

**9.7 ANIMAL HUSBANDRY/EXPOSURE:**

- 9.7.1 Housing:** Individually in cages.
- 9.7.2 Food:** None.
- 9.7.3 Water:** None.

**9.8 SELECTION FOR STUDY:**

More animals than required for the study will be purchased and equilibrated. Animals considered suitable for study on the basis of pretest physical examinations will be incorporated into the mating phase of the study. Females which mate will be assigned to groups daily in such a way as to most nearly equalize both the Day 0 mean body weights between groups and the distribution of animal into groups. This will be a manual sorting procedure. Disposition of all animals not utilized in the study will be maintained in the study file.

**9.9 ANIMAL IDENTIFICATION:**

Each female will be assigned a temporary identification number upon receipt. Mated animals assigned to the study will have affixed a metal eartag with a number assigned by the Testing Facility. This number plus the study number will comprise the unique identification for each animal. Each non-exposure cage will be provided with a label which will be color-coded for exposure level identification and will contain the study number and animal number.

**9.10 MATING PROCEDURE:**

Females selected for mating will be placed with the male rats nightly in a 1:1 ratio. Vaginal smears will be taken early in the morning following nightly intervals of co-housing and females will be considered to have mated if sperm is observed microscopically in the vaginal smear and/or a vaginal plug is observed. The day on which evidence of mating is observed will be defined as Day 0 of gestation.

## 10 TEST MATERIAL ADMINISTRATION:

### 10.1 ROUTE OF ADMINISTRATION:

Inhalation via whole-body exposures.

### 10.2 JUSTIFICATION FOR ROUTE OF ADMINISTRATION:

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

### 10.3 FREQUENCY OF ADMINISTRATION:

Once daily, 6 hours/day.

### 10.4 DURATION OF ADMINISTRATION:

Females will be exposed via inhalation for 6 hours/day on Days 6-19 of gestation. Control animals will be chamber-housed for 6 hrs/day and receive untreated, clean air only over the Day 6-19 gestation period.

### 10.5 ADMINISTRATION OF TEST MATERIAL:

The test material will be administered as a vapor in the breathing air of the animals. The test atmosphere will be generated using a counter-current volatilization chamber. Pretest trials will be performed to evaluate the optimal set of conditions and equipment to generate a stable atmosphere at the target exposure levels. The complete generation method will be described in the raw data of the study and in the final report.

The whole-body exposure chambers will each have a volume of approximately 1000 liters. Each chamber (control and exposure groups) will be operated at a minimum flow rate of 200 liters per minute. The final airflow will be set to provide at least one air change in 5.0 minutes (12 air changes/hour) and a  $T_{99}$  equilibrium time of at most 23 minutes. This chamber size and air flow rate is considered adequate to maintain the oxygen level above 19% and the animal loading factor below 5%. At the end of the exposure, all animals (control and treated) will remain in the chamber for a minimum of 30 minutes. During this time the chamber will be operated at approximately the same flow rate using clean air only.

**10.6 EXPOSURE CONCENTRATION DETERMINATION:**

*See p 22 Q8 1/20/95*  
A nominal exposure concentration will be calculated if possible. The flow of air through the chamber will be monitored using appropriate calibrated equipment. The test material consumed during the exposure will be divided by the total volume of air passing through the chamber (volumetric flow rate times total exposure time) to give the nominal concentration.

During each exposure, measurements of airborne concentrations will be performed at least six times/exposure level using an infrared spectrophotometer (Miran) sampling procedure. Additionally, one sample will be analyzed by gas chromatography to fingerprint for the 15 major components. One fingerprint will be analyzed daily rotating among the three exposure groups throughout the exposure period. Also prior to initiation of animal exposures additional samples will be taken to determine the distribution of the test material in the exposure chamber.

**10.7 PARTICLE SIZE DISTRIBUTION ANALYSIS:**

During each exposure, particle size determinations will be performed using a cascade impactor or other appropriate device, to characterize the aerodynamic particle size distribution of any aerosol present.

**10.8 CHAMBER ENVIRONMENT:**

Temperature, humidity and air flow rate will be recorded every 30 minutes during exposure. Chamber temperature and relative humidity will be maintained, to the extent possible, between 20 to 24°C and 40 to 60%, respectively.

## 10 TEST MATERIAL ADMINISTRATION:

### 10.1 ROUTE OF ADMINISTRATION:

Inhalation via whole-body exposures.

### 10.2 JUSTIFICATION FOR ROUTE OF ADMINISTRATION:

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### 10.3 FREQUENCY OF ADMINISTRATION:

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### 10.5 ADMINISTRATION OF TEST MATERIAL:

The test material will be administered as a vapor in the breathing air of the animals. The test atmospheres will be generated using a counter-current volatilization chamber. Pretest trials will be performed to evaluate the optimal set of conditions and equipment to generate a stable atmosphere at the target exposure levels. The complete generation method will be described in the raw data of the study and in the final report.

The whole-body exposure chambers will each have a volume of approximately 1000 liters. Each chamber (control and exposure groups) will be operated at a minimum flow rate of 200 liters per minute. The final airflow will be set to provide at least one air change in 5.0 minutes (12 air changes/hour) and a  $T_{99}$  equilibrium time of at most 23 minutes. This chamber size and air flow rate is considered adequate to maintain the oxygen level above 19% and the animal loading factor below 5%. At the end of the exposure, all animals (control and treated) will remain in the chamber for a minimum of 30 minutes. During this time the chamber will be operated at approximately the same flow rate using clean air only.

### 10.9 SUMMARY OF CHAMBER ACTIVITY:

The minimum frequency of chamber activity is summarized below.

<u>Activity</u>	<u>Frequency/chamber/day</u>
Measured Test Material Concentration (Miran)	6X
GC (major components)	one fingerprint/day rotating among the three exposure groups
Particle Size	1X
Temperature	13X
Relative Humidity	13X
Airflow Rate	13X
Nominal Test Material Concentration (excluding the air control chamber)	1X
Rotation Pattern of Exposure Cages	1X
Loading/Unloading Verification	1X

### 11 EXPERIMENTAL EVALUATION:

#### 11.1 OBSERVATIONS:

##### 11.1.1 Viability Checks (In-Cage):

Observations for mortality, general appearance and signs of severe toxic or pharmacologic effects will be made at least twice daily. Animals in extremely poor health or in a possible moribund condition will be identified for further monitoring and possible euthanasia.

##### 11.1.2 Physical Examinations:

Each animal will be removed from its cage and examined twice pretest and on Days 0, 6-20 of gestation. Examinations will include observations of general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia as well as evaluations of respiration. During the exposure period animals will be examined pre- and post-exposure.

**11.1.3 In-chamber Observations:**

All visible animals will be observed as a group at least once during each exposure for appearance, activity or other unusual findings.

**11.2 BODY WEIGHTS:**

Body weights will be recorded on Days 0, 3, 6, 9, 12, 15, 18 and 20 of gestation.

**11.3 FOOD CONSUMPTION:**

Food consumption will be recorded for each female during the following intervals of gestation: Days 0-3, 3-6, 6-9, 9-12, 12-15, 15-18 and 18-20.

**12 POSTMORTEM:**

**12.1 MACROSCOPIC POSTMORTEM EXAMINATIONS:**

Complete macroscopic postmortem examinations will be performed on all mated rats, including those dying spontaneously or sacrificed in a moribund condition and on females sacrificed after aborting or premature delivery of a litter. Only gross lesions will be saved in 10% neutral buffered formalin. Dams showing signs of abortion or premature delivery (expulsion of concepti) will be euthanatized (overdose of inhaled carbon dioxide) on the day such evidence is observed. Reproductive tracts will be examined and fetuses obtained 19 days or later will be weighed and given an external examination. These fetuses/delivered pups if still viable will be sacrificed with an overdose of carbon dioxide, eviscerated and processed for staining of the skeletal structures with Alizarin Red S and examined for skeletal malformations. Fetuses obtained earlier than Day 19 will be evaluated for external malformations and saved (10% neutral buffered formalin) at the discretion of the Study Director.

All females surviving on Day 20 of gestation will be sacrificed by exsanguination following anesthesia with carbon dioxide. The following examinations will be made for females sacrificed on Day 20 of gestation:

**12.1.1 Reproductive System:**

The intact uteri (ovaries and oviduct attached) will be examined *in-situ* for signs of hemorrhage and then removed from the abdominal cavity and weighed. The ovaries will then be dissected free and the uterus dissected longitudinally along the antimesometrial border and the number and location of the following will be recorded for each uterine horn.

**12.1.1 Reproductive System:**

- 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)
- implantation sites

Uteri without grossly visible implantations will be stained according to the procedure of Salewski<sup>a</sup>. If stained foci are present, the female will be considered pregnant for purposes of calculating pregnancy rates. The number of foci will not be used in the calculation of uterine implantation data.

**12.1.2 Ovaries:**

Corpora lutea of pregnancy will be counted.

**12.2 FETAL EVALUATIONS:**

**12.2.1 External Evaluations:**

All fetuses will be weighed and individually identified. Each fetus will be given a gross external examination for defects to include observation of the palate and external sex determinations will be made (to be confirmed by internal inspection of the gonads at subsequent examination).

**12.2.2 Fetal Skeletal Evaluations:**

Approximately one-half of the fetuses in each litter (alternating fetuses within the litter) will be processed for Alizarin Red S staining of the skeletal structures. The intact fetuses will be sacrificed with an overdose of carbon dioxide, eviscerated (internal sex noted) and processed for staining of the skeletal structures. Subsequently, these fetuses will be evaluated for skeletal malformations and/or ossification variations.

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<sup>a</sup>Salewski, E. (1964). Farbmethode zum makroskopischen nachweis von implantationsstellen am uterus der ratte. Archiv. Path. Exp. Pharmacol. 247:367.

#### 12.2.3 Fetal Soft Tissue Evaluation:

The remaining fetuses in each litter will be processed for soft tissue examination using a microdissection technique similar to that described by Staples<sup>a</sup>. The evaluations will be performed on the fresh fetal specimens soon after removal from the uterus. The fetuses designated for soft tissue evaluation will be decapitated (head placed in Bouin's solution for later evaluation). The fetal specimens will then be secured beneath a dissecting microscope (10-20X) and dissected so as to permit evaluation of tissues in the thoracic and abdominal cavities. At completion of the examination, the decapitated fetal specimen with viscera intact will be placed in individual cassettes and preserved in a 10% neutral buffered formalin solution. Such fetuses could be stained with Alizarin Red S and subsequently evaluated for skeletal malformations or ossification variations, if necessary (additional cost required).

Fetal heads, preserved in Bouin's solution, will be sectioned with a razor blade. The serial, transverse sections generated during this procedure will be evaluated for malformations of the palate, eyes and brain.

All fetal evaluations (microdissection, stained specimens and head sections) will be performed under a dissecting microscope (10-20X).

#### 12.2.4 Resorptions:

Late resorptions will be weighed and examined for external malformations. Only late resorptions with obvious external malformations will be saved (10% neutral buffered formalin) for future possible examination. Late resorptions that are externally unremarkable will be discarded. Early resorptions will be discarded.

### 13 PRESERVATION OF RECORDS AND SPECIMENS:

All data documenting experimental details and study procedures and observations will be recorded and maintained as raw data.

At the completion of the study, all reports, raw data, preserved specimens and retained samples will be maintained in the Testing Facility's Archives for a period of one year after submission of the signed final report.

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<sup>a</sup>Staples, R.E. (1974). Detection of Visceral Alterations in Mammalian Fetuses. *Teratology*, 9:437 (Abstract).

### **13 PRESERVATION OF RECORDS AND SPECIMENS:**

The Sponsor will be contacted in order to determine the final disposition of these materials. The Sponsor is responsible for all costs associated with the storage of these materials beyond one year from the issuance of the final report and for any costs associated with the shipment of these materials to the Sponsor or to any other facility designated by the Sponsor.

### **14 STATISTICAL EVALUATIONS:**

The following items will be analyzed statistically in the final report:

#### **14.1 CONTINUOUS DATA:**

Mean body weights (all recorded intervals to include the actual and corrected Day 20 gestation weights). The corrected Day 20 gestation weight will be derived by subtracting the gravid uterine weight from the actual Day 20 gestation weight;

Mean body weight change (between all recorded interval and cumulative change to include the Day 0-6, 6-20 gestation intervals. The later will be calculated using the actual and corrected Day 20 gestation weights;

Mean food consumption (all recorded intervals);

Mean number of corpora lutea of pregnancy;

Uterine implantation data;

Mean pre-implantation loss ratio;

Ratio of resorptions to implantations;

Mean number of male and female fetuses;

Mean fetal weight (composite for both sexes and distinguished by sex);

Mean number of male and female fetuses per litter.

#### **14.2 CONTINUOUS DATA - MULTIPLE GROUP ANALYSIS:**

Statistical evaluation of equality of means will be made by the appropriate one way analysis of variance technique, followed by a multiple comparison procedure, if needed. Bartlett's test will be performed to determine if groups have equal variance. If the variances are equal, parametric procedures will be used; if not, nonparametric procedures will be used. The parametric procedures will be the standard one way ANOVA using the F distribution to assess significance. If significant differences among the means are indicated, Dunnett's test will be used to determine which means are significantly different from the control. If a nonparametric procedure for testing equality of means is needed, the Kruskal-Wallis test will be used, and if differences are indicated, a summed rank test (Dunn) will be used to determine which treatments differ from control.

#### 14.2 CONTINUOUS DATA - MULTIPLE GROUP ANALYSIS:

A statistical test for trend in the dose levels will also be performed. In the parametric case (i.e., equal variance), standard regression techniques with a test for trend and lack of fit will be used. In the non-parametric case, Jonckheere's test for monotonic trend will be used.

All ratios will be transformed via Bartlett's transformation followed by the arc-sine transformation prior to analysis. Data will be presented untransformed.

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

References for these techniques are Snedecor, G.W., Cochran, W.G., *Statistical Methods*, 6<sup>th</sup> edition, Iowa State Univ. Press (1967); Hollander and Wolfe, *Nonparametric Statistical Methods*, John Wiley and Sons, New York (1973); Dunnett, C.W., *J. Am. Sta. Assn.* 50: 1096-1121 (1955) and *Biometrics* 20: 482 (1964).

Bartlett's Test	pp. 256-298	Snedecor & Cochran
ANCOVA	pp. 277-279	Snedecor & Cochran
Dunnett's Test	pp. 1096-1121	Dunnett
	pp. 482-491	Biometrics
Kruskal-Wallis	pp. 114-116	Hollander & Wolfe
Summed Rank Test (Dunn)	p. 131	Hollander & Wolfe
Regression Analysis - Trend	pp. 135-153	Snedecor & Cochran
Lack of fit	pp. 456-459	Snedecor & Cochran
Arc Sine Transformation	pp. 327-329	Snedecor & Cochran
Jonckheere's Statistic	pp. 120-123	Hollander & Wolfe

#### 14.3 INCIDENCE DATA:

Mortality rate;

Pregnancy rates;

Incidence of fetuses with malformations/variations (external, soft tissue and skeletal) analyses will be based on the total number of fetuses with malformations for each evaluation - external, soft tissue and skeletal;

Incidence of litters containing fetuses with malformations/variations (external, soft tissue and skeletal) analyses will be based on the total number of litters containing one or more fetuses with malformation for each evaluation - external, soft tissue and skeletal);

Incidence of fetuses with at least one ossification variation;

Incidence of females with resorptions.

#### 14.4 INCIDENCE DATA ANALYSIS:

Statistical analysis of incidence data will be performed using contingency tables. First, a standard chi-square analysis will be performed to determine if the proportion of incidences differed between the groups tested. Next, each treatment group will be compared to the control group using a 2 x 2 Fisher Exact Test; the significance level will be corrected via the Bonferroni inequality to assure an overall test of the stated significance level. Thirdly, Armitage's test for linear trend in the dosage groups will be performed. In keeping with standard statistical practice, if any one cell has an expected value less than 5, the chi-square and Armitage's tests will not be reported. When this occurs, only the Fisher Exact test (corrected via Bonferroni inequality) will be performed and reported:

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

References for the techniques are as follows: Snedecor, G.W., and Cochran, W.G., *Statistical methods*, 6<sup>th</sup> ed., Iowa State University Press, Ames, Iowa (1971); Bradley, J.V., *Distribution Free Statistical Tests*, Prentice-Hall, Englewood Cliffs, New Jersey (1968); Miller, R.G., Jr., *Simultaneous Statistical Inference*, McGraw-Hill Book Co., New York (1966); Armitage, P., "Tests for Linear Trends in Proportions and Frequencies", *Biometrics*, (Sept. 1955).

Chi-square	pp. 250-253	Snedecor & Cochran
Fisher Exact Test	pp. 195-203	Bradley
Bonferroni Inequality	p. 15	Miller
Armitage's Test	pp. 375-386	Armitage

#### 15 REPORT:

One copy of a draft report will be submitted following termination of the study. After receipt and review of the Sponsor's comments, appropriate changes will be made and two copies of a signed, final report will be issued. (Additional copies will be provided at additional cost). The report will include:

Abstract;  
Introduction;  
Experimental Design;  
Materials and Methods;  
Discussion of Study Results;  
Conclusion and No Observed Adverse Effect Level (NOAEL) Statement;  
Tables of daily mean exposure concentrations;  
Mortality data;

## 15 REPORT:

### Pregnancy data:

Mean maternal body weight and weight gain data;  
Mean maternal food consumption data;  
Mean uterine weight data and corrected Day 20 gestation weights;  
Mean number of corpora lutea, implantation sites, resorptions and fetuses;  
Mean pre-implantation loss ratio;  
Mean ratio of resorptions to implants;  
Incidence of females with resorptions;  
Mean fetal weight (composite for both sexes and distinguished by sex);  
Mean number of male and female fetuses per litter;  
Incidence of fetuses with at least one ossification variation;  
Incidence of fetuses with malformations/variations (external, soft tissue and skeletal);  
Incidence of litters containing fetuses with malformations/variations (external, soft tissue and skeletal);  
Summary statement on physical in-life observation;  
Summary statement on ossification variation data;  
Summary of maternal gross postmortem examination data.

Appended data will include but not be limited to the following:

Individual maternal body weight data and weight gain;  
Individual maternal food consumption data;  
Individual female uterine implantation data and corpora lutea data;  
Individual female uterine weight data and corrected Day 20 gestation weights;  
Summarized and individual data for the detailed physical examinations;  
Daily in-chamber observation data;  
Maternal gross postmortem observations;  
Summary of type/incidence of ossification variation data and individual fetal observations;  
Malformations/variation observations - individual fetal data for external soft tissue and skeletal observations;  
Chamber exposure data;  
Recent historical control data for this laboratory;  
Statistical procedures;  
Analytical data for the water supplied to the facility during study period;  
Certification records for the feeds used on study;  
Personnel involved in the study;  
Quality Assurance statement;  
Compliance statement.

**16 SIGNATURES:**

**16.1 INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC):**

The IACUC Protocol Review Subcommittee has reviewed this protocol and found it to be in compliance with all appropriate regulations.

BY: Tim S. [Signature] DATE: 25 Sep 95

BY: [Signature] DATE: 25 Sep 95

FOR: Pharmaco LSR  
Toxicology Services Worldwide  
Institutional Animal Care and Use Committee

**16.2 PROTOCOL REVIEWED AND ACCEPTED:**

BY: Raymond E. Schroeder [Signature] DATE: 26 Sept 95

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

BY: Paul E. Newton [Signature] DATE: 26 Sept 95

Paul E. Newton, Ph.D., D.A.B.T.  
TITLE: Inhalation Toxicologist

FOR: Pharmaco LSR  
Toxicology Services Worldwide

BY: Richard A. Rhoden [Signature] DATE: 9/22/95

Richard A. Rhoden, Ph.D.  
TITLE: Sponsor's Representative  
FOR: American Petroleum Institute

PROTOCOL AMENDMENT

Page 1 of 2

PHARMACO LSR INC. PROJECT NO.: 95-6083  
SPONSOR PROJECT NO.: 08200-0601-SH9343

AMENDMENT NO.: 1

**STUDY TITLE:** An Inhalation Developmental Toxicity Study of Unleaded Gasoline in the Rat via whole-body Exposure.

**TEST MATERIAL:** Unleaded Gasoline (130F fraction)

**Change:** \_\_\_\_\_

**1. Page 12, 10 TEST MATERIAL ADMINISTRATION:**

**10.6 EXPOSURE CONCENTRATION DETERMINATION:**

Change the following statement -

From:

Additionally, one sample will be analyzed by gas chromatography to fingerprint for the 15 major components."

To:

Additionally, one sample will be analyzed by gas chromatography to fingerprint for the 12 major components which comprise 80% of the test material."

**Reason for Change:** \_\_\_\_\_

1. In the pilot study (95-6082) 10 major components were identified from the charcoal and syringe samples. The protocol for this pilot study was not specific on the number of components that would be analyzed. Since these 10 components comprised approximately 78% of the test material, we proposed amending the protocol for the definitive study to analyze for these same 10 components. The components identified for analysis and a chromatogram of the test material were sent to Dr. Russ White (Chevron) for review (28 September 1995). Dr. White reported back that the taskforce would like us to account for at least 80% of the test material and requested that benzene be included in our analyses. We included benzene and 2,2,4-trimethylpentane in our analyses and these 12 components account for 80.35% of the test material.

PROTOCOL AMENDMENT

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PHARMACO LSR INC. PROJECT NO.: 95-6083  
SPONSOR PROJECT NO.: 08200-0601-SH9343

AMENDMENT NO.: 1

STUDY TITLE: An Inhalation Developmental Toxicity Study of Unleaded Gasoline in the Rat via whole-Body Exposure.

TEST MATERIAL: Unleaded Gasoline (130F fraction)

Additional Cost Required:      Yes   x   No

Amendment approved by:

Raymond E. Schroeder  
Raymond E. Schroeder, M.S., D.A.B.T  
Title: Study Director  
Toxicology Services Worldwide  
For: Pharmaco LSR Inc.

23 Oct 95  
Date

Richard A. Rhoden  
Richard A. Rhoden, Ph.D.  
Title: Sponsor Representative  
For: American Petroleum Institute

10/19/95  
Date

### CERTIFICATE OF AUTHENTICITY

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