

ABSTRACT

In compliance with the Clean Air Act Section 211(b) for fuel and fuel additive registration, the petroleum industry and oxygenate manufacturers have conducted comparative chronic toxicology testing of evaporative emissions from unleaded gasoline (baseline gasoline vapor condensate [BGVC]) and unleaded gasoline containing MTBE (gasoline/MTBE vapor condensate [GMVC]). This is the final report of the BGVC chronic study.

Groups of 50 male/50 female CDF(F344)CrIBR rats were exposed in H2000 whole-body inhalation chambers at BGVC vapor concentrations of 2 g/m³ (low level), 10 g/m³ (mid level), and 20 g/m³ (high level) 6 hours/day, 5 days/week for 104 weeks (518 exposure days).

There were no clinical signs of toxicity attributable to BGVC inhalation. Survival of the BGVC-exposed rats was not significantly different from that of control rats. Body weights of males in the mid- and high-level BGVC groups were significantly below control values throughout the study. Body weights of high-level and mid-level females were below control values after approximately 1 and 3 months of exposure, respectively. Body weights of low-level females were sporadically affected. At the final sacrifice, body weights of high-level males and high- and mid-level female rats were significantly below control values. Among mid-level males, the absolute epididymis weight, percent epididymis-to-body and percent epididymis-to-brain weights were significantly below the corresponding control values. The adrenal-to-body and brain-to-body weight values of high-level males were significantly greater than control values. The kidney weights of high- and mid-level females were significantly increased compared to controls, however they did not correlate with kidney pathology findings. The spleen weights of mid-level females were significantly increased compared to controls, which is likely a result of the high incidence of age-related mononuclear cell leukemia (MCL). Among mid-level females, the percent organ-to-body weight values for brain, heart, kidney, liver, lungs, and spleen were significantly greater than corresponding control values. Among high-level females, the percent organ-to-body values for adrenals, brain, heart, liver, kidney, and lungs were significantly greater than control values. The percent kidney-to-brain weights for mid- and high-level females and the percent spleen-to-brain weight for the mid-level females were significantly greater than the

corresponding control values. It is likely that many of the organ-to-body weights increases were artifacts of the treatment-related depression in body weight. For females, there were significantly fewer numbers of total WBCs, neutrophils, lymphocytes, eosinophils, and nucleated red blood cells (RBCs) in the high-level group at the 12 month time-point, only. In males, only the absolute number of lymphocytes was significantly elevated in the high-level group at the final sacrifice time-point. Due to blood sampling difficulties at the 12 month time point and the high, age-related incidence of mononuclear cell leukemia in all groups, the relationship of treatment to these changes in hematology values is unknown. BGVC exposure was associated with an increase in the incidence of respiratory epithelial degeneration and a significant decrease in the incidence of olfactory epithelial degeneration in the nasal passages of both male and female rats. The respiratory epithelial degenerative effects were most likely caused by exposure to the test material.

Exposure did not enhance the incidence of chronic progressive nephropathy in male or female rats, but did increase the severity of this lesion among mid- and high-level groups of males. The increased severity of chronic progressive nephropathy in males is consistent with previous studies on wholly vaporized unleaded gasoline, and is attributable to male rat specific alpha-2u globulin overload as a result of BGVC exposure. Consistent with previous studies and the alpha-2u globulin overload mechanism of male rat kidney damage, BGVC induced increases in male renal adenoma (1/50 in control; 1/50 in 2 g/m³ group; 4/50 in 10 g/m³ group; and 0/50 in 20 g/m³ group) and carcinoma (0/50 in controls; 0/50 in 2 g/m³ group; 3/50 in 10 g/m³ group; and 0/50 in 20 g/m³ group). Although the incidences of renal adenomas and carcinomas taken separately were not statistically significant, combined incidences for the control (1/50; 2%), low (1/50, 2%), mid (7/50, 14%) and high (0/50; 0%) exposure groups were significant ($p = 0.004$, Fisher's Exact Test). Pair-wise tests did not demonstrate any significant difference among the individual dose groups. These neoplasms may be considered to be exposure related.

The incidence of squamous cell carcinoma in the nasal passages (turbinate levels 2-4) was elevated in the high-level group of male rats, with tumor incidence of 1/50, 3/50, and 3/50 for levels 2, 3 and 4, respectively. The level 3 incidence gave a statistically significant trend for increased incidence with increased exposure concentration ($p = 0.019$, Cochran-Armitage [CA]

test). Squamous cell carcinoma was noted in the nasal specimens of only one female control rat of the current study. The squamous cell carcinomas in all of the male nasal specimens had morphologies consistent with origination from the oral mucosa and had varying degrees of invasion into the nasal passages. By comparison, in the concurrent Gasoline MTBE Vapor Condensate study (FY01-013), squamous cell carcinoma of oral origin occurred in one male control, two male GMVC low dose and two male high GMVC dose, but in no female rats. While the fact that the nasal tumors were of oral origin might suggest that the inhalation exposure was not causal, spontaneous oral squamous cell carcinomas in other studies have been nearly as rare as those originating from the nasal mucosa (~0.6%; Haseman et al., 1990; NTP, 1999). The historical incidence of squamous cell carcinoma of oral origin in a large cigarette smoke study conducted at LRRI in the 1990s was 0/118 (0%) in males and 3/119 (2.5% in females). Taken together, the data provide some evidence that the development of nasal squamous cell carcinomas in male rats may have resulted from test article exposure.

Similar to the incidence of renal neoplasms, there was an increased incidence of testicular mesothelioma in the mid-level group of male rats (4 of 50 examined). Fisher's exact test demonstrated that this incidence was greater than expected, but pair-wise testing between the groups and trend tests for exposure concentration-response effects did not indicate statistically significant effects of exposure. The incidence in the mid-level group is approximately 3-fold greater than the average incidence in male control F344 rats exposed to air in inhalation toxicology studies (28 tumors in 1,055 rats; NTP, 1999) and may be regarded as an effect of exposure to the test substance. As with this study, there were no mesotheliomas found in the testes or other tissues of the control male animals of the GMVC study (LRRI protocol FY01-013), although there was a statistically non-significant incidence (2 in 50 animals or 4%) of the tumor in the testes of the high-level GMVC-exposed rats and sporadic incidence of the tumor in other tissues of GMVC-exposed male rats. The absence of testicular mesothelioma in control animals of the present study, where 1 or 2 tumors might normally be expected to be found in 50 animals (NTP, 1999), may have influenced the statistical analysis to indicate a significant exposure-related incidence in the mid-level BGVC-exposed male rats, when no causal increase is actually present.

There was a trend for increased incidence of thyroid follicular cell carcinoma in male animals ($p < 0.05$, FE test over all groups; see Table 3-12) but there were no significant differences among the groups. There were two tumors in the 27 mid-dose glands examined and no tumors in any other male control or exposed group. Whether the incidence of thyroid follicular carcinoma was a causal effect of exposure to the test substance or a sporadic occurrence remains a question, especially given that there is no apparent exposure concentration-response effect. Pair-wise testing suggests no statistically significant differences existed among the groups ($p > 0.008$, FE test). However, the incidence (7%) in the mid-level group of males is considerably higher than that reported in control F344 rats exposed to air (average incidence of 1% in 21 studies, range of 0 carcinomas in 52 rats to 1 in 45; NTP, 1999). While the incidence of thyroid follicular adenomas reported here are similar to or slightly higher than those previously reported for control animals, the incidence of thyroid follicular carcinomas and adenomas combined are higher than what might be expected (NTP, 1999). However, there are no statistically significant differences in the combined incidence of thyroid follicular adenomas and carcinomas among the exposure groups. In the concurrent Gasoline MTBE Vapor Condensate study, there were no significant increases in thyroid follicular cell adenomas, carcinomas, or combined adenomas and carcinomas in males or females. Thyroid follicular carcinoma might be considered to be caused by the BGVC exposure despite the lack of an apparent exposure concentration-response effect.

In summary, chronic BGVC inhalation suppressed body weight in males, and to a greater extent in females, increased the severity of chronic progressive nephropathy in males and caused epithelial degeneration in the nasal passages of both sexes. The degenerative nasal effects were most likely caused by the test material. Chronic exposure to BGVC did not enhance the development of proliferative lesions (hyperplastic lesions, neoplasms) in female rats. However, there was an increased incidence in mononuclear cell leukemia in low- and mid-level exposed females, most likely due to protocol-driven sampling bias in these groups. Consistent with previous studies and the concurrent GMVC study, chronic exposure to BGVC did enhance the development of renal adenomas and carcinomas in male rats. In contrast with the concurrent GMVC chronic study, BGVC may also have enhanced squamous cell carcinoma in the nasal specimens, testicular mesothelioma, and thyroid follicular carcinoma. Consequently, due

primarily to treatment-related effects in the male kidney, nose, testes, and thyroid, chronic inhalation of Baseline Gasoline Vapor Condensate was determined to be carcinogenic in male rats in this study. Chronic inhalation of Baseline Gasoline Vapor Condensate was determined not to be carcinogenic in female rats in this study.

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LABORATORY TITLE PAGE

211(b) CHRONIC CARCINOGENICITY STUDY
BASELINE GASOLINE VAPOR CONDENSATE (BGVC)

LRRI Study Number: FY01-027

Laboratory: Lovelace Respiratory Research Institute (LRRI)
2425 Ridgecrest Dr. SE
Albuquerque, NM 87108

Courier Address and Location of Laboratory:
Bldg. 9217, Area Y
Kirtland Air Force Base
Albuquerque, NM 87115

Sponsor: American Petroleum Institute (API)
1220 L Street, NW
Washington, DC 20005

Study Initiation Date: August 12, 2001

Study Completion Date: TBD

September 2009

Total number of pages: ____

KEY STUDY PERSONNEL

Study Director	Janet M. Benson, PhD, DABT Lovelace Respiratory Research Institute
Veterinary Pathologist	Thomas F. March, DVM, PhD, DACVP Lovelace Respiratory Research Institute
Aerosol Engineers	Quint H. Powell, MS Lovelace Respiratory Research Institute Edward B. Barr, MSEE Lovelace Respiratory Research Institute
Laboratory Animal Veterinarian	David G. Burt, DVM, ACLAM Lovelace Respiratory Research Institute
Director of Quality (Until 2007)	Stephanie Taulbee, MSPH Lovelace Respiratory Research Institute
Quality Assurance Manager (Until 2006)	Dorothy L. Harris, MS, CRM, RQAP-GLP Lovelace Respiratory Research Institute
Quality Assurance Manager	Joan Gallis, BS Lovelace Respiratory Research Institute

SUBCONTRACTOR

Statistician	Betty Skipper, PhD Department of Family and Community Medicine, University of New Mexico
--------------	--

LABORATORY SIGNATURE PAGE

Study Number FY01-027
211(b) Chronic Carcinogenicity Study
Baseline Gasoline Vapor Condensate (BGVC)

Janet M. Benson, PhD, DABT
Study Director

Date

Edward B. Barr, MSEE
Aerosol Scientist

Date

Thomas H. March, DVM, PhD, DACVP
Veterinary Pathologist
(Clinical Pathology and Histopathology)

Date

Betty Skipper, PhD
Director, Biostatistics
Department of Family and Community Medicine
University of New Mexico

Date

LABORATORY QA STATEMENT

Study Title	211(b) Chronic Carcinogenicity Study – Baseline Gasoline Vapor Condensate (BGVC)
LRRI Study Number	FY01-027
Sponsor Study Number	N/A
Study Director	J. Benson

This study was inspected by the LRRI Quality Assurance Unit. The final report accurately reflects the raw data. Findings were reported to the Study Director and Test Facility Management as follows:

Study Phase	Performed by	Date(s) of Inspection or Audit	Testing Facility Management	Study Director
Miran spectrophotometer calibration	R. Marr	17-Jul-01	18-Jul-01	18-Jul-01
Animal Conditioning, Chamber maintenance, Husbandry process	D. Harris	3-Aug-01	12-Aug-01	12-Aug-01
Day -7 randomization. Body weights and observations	D. Harris	6-Aug-01	12-Aug-01	12-Aug-01
Data inspection for conditioning room	R. Marr	8-Aug-01	8-Aug-01	8-Aug-01
Protocol	D. Harris	12-Aug-01	12-Aug-01	12-Aug-01
Day -1 body weights and observations	D. Harris	12-Aug-01	12-Aug-01	12-Aug-01
TA logbook data audit	R. Marr	18-Oct-01	18-Oct-01	18-Oct-01
Environmental, Miran and GC data audit	R. Marr / D. Harris	18-Oct-01	30-Oct-01	30-Oct-01
Environmental data audit, Body weight and observation data, Miran calibrations	R. Marr	4-March-02	7-Mar-02	7-Mar-02

Study Number FY01-027
211(b) Chronic Carcinogenicity Study
Baseline Gasoline Vapor Condensate (BGVC)
September 2009

Study Phase	Performed by	Date(s) of Inspection or Audit	Testing Facility Management	Study Director
Environmental data, exposure data, body weight and observation printouts	R. Marr	2-May-02	6-May-02	6-May-02
Environmental data, exposure data, TA characterization, chamber atmosphere, TA use/fill logs, body weight and observation data, exposure chamber maps and checklists	R. Marr	14-Aug-02	6-Sept-02	6-Sept-02
Body weight, observation and blood smear procedures	R. Marr	16-Aug-02	21-Aug-02	21-Aug-02
IANR forms, body weight and observation data, environmental data, chamber atmosphere, exposure data, TA use/fill logs, Miran calibrations	R. Marr	6-Nov-02	25-Nov-02	25-Nov-02
IANR forms, animal removal, chain of custody records, animal exposure room log, sick animal observations, exposure chamber checklist, chamber maps, environmental data, exposure data, body weight and observation data, chamber atmospheres	R. Marr / D. Harris	19-Dec-02	20-Dec-02	20-Dec-02
Exposure data, TA, body weight and observations, chamber atmosphere, environmental data, Miran calibrations, IANR forms, GC data, animal removal records	R. Marr	18-Mar-03	27-Mar-02	27-Mar-02

Study Number FY01-027
211(b) Chronic Carcinogenicity Study
Baseline Gasoline Vapor Condensate (BGVC)
September 2009

Study Phase	Performed by	Date(s) of Inspection or Audit	Testing Facility Management	Study Director
Terminal sacrifices	D. Harris	12-Aug-03	19-Aug-03	19-Aug-03
Terminal sacrifices	D. Harris	18-Aug-03	19-Aug-03	19-Aug-03
IANR and N module data	J. Godbe	13-Nov-03	17-Nov-03	17-Nov-03
Draft final report	D. Harris	17-Aug-04	2-Sept-04	2-Sept-04
Statistical Data	C. Storch	28-Jan-09	9-Feb-09	9-Feb-09
Pathology Report	C. Storch	27-Apr-09	13-May-09	13-May-09
Final Report Text	C. Storch	4-May-09	13-May-09	13-May-09
Final Report	C. Storch	30-Sept-09	30-Sept-09	30-Sept-09

Christa R. Storch, BS, RQAP-GLP
Senior Quality Assurance Specialist
LRRI Quality Assurance Unit

Date

LABORATORY COMPLIANCE STATEMENT

Study Number FY01-027

211(b) Chronic Carcinogenicity Study

Baseline Gasoline Vapor Condensate (BGVC)

This study was conducted in compliance with Alternative Tier 2 testing requirements under Section 211(b) of the Clean Air Act and the EPA Health Effects Test Guidelines OPPTS 870.4200, "Carcinogenicity." The stipulations of this protocol were implemented in conformance with EPA regulations as specified in 40 CFR 79.60, "Good Laboratory Practices (GLP) Standards for Inhalation Exposure Health Effects Testing."

The following were not conducted under GLP guidelines:

Serological health assessments were conducted throughout the study. Of the five assessments, two were conducted under GLP guidelines, three were not. This was because LRRI did not specify the need to run three of the shipments GLP in our communications with BioReliance.

Light and noise measurements were not conducted under GLP guidelines. No Standard Operating Procedure was in place for these endpoints.

It was the Sponsor's responsibility to maintain records of the method of synthesis, fabrication, or derivation of the test substance. The method of fabrication was not available at the time the study was initiated. It is, however, presently available with the sponsor.

Janet M. Benson, PhD, DABT
Study Director
Lovelace Respiratory Research Institute

Date

Thomas M. Gray, MS, DABT
Sponsor Representative
American Petroleum Institute

Date

SUMMARY

In compliance with the Clean Air Act Section 211(b) for fuel and fuel additive registration, the petroleum industry and oxygenate manufacturers have conducted comparative chronic toxicology testing of evaporative emissions from unleaded gasoline (baseline gasoline vapor condensate [BGVC]) and unleaded gasoline containing MTBE (gasoline/MTBE vapor condensate [GMVC]). This is the final report of the BGVC chronic study.

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There were no clinical signs of toxicity attributable to BGVC inhalation. Survival of the BGVC-exposed rats was not significantly different from that of control rats. Body weights of males in the mid- and high-level BGVC groups were significantly below control values throughout the study. Body weights of high-level and mid-level females were below control values after approximately 1 and 3 months of exposure, respectively. Body weights of low-level females were sporadically affected. At the final sacrifice, body weights of high-level males and high- and mid-level female rats were significantly below control values. Among mid-level males, the absolute epididymis weight, percent epididymis-to-body and percent epididymis-to-brain weights were significantly below the corresponding control values. The adrenal-to-body and brain-to-body weight values of high-level males were significantly greater than control values. The kidney weights of high- and mid-level females were significantly increased compared to controls, however they did not correlate with kidney pathology findings. The spleen weights of mid-level females were significantly increased compared to controls, which is likely a result of the high incidence of age-related mononuclear cell leukemia (MCL). Among mid-level females, the percent organ-to-body weight values for brain, heart, kidney, liver, lungs, and spleen were significantly greater than corresponding control values. Among high-level females, the percent

organ-to-body values for adrenals, brain, heart, liver, kidney, and lungs were significantly greater than control values. The percent kidney-to-brain weights for mid- and high-level females and the percent spleen-to-brain weight for the mid-level females were significantly greater than the corresponding control values. It is likely that many of the organ-to-body weights increases were artifacts of the treatment-related depression in body weight. For females, there were significantly fewer numbers of total WBCs, neutrophils, lymphocytes, eosinophils, and nucleated red blood cells (RBCs) in the high-level group at the 12 month time-point, only. In males, only the absolute number of lymphocytes was significantly elevated in the high-level group at the final sacrifice time-point. Due to blood sampling difficulties at the 12 month time point and the high, age-related incidence of mononuclear cell leukemia in all groups, the relationship of treatment to these changes in hematology values is unknown. BGVC exposure was associated with an increase in the incidence of respiratory epithelial degeneration and a significant decrease in the incidence of olfactory epithelial degeneration in the nasal passages of both male and female rats. The respiratory epithelial degenerative effects were most likely caused by the test material.

Exposure did not enhance the incidence of chronic progressive nephropathy in male or female rats, but did increase the severity of this lesion among mid- and high-level groups of males. The increased severity of chronic progressive nephropathy in males is consistent with previous studies on wholly vaporized unleaded gasoline, and is attributable to male rat specific alpha-2u globulin overload as a result of BGVC exposure. Consistent with previous studies and the alpha-2u globulin overload mechanism of male rat kidney damage, BGVC induced increases in male renal adenoma (1/50 in control; 1/50 in 2 g/m³ group; 4/50 in 10 g/m³ group; and 0/50 in 20 g/m³ group) and carcinoma (0/50 in controls; 0/50 in 2 g/m³ group; 3/50 in 10 g/m³ group; and 0/50 in 20 g/m³ group). Although the incidences of renal adenomas and carcinomas taken separately were not statistically significant, combined incidences for the control (1/50; 2%), low (1/50, 2%), mid (7/50, 14%) and high (0/50; 0%) exposure groups were significant (p = 0.004, Fisher's Exact Test). Pair-wise tests did not demonstrate any significant difference among the individual dose groups. These neoplasms may be considered to be exposure related. The highest incidence of renal neoplasms in males was in the 10 g/m³ exposure group.

The incidence of squamous cell carcinoma in the nasal passages (turbinate levels 2-4) was elevated in the high-level group of male rats, with tumor incidence of 1/50, 3/50, and 3/50 for levels 2, 3 and 4, respectively. The level 3 incidence gave a statistically significant trend for increased incidence with increased exposure concentration ($p = 0.019$, Cochran-Armitage [CA] test). Squamous cell carcinoma was noted in the nasal specimens of only one female control rat of the current study. The squamous cell carcinomas in all of the male nasal specimens had morphologies consistent with origination from the oral mucosa and had varying degrees of invasion into the nasal passages. By comparison, in the concurrent Gasoline MTBE Vapor Condensate study (FY01-013), squamous cell carcinoma of oral origin occurred in one male control, two male GMVC low dose and two male high GMVC dose, but in no female rats. While the fact that the nasal tumors were of oral origin might suggest that the inhalation exposure was not causal, spontaneous oral squamous cell carcinomas in other studies have been nearly as rare as those originating from the nasal mucosa (~0.6%; Haseman et al., 1990; NTP, 1999). The historical incidence of squamous cell carcinoma of oral origin in a large cigarette smoke study conducted at LRRI in the 1990s was 0/118 (0%) in males and 3/119 (2.5% in females). Taken together, the data provide some evidence that the development of nasal squamous cell carcinomas in male rats may have resulted from test article exposure.

Similar to the incidence of renal neoplasms, there was an increased incidence of testicular mesothelioma in the mid-level group of male rats (4 of 50 examined). Fisher's exact test demonstrated that this incidence was greater than expected, but pair-wise testing between the groups and trend tests for exposure concentration-response effects did not indicate statistically significant effects of exposure. The incidence in the mid-level group is approximately 3-fold greater than the average incidence in male control F344 rats exposed to air in inhalation toxicology studies (28 tumors in 1,055 rats; NTP, 1999) and may be regarded as an effect of exposure to the test substance. As with this study, there were no mesotheliomas found in the testes or other tissues of the control male animals of the GMVC study (LRRI protocol FY01-013), although there was a statistically non-significant incidence (2 in 50 animals or 4%) of the tumor in the testes of the high-level GMVC-exposed rats and sporadic incidence of the tumor in other tissues of GMVC-exposed male rats. The absence of testicular mesothelioma in control

animals of the present study, where 1 or 2 tumors might normally be expected to be found in 50 animals (NTP, 1999), may have influenced the statistical analysis to indicate a significant exposure-related incidence in the mid-level BGVC-exposed male rats, when no causal increase is actually present.

There was a trend for increased incidence of thyroid follicular cell carcinoma in male animals ($p < 0.05$, FE test over all groups; see Table 3-12), but there were no significant differences among the groups. There were two tumors in the 27 mid-dose glands examined and no tumors in any other male control or exposed group. Whether the incidence of thyroid follicular carcinoma was a causal effect of exposure to the test substance or a sporadic occurrence remains a question, especially given that there is no apparent exposure concentration-response effect. Pair-wise testing suggests no statistically significant differences existed among the groups ($p > 0.008$, FE test). However, the incidence (7%) in the mid-level group of males is considerably higher than that reported in control F344 rats exposed to air (average incidence of 1% in 21 studies, range of 0 carcinomas in 52 rats to 1 in 45; NTP, 1999). While the incidence of thyroid follicular adenomas reported here are similar to or slightly higher than those previously reported for control animals, the incidence of thyroid follicular carcinomas and adenomas combined are higher than what might be expected (NTP, 1999). However, there are no statistically significant differences in the combined incidence of thyroid follicular adenomas and carcinomas among the exposure groups. In the concurrent Gasoline MTBE Vapor Condensate study, there were no significant increases in thyroid follicular cell adenomas, carcinomas, or combined adenomas and carcinomas in males or females. Thyroid follicular carcinoma might be considered to be caused by the exposure despite the lack of an apparent exposure concentration-response effect.

In summary, chronic BGVC inhalation suppressed body weight in males, and to a greater extent in females, increased the severity of chronic progressive nephropathy in males and caused epithelial degeneration in the nasal passages of both sexes. The degenerative nasal effects were most likely caused by the test material. Chronic exposure to BGVC did not enhance the development of proliferative lesions (hyperplastic lesions, neoplasms) in female rats. However, there was an increased incidence in mononuclear cell leukemia in low- and mid-level exposed females, most likely due to protocol-driven sampling bias in these groups. Consistent with

previous studies and the concurrent GMVC study, chronic exposure to BGVC did enhance the development of renal adenomas and carcinomas in male rats. In contrast with the concurrent GMVC chronic study, BGVC may have enhanced squamous cell carcinoma in the nasal specimens, testicular mesothelioma, and thyroid follicular cell carcinoma. Consequently, due primarily to treatment-related effects in the male kidney, nose, testes, and thyroid, chronic inhalation of Baseline Gasoline Vapor Condensate was determined to be carcinogenic in male rats in this study. Chronic inhalation of Baseline Gasoline Vapor Condensate was determined not to be carcinogenic in female rats in this study.

INTRODUCTION

The purpose of this study was to evaluate the chronic toxicity, potential carcinogenicity, and exposure concentration-response relationships associated with inhalation of baseline gasoline vapor condensate (BGVC). The study was conducted in compliance with Alternative Tier 2 testing requirements under Section 211(b) of the Clean Air Act and the EPA Health Effects Test Guidelines OPPTS 870.4200m, "Carcinogenicity." The stipulations of this protocol were implemented in conformance with EPA regulations as specified in 40CFR 79.60, "Good Laboratory Practices (GLP) Standards for Inhalation Exposure Health Effects Testing." The study protocol and amendments are provided in Appendix A.

MATERIALS, METHODS, AND PROCEDURES

TEST SUBSTANCE

Identification, Source, and Storage

BGVC was prepared and supplied in 420-pound and 20-pound gas cylinders by Chevron Research and Technology Center (CRTC; Richmond, CA). Two lots of BGVC were used in the study. API 99-01 was used from the first day of exposures through the week of May 19, 2003. Starting the week of May 27, 2003 and running through the last day of exposures, API 02-08 was used. It was necessary to prepare a second lot of test material because the first attempt to conduct this study was aborted due to inaccurate dose calibrations resulting in chamber concentrations up to 40% greater than target concentrations. While there were some minor differences in the relative concentrations of various alkanes and isoalkane components between the lots, these differences did not influence the outcome of the study (see Table 2-1). Original characterization of both lots of the test substance was performed by CRTC and ExxonMobil Biomedical Sciences, Inc. (EMBSI), Annandale, NJ.

Table 2-1. Analytical Comparison of Test Material Lots

Compound	BGVC Lot # API 99-01 (area percent)	BGVC Lot # API 02-08 (area percent)
Isobutane	3.6	2.1
n-Butane	15.2	19.9
Isopentane	35.1	32.0
n-Pentane	13.2	5.4
Trans-2-pentene	2.5	2.4
2-Methyl-2-butene	3.8	3.3
2,3-Dimethylbutane	1.6	6.2
2-Methylpentane	6.3	9.6
3-Methylpentane	3.6	6.8
n-Hexane	3.0	1.2
Methylcyclopentane	1.5	0.7
2,4-Dimethylpentane	1.0	0.6
Benzene	2.1	1.9
2-Methylhexane	1.1	1.6

Table 2-1. Analytical Comparison of Test Material Lots (Concluded)

Compound	BGVC Lot # API 99-01 (area percent)	BGVC Lot # API 02-08 (area percent)
2,3-Dimethylpentane	1.1	1.0
3-Methylhexane	1.3	2.2
Isooctane	1.3	0.7
Toluene	3.0	2.6

Twenty-pound cylinders and some 420-pound cylinders were stored at ambient temperature in a storage building dedicated for that purpose at LRRI. The remaining 420-pound cylinders were stored in an outside, controlled area at ambient temperature. The test substance was transferred, as needed, from the 420-pound to the 20-pound cylinders. Only authorized personnel were allowed access to the test substance. Receipt, use, and inventory of this test substance were documented.

Analysis

Before dispensing BGVC from each 420-pound tank, a sample was removed from the tank and analyzed by gas chromatography (GC) with flame ionization detection (FID) at LRRI using a Shimadzu Model GC-17A/FID (Shimadzu Scientific Instruments, Columbia, MD). The gas chromatographic profile of the 18 major peaks (retention time and relative peak area) was compared with that originally determined for the BGVC by ExxonMobil Biomedical Sciences, Inc. (EMBSI, Annandale, NJ). Tank BG22, used September 24 thru November 18, 2002, was inadvertently not analyzed prior to use. Additionally, there were differences in the relative concentrations of various alkanes and isoalkane components between the two lots of test material. The absence of analysis of tank BG22 and test material lot API 99-01 vs. lot API 02-08 were considered minor issues and were judged to have no significant effect on the outcome of the study. Results are provided in Appendix B.

Expiration Date

An expiration date is not available. The test material is stable per MSDS. The test substance stability was tested concurrently with the study with the analysis of the exposure chamber

atmospheres and each #420 pound tank before its use. Results, provided in Appendix E, showed it to be stable for the duration of the study.

Reserve Sample

The Sponsor-contracted archives have retained samples of the test substance.

ANIMALS AND ANIMAL ASSIGNMENTS

Animal Receipt, Housing, and Quarantine

Four hundred-forty CDF(F344)CrIBR rats (5–6 weeks old when received) were purchased from Charles River Laboratories (Raleigh, NC). The rats were examined by a veterinarian upon their arrival. All animals were quarantined and acclimated to whole-body inhalation chambers for 20 days. Healthy animals were randomly assigned by weight to the core exposure groups (400 total; 50 rats/sex/exposure level). The weight range of male rats assigned to study at randomization was 152.0–194.4 g. For females, the weight range at randomization was 112.2–138.7 g. Following randomization, the rats assigned to study were identified by tail tattoo. Five male and five female rats were assigned as sentinels and housed in the control chamber. Five unassigned male and female rats were sacrificed before exposures began to evaluate their health status as an indicator of the health status of the population on study. The remaining unassigned rats were euthanized. Receipt and initiation of exposures of male and female rats were staggered by one week.

Animal Disease Screening Program

Five male and five female rats not assigned to study were sacrificed, bled and received a complete necropsy. No gross lesions were found, and tissues were subsequently discarded because there were no subsequent questions regarding the health status of the animals on study. Sera from these rats were submitted to BioReliance, Rockville, MD, for analysis of antibodies common to rodents. The following tests were run: Cilia-Associated Respiratory Bacillus (CARB), Mycoplasma pulmonis (M. PUI), Pneumonia virus of mice (PVM), Rat Coronavirus/Sialodacryoadenitis Virus (RCV/SDA), Reovirus (Reo), Sendai Virus (Sendai), Lymphocytic Choriomeningitis Virus (LCM), Parvovirus (Parvo), Toolan's H-1 Virus (H-1), and Kilham rat Virus (KRV). The five male and five female sentinel rats in the control chamber were bled retro-

orbitally after 26, 52, and 78 weeks of exposure. Blood was also obtained from sentinels surviving to 104 weeks. Sera were analyzed by BioReliance, as described above. The data are provided in Appendix C.

Justification of Test Animals

Rats were used in this study because of the large database available on the inhalation toxicity and carcinogenicity of toxic materials in rats. The study design was justified because it provided exposure concentration-response information on the possible carcinogenicity associated with repeated inhalation of BGVC.

Environmental Conditions

The rats were housed 24 hours per day during quarantine and exposure in Hazleton 2000 whole-body inhalation chambers. Initially, all rats were housed separately in 3.8-inch wide by 11-inch long by 8-inch high compartments within stainless steel baskets. When the male rats reached 400 g they were transferred to baskets with 5.7-inch by 11-inch by 8-inch compartments. Each chamber contained six baskets.

The chambers and cage racks were washed weekly. The cage racks were rotated clockwise weekly when the rats were transferred from the dirty to the clean chambers.

The chambers were held at approximately 1 inch of water negative pressure with respect to the exposure room, and the chamber flow rates were maintained at 12 to 15 air changes per hour (400–500 liters per minute [LPM]). Chamber temperatures were maintained at 20° to 24°C. Temperature, relative humidity, and chamber air flow rates were continuously monitored, 24 hours per day. Values for the three parameters were recorded at 30-minute intervals. Oxygen concentration in the chambers was maintained at 19%. This parameter was monitored, but not recorded.

A 12-hour light/dark cycle was maintained with lights on at 0600. Light levels in the exposure room and noise levels in the chambers were determined periodically.

Diet and Drinking Water

Unlimited municipal tap water was available at all times. Rats were fed Teklad Certified Rodent Diet (8728C; Harlan Teklad, Madison, WI). Food was available at all times except during the daily exposure period. Certified diet was analyzed for heavy metals, aflatoxin, organophosphates and chlorinated hydrocarbons, and values present were below maximum allowable values established by the manufacturer. Water was analyzed by an independent laboratory for metals, ions, microbes, and pesticides. Analytes were either present at non-detectable concentrations or sufficiently low concentrations as to not interfere with the outcome of the study. Feed and water analyses results are provided in Appendix D.

EXPERIMENTAL DESIGN

Group Assignment

The experimental design is shown in Table 2-2.

Table 2-2. Experimental Design

Rat Strain and Sex	CDF(F344)CrIBR; 200 males/200 females assigned to core study
Animal Source	Charles River Laboratories, Raleigh, NC
Time Held Before Exposure	14 days (males and females)
Age When Placed on Study	7 to 8 weeks
Study Dates	
Study Initiation Date	August 12, 2001
Initiation of Exposures (Males)	August 13, 2001
Initiation of Exposure (Females)	August 20, 2001
Last Exposure Day (Males)	August 8, 2003
Last Exposure Day (Females)	August 15, 2003
Final Sacrifice (Males)	August 12–15, 2003
Final Sacrifice (Females)	August 18–22, 2003
Target Exposure Concentrations	
Control	0 mg/m ³ BGVC
Low Level	2 g/m ³ BGVC
Mid Level	10 g/m ³ BGVC
High Level	20 g/m ³ BGVC

Table 2-2. Experimental Design (Concluded)

Animal Identification Scheme(by tail tattoo)	
Control	I001-I050 (M); I051-I100 (F)
Low Level	J101-J150 (M); J151-J200 (F)
Mid Level	K201-K250 (M); K251-K300 (F)
High Level	L301-L350 (M); L351-L400 (F)
Exposure Duration	6 hours/day, 5 days/week for 104 weeks (518 exposure days)
In-Life Monitoring	Daily observations for morbidity and mortality. Weekly body weights for first 13 weeks, then every 4 th week. Weekly detailed clinical observations.
Sentinel Animal Bleeds for Serology	Prior to study start and at six-month intervals thereafter, including prior to final sacrifice.
Hematology Evaluations (Control and High-Level Exposure Groups)	At 12 and 18 months and at final sacrifice.
Histopathology	All protocol-required tissues collected.

The Laboratory Animal Veterinarian visually examined all rats before they were placed on study. Only animals judged to be of acceptable health were used. Animals were weighed and randomly assigned to a test group using a computerized data acquisition system (Path-Tox[®]; Xybion, Cedar Knolls, NJ) operated according to LRRI Standard Operating Procedures (SOPs).

Exposure System

A schematic of the vapor exposure system is shown in Figure 2-1.

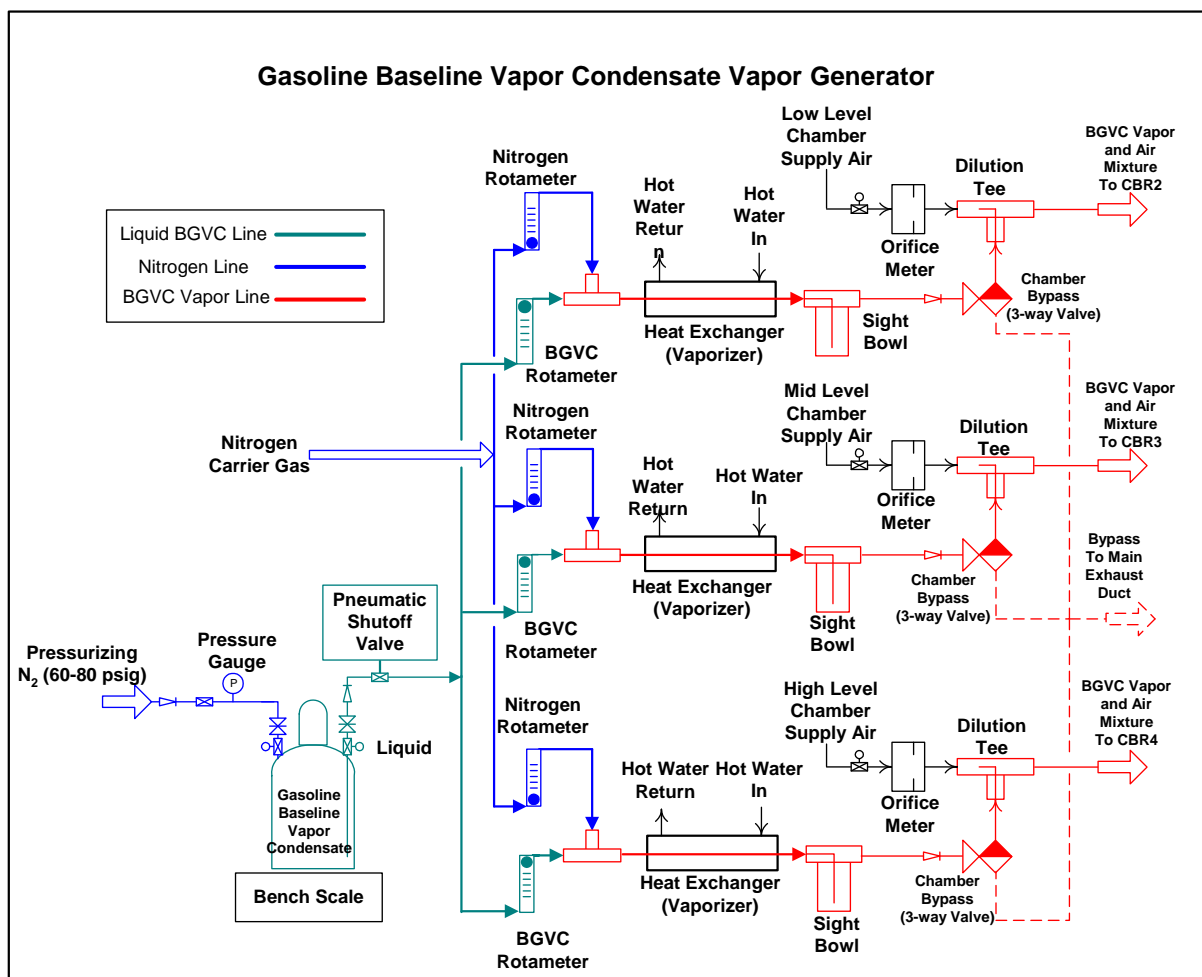


Figure 2-1. Vapor Generation System for BGVC

The daily supply of BGVC for each exposure chamber was contained in 20-pound gas storage cylinders. Exposure atmospheres were generated by controlling the flow of pressurized BGVC through a rotameter, into a heated stainless steel transfer line where the BGVC was completely vaporized. Chamber concentrations were controlled by adjusting the flow rates of the BGVC and dilution air rate. Chamber exhaust was carried to an oxidizer on the roof of the exposure facility where it was burned.

Pre-Test Characterization. The exposure system was tested prior to animal exposures.

Characterization included the following: 1) uniformity of the distribution of total vapor within each chamber; 2) within-day and between-day stability of vapor concentration; 3) within-day and between-day consistency of the hydrocarbon profile as determined by gas chromatography; and

4) determination of the time for vapor concentration to achieve 90% of the equilibrium target value (T90). The exposure atmosphere in the animals' breathing zone was also examined for the presence of aerosol particles using a TSI Scanning Mobility Particle Sizer (TSI Industries, Shoreview, MN).

Once, prior to the initiation of animal exposures, the concentrations of the test substance in the generator control box and in the exposure room were determined to ensure that the generator containment hood was operating satisfactorily.

Chamber Distribution Evaluation During Exposures. During the second week of exposure, the uniformity of vapor distribution was re-evaluated to determine the distribution in the presence of the test animals.

Quantitation of Exposure Atmospheres. Vapor concentrations in the exposure chambers were continuously monitored using Miran 1A infrared analyzers (Foxboro Wilks, Foxboro, CT). The high-, mid-, and low- level exposure chambers were each monitored with their own analyzer. The analyzers for the high-, mid-, and low-level chambers underwent weekly five-point calibrations and daily one-point calibration checks using test substance. A fourth analyzer was devoted to monitoring the control chamber, the room air, and the hood enclosing the 20 pound tank of test substance. This Miran 1A was calibrated approximately quarterly until December 2001, and then once before exposures ended. The Miran 1A analyzers dedicated to the mid- and high-level chambers were calibrated over a range of 6–35 g/m³. The Miran 1A analyzers for the control chamber and the low-level chamber were calibrated over a concentration range of 1–7 g/m³.

The absorbance signals were recorded continuously. Average values were obtained for the first, second and third 2-hour segments of each exposure day. The mean of the three values from each exposure chamber was reported as the day's exposure concentration for that chamber.

Qualitative Assessment of Exposure Atmospheres. The qualitative composition of the exposure atmosphere in each chamber was determined weekly by gas chromatography using a Shimadzu Model GC-17A/FID. The percent peak area of each of 18 major components was determined and recorded. The retention times of eight representative components were verified against

EMBSI values each week and, one-point performance qualifications were performed on any day in which chamber profiles were analyzed. The one-point check was performed using a certified standard of 840 ppm butane in N₂ (Matheson Tri-Gas, Irving, TX).

Determination of Nominal Concentration. Daily nominal or “anticipated” usage was calculated by multiplying the average BGVC concentration in each chamber (low, mid, high; g/m³) by the total flow through each respective chamber ([L/min * min]/1000 m³) and then summing the values for all three chambers. This value was compared to the actual BGVC usage determined by taking the difference between the weight of the 20-pound cylinder before and after each exposure.

Concentration of Test Substance in the Exposure Room. Concentration of the test substance in the exposure room was determined at approximately 60-day intervals using a Miran 1A Infrared Spectrometer (Foxboro Wilks, Foxboro, CT) operated using the same settings as were used to monitor the low-level exposure chamber.

In-Life Endpoints

Rats were exposed 6 hours/day (plus 14 minutes, the time for the vapor concentration to reach T90), 5 days/week for 104 weeks (518 exposure days). The following observations and measurements were made during the dosing phase.

Mortality and Morbidity. Laboratory animal technicians observed the rats twice daily throughout the study. Examinations were oriented toward identifying dead, weak, or moribund animals and documenting the onset and progression of any abnormal clinical signs. Appropriate actions were taken to minimize the loss of animals from study (e.g., necropsy or refrigeration of any rats found dead and sacrifice of weak or moribund animals).

Body Weight. All animals were individually weighed using the Path-Tox[®] data acquisition system (Xybion, Cedar Knolls, NJ) on study Day -7 (to randomly assign rats to groups by weight), Day -1, weekly for 13 weeks, and then every 4 weeks thereafter.

Clinical Signs of Toxicity. Thorough clinical examinations were made at randomization, on Day -1, and weekly thereafter. Observations were detailed and carefully recorded using Path-

Tox[®] software. Observations included evaluations of skin and fur; eyes and mucous membranes; respiratory and circulatory effects; autonomic effects such as salivation and central nervous system effects, including tremors and convulsions, and changes in the level of activity, gate, and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self-mutilation, walking backward).

Hematology. At 12 months, 18 months, and at final sacrifice, blood smears were prepared, and manual differential cell counts were made. For the 12 and 18 month time points, blood was obtained by tail nick from the control and high-level-exposed rats. At final sacrifice, blood was obtained by cardiac puncture from all rats and collected into Vacutainer tubes containing ethylenediamine tetra-acetic acid (EDTA). Parameters evaluated included total leukocyte estimates (WBC), nucleated red blood cell counts (cells/100 WBC), anisocytosis (0 to 4+), polychromasia (1+ to 4+), and relative differential leukocyte counts for segmented neutrophils, band neutrophils, lymphocytes, monocytes, eosinophils, basophils, atypical lymphocytes and blastocytes. Absolute leukocyte counts were calculated for segmented neutrophils, band neutrophils, lymphocytes, monocytes, eosinophils, basophils, atypical lymphocytes and blastocytes using the formula $WBC \times \text{relative percent} / 100$.

Post-Exposure Endpoints

Gross Necropsy. A complete gross examination was performed on all animals at final sacrifice and on those animals that died naturally or were sacrificed in a moribund condition. Sacrifices of rats surviving 518 days of exposure occurred during the week following the last exposure day for each sex. Animals were randomly assigned to a sacrifice day. Samples collected at necropsy are listed in Table 2-3.

Table 2-3. Organs and Representative Samples Taken for Examination

Cardiovascular/Hematopoietic System

1. Aorta
2. Bone marrow (and/or fresh aspirate)
3. Heart
4. Lymph nodes (mandibular, mesenteric, bronchial, mediastinal)
5. Spleen

Digestive System

1. Cecum
2. Colon
3. Duodenum
4. Esophagus
5. Ileum
6. Jejunum
7. Liver
8. Pancreas
9. Rectum
10. Salivary glands
11. Stomach

Glandular System

1. Adrenals
2. Parathyroid
3. Thyroid

Nervous System

1. Brain (including sections of medulla/pons, cerebellum, and cerebrum)
2. Eyes (retina, optic nerve)
3. Peripheral nerve (sciatic or tibial, preferably in close proximity to the muscle)
4. Pituitary
5. Spinal cord (three levels: cervical, mid-thoracic, and lumbar)

Other

1. All gross lesions and masses
2. Skin
3. Tail (for identification)

Respiratory System

1. Larynx
 2. Lung (infused with fixative)
 3. Nose
 4. Paranasal sinuses
 5. Pharynx (evaluated with larynx)
 6. Trachea
-

Table 2-3. Organs and Representative Samples Taken for Examination (Concluded)

Urogenital System

1. Epididymides
 2. Female mammary gland
 3. Kidneys
 4. Ovaries
 5. Prostate
 6. Seminal vesicle(s)
 7. Testes
 8. Urinary bladder (infused with fixative)
 9. Uterus
-

All study animals received a complete necropsy. Animals were euthanized with an overdose of intraperitoneally injected barbiturate anesthetic (Euthasol[®], Virbac AH Inc., Fort Worth, TX). Body weights and fresh organ weights were collected on lungs, liver, kidneys, adrenals, testes, epididymides, ovaries, uterus, spleen, brain and heart of final sacrifice and moribund sacrifice animals. Animals found dead received a complete necropsy with tissue collection, but blood and organ weight data were not routinely collected. Cardiac blood was collected from animals at final sacrifice for determination of total and differential white blood cell counts. Gross lesions, body weights, and organ weights were entered on pre-prepared forms and then the information was recorded on the Path-Tox[®] database.

Lungs were gently instilled via the trachea with 10% neutral buffered formalin (NBF) to approximate normal volume. Organs and tissues were immersion fixed in 10% NBF for subsequent histopathologic examination. Tissues were trimmed, processed routinely, paraffin embedded, sectioned at 5 μm and stained with hematoxylin and eosin for histopathologic examination.

Histopathology. All collected tissues and lesions were examined histologically in control (0 g/m³) animals, high-level (20 g/m³) animals, and dead or moribund animals of all groups. Respiratory tissues (lungs, larynx, trachea and nasal turbinate sections), potential target tissues (testes, kidneys of males) and gross lesions from non-target tissues were examined histologically from final sacrifice low-level (2 g/m³) and mid-level (10 g/m³) animals. Nomenclature of proliferative lesions was based on the international harmonized nomenclature recommended by the Rat Nomenclature Reconciliation Subcommittee of the Society of Toxicologic Pathologists

(see <http://www.toxpath.org>; Standardized Rat Nomenclature). Nomenclature of other lesions was routine, widely understood usage (see Boorman et al., 1990).

Statistics

Body and Organ Weights. Group mean body weight, organ weight, percent organ-to-body weight, and percent organ-to-brain weight data were tested for statistical significance using Path-Tox[®] software. After testing for an overall trend among test groups by an analysis of variance, Bartlett's test was used to establish the homogeneity of the data. If the data were homogeneous, group differences were evaluated using a modified Dunnett's test. If data were non-homogeneous, group differences were assessed using a modified t-test. Significance levels were set at $p \leq 0.05$.

Survival Analysis. The probability of survival was estimated by the Kaplan-Meier product-limit method using PROC LIFETEST in SAS Version 8.2 (SAS Institute, Cary, NC). Mean numbers of survival days and time to 25% mortality were estimated for each exposure group by the PROC LIFETEST program. Log-rank tests were used to test the hypothesis that there are differences among the four groups for each sex. The significance level was set at $p = 0.05$. All reported p-values for the survival analysis are two-sided.

Analysis of White Blood Cell Total and Differential Cell Counts. For the hematology data, medians and ranges are presented because the distributions are highly skewed for some variables. Preliminary analyses were done using the generalized estimating equation approach for longitudinal data analysis because there were three time points. For some variables these analyses showed significant interactions between time and group. Therefore, analyses were performed to compare exposure groups at each time point and to compare time points within each exposure group. The Kruskal-Wallis test was used for these comparisons.

Histopathology. The incidences of all neoplastic and non-neoplastic lesions are given as the ratio of the number of affected animals to the number of animals with the site examined microscopically. Three statistical evaluations were performed on the histopathology lesion incidence data: 1) Cochran-Armitage test, which tests whether the incidence of lesions shows a trend across exposure groups; 2) logistic regression test that takes death date into account when

assessing the presence of an exposure-dependent trend; and 3) the Fisher's exact test, which compares incidences among the four exposure groups. The two-sided significance level was set at $p = 0.05$. If a significant difference was detected by the Fisher's exact test, six possible pair-wise comparisons were calculated. Using the Bonferroni correction for pair-wise comparisons, each pair-wise comparison would be considered significant if $p < 0.008$.

Fisher's exact test and the Cochran-Armitage test do not use survival information and are appropriate in situations where survival is similar among exposure groups as is the case for this study. The Fisher's exact test tests the null hypothesis of equality of prevalences across exposure groups against the alternate hypothesis that the prevalences are not equal while the Cochran-Armitage test tests the null hypothesis of equality across exposures against the alternate hypothesis of a monotonic increasing or decreasing trend.

Additionally, differences between groups with regard to both the severity and incidence of non-proliferative lesions were analyzed by the Kolmogorov-Smirnov two-sample, one-tailed test as performed by the Path-Tox system (*c.f.*, Algorithms used in Path/Tox System 4.2.2; Vol. I, Chap. 7, pp. 24-25). These analyses were performed by the pathologist. The significance level was set at $p = 0.05$.

RESULTS AND CONCLUSIONS

EXPOSURE ATMOSPHERE

Pre-Test Chamber Trials

Three-Day Stability Evaluation and Nominal BGVC Usage. Before exposures were initiated the system was operated for 6 hours/day for 3 consecutive days. The concentration of vapor in each chamber was close to target and constant within each day, and the concentrations were reproducible from day to day (Table 3-1). Gas chromatographic analysis of the chamber atmospheres indicated that the relative percentages of the major components were constant over the 3-day period. The percentages of actual BGVC usage/anticipated usage (nominal usage) on the 3 days of stability testing were 96, 95, and 96% for Days 1, 2, and 3, respectively (see Appendix E).

Table 3-1. Results of 3-Day Stability Evaluations

Target Exposure Concentration (g/m ³)/ Chamber Number	Achieved Concentration \pm SD (n = 3)		
	Day 1 (7/23/01)	Day 2 (7/24/01)	Day 3 (7/25/01)
2 (Chamber 2)	2.03 \pm 0.03	2.07 \pm 0.04	2.09 \pm 0.09
10 (Chamber 3)	9.64 \pm 0.47	9.21 \pm 0.13	10.0 \pm 0.29
20 (Chamber 4)	20.3 \pm 0.26	20.7 \pm 0.96	20.6 \pm 0.89

Homogeneity of Vapor Concentration. The homogeneity of vapor concentration throughout the exposure chambers was determined by conducting a chamber distribution study. The concentrations of BGVC were measured from four sampling ports located in front and four sampling ports located in the back of the chambers during a pre-test chamber trial day and compared to the concentration obtained in a reference location. The spatial variation measured in Chambers 2 (2 g/m³), 3 (10 g/m³) and 4 (20 g/m³) was 1.6%, 1.0%, and 0.2%, respectively. These results indicate the vapor is distributed evenly within each of the chambers (Appendix E).

T90 Determination. The amounts of time required to reach T90 were 14.2, 12.1, and 13.7 minutes for Chambers 2, 3, and 4, respectively. A T90 of 14 minutes was chosen for use with this study. Daily exposures periods to BGVC were then 6 hours and 14 minutes.

Confirmation of the Absence of Aerosols. The particle concentrations of air inside Chamber 4, operated at a target concentration of 20 g BGVC/m³ and the control chamber (Chamber 1) were determined using a TSI Aerodynamic Particle Sizer. There was less than one particle per cubic centimeter air in the control and high-level chambers, indicating that there were no BGVC aerosols at the highest target vapor concentration used in this study.

In-Study Data

Chamber Concentrations and Nominal Usage. The overall study means of the daily vapor concentrations achieved for the 2, 10, and 20 g BGVC/m³ vapors are provided in Table 3-2. The overall achieved means were within 2% of target for each exposure concentration. Thus, all future references to exposure concentration are in terms of target concentration. Concentrations of vapor in the control chamber were below the lowest concentration on the standard curve used to calibrate the control chamber Miran 1A analyzer (1 g BGVC/m³). The overall average of the daily percent nominal usage is 98% ± 6%, indicating excellent agreement between anticipated and actual BGVC usage. Daily vapor concentrations and nominal usage are provided in Appendix E.

Table 3-2. Summary of BGVC Vapor Concentrations^a

Target g BGVC/m ³	Achieved Concentrations	Percent of Target
0	< 1 ^b	NA
2	2.0 ± 0.07	101
10	10.1 ± 0.34	101
20	20.3 ± 0.74	101

^aResults are the mean ± SD of vapor concentrations obtained in 518 exposure days (combined days for males and females).

^bConcentrations of “zero” were obtained on all but 13 exposure days. On these 13 days, the readings were below the lowest concentration on the standard curve used to calibrate the control chamber Miran 1A (1 g BGVC/m³).

Homogeneity of Vapor Concentration. An evaluation of the homogeneity of vapor concentration in each BGVC chamber was conducted after animal exposures began. The spatial variations measured in Chambers 2 (2 g/m³), 3 (10 g/m³), and 4 (20 g/m³) were 0.49%, 0.59%, and 5.39%, respectively. These results indicate the vapor was distributed evenly within each of the chambers.

Gas Chromatographic Profiles of BGVC Exposure Atmospheres. Gas chromatographic profiles were obtained from each exposure chamber weekly to assess whether the animals were exposed to the relative amounts of the same 18 major components throughout the study. The peak areas of the 18 major components were summed to provide a “total peak area” for those components. The peak area of each of the components was divided by the total peak area for the components to obtain a relative peak area (in percent) for each of the components. These values were compared to the reference values provided by EMBSI. Profiles remained acceptably constant throughout the study. Weekly results are provided in Appendix F.

Chamber Environmental Data. The temperature, relative humidity, chamber flow rate, and pressures were maintained within acceptable ranges with only occasional excursions outside acceptable limits. Environmental data are provided in Appendix G.

Survival Analysis. There were 50 animals per group for males and 48 animals per group for the females. The following eight females were omitted from the survival analysis because they were removed from the study for reasons not related to the BGVC exposure: I055 (malocclusion); I074 (malocclusion), J176 (missing), J184 (nose injury), K274 (nose injury), K298 (accidental trauma), L356 (nose injury), and L357 (nose injury).

Figures 3-1 and 3-2 show the survival curves for male and female animals, respectively. The differences among the groups are not statistically significant for male ($p = 0.24$) or female ($p = 0.53$) animals. Table 3-3 shows means, standard errors, and day of 25% mortality for male and female animals under each experimental condition. The day of 25% mortality is the day of the study at which 25% of the animals had died in each group. This provides a way of comparing mortality at a single time point among groups within and between sexes. For the calculation of mean and standard errors of survival times, the last day of the study was used as the value of the survival time for animals that were sacrificed at the time that the study was terminated.

Therefore, the means and standard errors may be underestimated since some of the animals may have lived longer if the protocol had included following them for a longer period of time. The results indicate that chronic BGVC inhalation did not shorten the lifespan of the exposed rats compared to the controls.

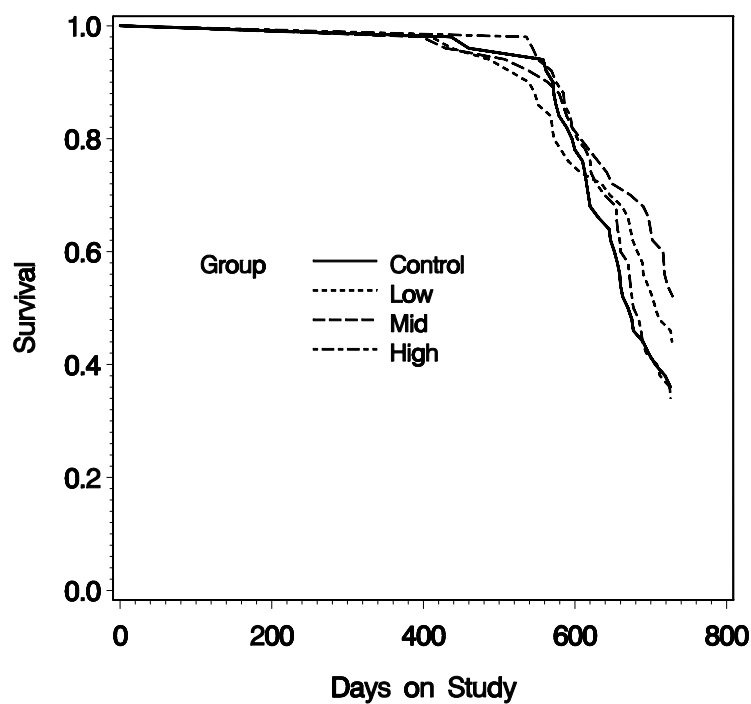


Figure 3-1. Survival of Male Animals

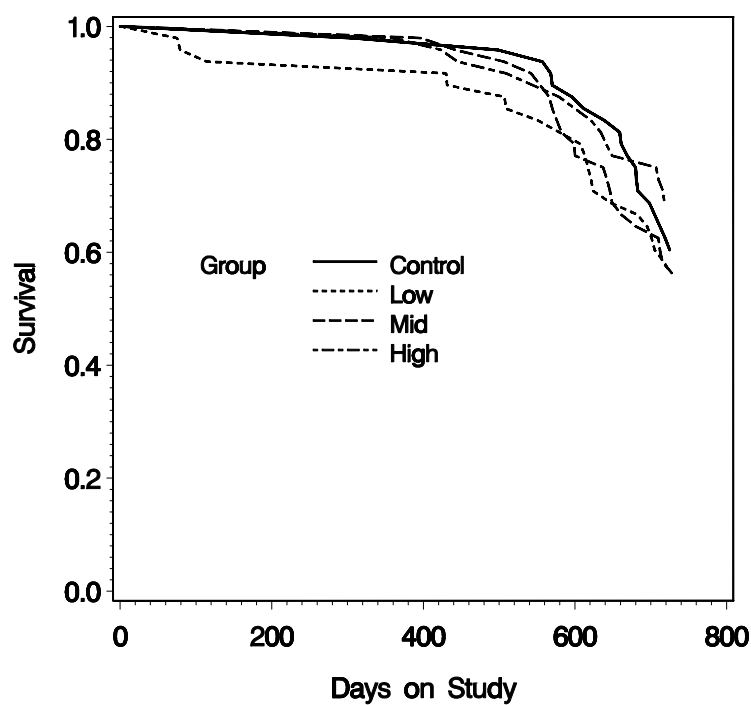


Figure 3-2. Survival of Female Animals

Table 3-3. Mean Survival Days and Day of 25% Mortality
for Male and Female Rats

Group	Male		Female	
	Mean Days of Survival (SE)	Estimated Day of 25% Mortality	Mean Days of Survival (SE)	Estimated Day of 25% Mortality
Control	660.5 (10.2)	613	686.8 (11.4)	680
Low	666.5 (12.1)	607	642.9 (24.1)	619
Mid	679.9 (11.6)	642	674.8 (12.2)	640
High	669.6 (8.4)	621	678.9 (12.6)	708

Animal Disposition. The disposition of all animals on study is provided in Tables 3-4. A small percentage of animals died naturally. A majority of males underwent moribund sacrifices. Individual animal disposition is provided in Appendix H.

Table 3-4. Disposition of Rats on Study

Death Type	Control		2 g BGVC/m ³		10 g BGVC/m ³		20 g BGVC/m ³	
	Male	Female	Male	Female	Male	Female	Male	Female
Moribund Sacrifice	30	17	25	18	19	17	26	12
Natural Death	2	2	3	3	4	4	7	3
Final Sacrifice	18	29	22	27	26	27	17	33
Other	0	2 ^a	0	2 ^{b,c}	1 ^d	2 ^{c,e}	0	2 ^c
Total	50	50	50	50	50	50	50	50

^aMalocclusion.

^bMissing, presumed dead.

^cNose injury.

^dAccidental removal through misidentification (included in survival analysis).

^eAccidental death due to trauma.

Body Weight Gain. Mean group body weights of male and female rats are provided in Tables 3-5 and 3-6, respectively. Growth curves are provided in Figures 3-3 and 3-4 for males and females, respectively. All animals gained weight during the study. However, body weights of males in the mid- and high-level BGVC exposure groups were significantly below control values throughout the study. Body weights of high-level females were below control weights from approximately Day 31 to the end of the study. Body weights of mid-level females were significantly below control values from Day 122 to the end of the study (except days 290, 318 and 378), while body weights of low-level females were only sporadically below control values. At terminal sacrifice, male percent of treated: control weights were 99.3%, 93.4% and 91.7% for low-, mid- and high-level groups, respectively. At terminal sacrifice, female percent treated:control weights were 97.4%, 91.2% and 89.6% for low-, mid- and high-level groups, respectively. Individual animal body weights are provided in Appendix I.

Clinical Signs of Toxicity. Rats exposed to BGVC displayed no clinical signs of toxicity-related BGVC exposure. As this was a chronic toxicity evaluation, many of the observations observed during the second year of the study were related to aging (mammary masses, jaundice). A summary of clinical observations by exposure group is provided in Appendix J.

Final Body Weights

Body weights at final sacrifice of high-level male and mid- and high- level female rats were significantly below control values (Table 3-7). Individual animal data are provided in Appendix K.

Absolute Organ Weights at Final Sacrifice

Only the mean epididymis weights of mid-level males were significantly different (reduced) from the mean control values. The kidney weights for mid- and high-level female rats were significantly greater than the mean control values, however they did not correlate with kidney pathology findings. In addition the spleen weight of mid-level females was significantly increased compared to controls. The mean value was strongly influenced by a few large spleens (>10 g) from animals having mononuclear leukemia. The effects on absolute organ weights were not considered to be treatment related. Summary statistics are provided in Table 3-8. Individual animal data are provided in Appendix K-1.

Table 3-5. Body Weight Summary for Male Rats^a

Study Day	Control	2 g/m ³	Percent Control (2 g/m ³)	10 g/m ³	Percent Control (10 g/m ³)	20 g/m ³	Percent Control (20 g/m ³)
-1	174.5	173.1	99.2	172.1	98.6	173.1	99.2
10	207.0	206.0	99.5	203.6	98.4	202.3	97.7
17	221.8	223.6	100.8	215.4	97.1	213.4	96.2
24	233.9	234.9	100.4	226.4	96.8	222.6	95.2
31	237.4	241.6	101.8	230.8	97.2	227.2	95.7
38	246.7	249.6	101.2	238.3	96.6	233.6	94.7
45	257.0	262.4	102.1	249.1	96.9	242.6	94.4
52	266.3	271.8	102.1	258.2	97.0	249.3	93.6
59	273.3	278.3	101.8	264.3	96.7	254.1	93.0
66	281.8	283.1	100.5	271.4	96.3	261.7	92.9
73	291.3	293.2	100.7	281.8	96.7	271.4	93.2
80	302.7	302.3	99.9	293.1	96.8	281.8	93.1
87	311.8	310.8	99.7	300.0	96.2	288.6	92.6
94	316.3	316.3	100.0	304.6	96.3	294.5	93.1
122	340.5	336.5	98.8	325.7	95.7	314.7	92.4
150	358.0	355.0	99.2	341.1	95.3	331.6	92.6
178	369.5	367.0	99.3	354.6	96.0	342.0	92.6
206	379.1	375.1	98.9	366.4	96.6	355.3	93.7
234	393.7	390.8	99.3	380.4	96.6	367.9	93.4
262	406.3	398.3	98.0	389.7	95.9	376.9	92.8
290	411.8	407.9	99.1	395.8	96.1	384.7	93.4
318	422.8	416.1	98.4	400.8	94.8	393.9	93.2
346	425.0	417.0	98.1	406.6	95.7	392.6	92.4
374	428.0	422.2	98.6	409.8	95.7	393.4	91.9
405	437.1	433.0	99.1	416.7	95.3	400.8	91.7
433	440.9	437.3	99.2	418.6	94.9	404.3	91.7
458	427.9	425.3	99.4	405.6	94.8	393.6	92.0
486	428.7	421.4	98.3	402.9	94.0	390.3	91.0
514	431.5	424.9	98.5	404.2	93.7	389.8	90.3
542	424.1	418.8	98.8	400.8	94.5	386.0	91.0
570	419.5	415.4	99.0	397.1	94.7	382.5	91.2
598	422.7	414.9	98.2	396.7	93.8	382.8	90.6
626	418.2	411.7	98.4	395.4	94.5	377.5	90.3
661	402.9	398.8	99.0	381.6	94.7	369.6	91.7
682	400.3	395.9	98.9	377.8	94.4	359.9	89.9
710	392.2	389.6	99.3	366.4	93.4	359.5	91.7

^aBolded and italicized values are significantly different from controls at that time point; $p \leq 0.05$. Group mean body weight, organ weight, and percent organ-to-body weight data were tested for statistical significance using Path-Tox[®] software. After testing for an overall trend among test groups by an analysis of variance, Bartlett's test was used to establish the homogeneity of the data. If the data were homogeneous, group differences were evaluated using a modified Dunnett's test. If data were non-homogeneous, group differences were assessed using a modified t-test. Significance levels were set at $p \leq 0.05$.

Table 3-6. Body Weight Summary for Female Rats^a

Study Day	Control	2 g/m ³	Percent Control (2 g/m ³)	10 g/m ³	Percent Control (10 g/m ³)	20 g/m ³	Percent Control (20 g/m ³)
-1	126.8	126.2	99.5	125.0	98.6	124.5	98.2
10	135.8	136.4	100.4	135.6	99.9	133.6	98.4
17	141.5	143.5	101.4	141.0	99.7	138.5	97.9
24	144.8	148.1	102.3	144.5	99.8	143.0	98.8
31	149.9	152.0	101.4	148.7	99.2	145.7	97.2
38	153.7	157.1	102.2	151.9	98.8	150.0	97.6
45	157.1	161.1	102.5	155.3	98.9	153.7	97.8
52	161.4	163.9	101.5	156.2	96.8	153.8	95.3
59	163.0	167.0	102.5	160.8	98.7	158.7	97.4
66	168.0	173.0	103.0	166.7	99.2	163.5	97.3
73	171.8	173.6	101.1	169.7	98.8	167.5	97.5
80	174.2	177.1	101.7	171.9	98.7	169.0	97.0
87	177.7	180.5	101.6	174.6	98.3	171.1	96.3
94	179.0	182.5	102.0	176.8	98.8	173.1	96.7
122	187.3	187.7	100.2	182.8	97.6	179.0	95.6
150	192.6	193.5	100.5	188.5	97.9	184.9	96.0
178	197.5	196.9	99.7	192.2	97.3	187.3	94.8
206	204.9	206.7	100.9	200.2	97.7	193.7	94.5
234	206.8	204.2	98.7	200.3	96.9	194.6	94.1
262	206.4	206.6	100.1	196.8	95.5	196.6	95.3
290	211.4	211.2	99.9	206.8	97.8	199.9	94.6
318	215.1	214.2	99.6	209.9	97.6	205.3	95.4
346	221.2	219.7	99.3	213.0	96.3	207.4	93.8
378	228.2	227.2	99.6	222.0	97.3	216.8	95.0
405	236.6	235.9	99.7	226.7	95.8	222.0	93.8
431	237.6	233.0	98.1	227.1	95.6	221.3	93.1
458	241.0	235.2	97.6	225.4	93.5	220.5	91.5
486	245.6	237.5	96.7	229.2	93.3	218.7	89.0
514	252.7	241.6	95.6	232.9	92.2	227.3	89.9
542	260.0	247.0	95.0	238.4	91.7	231.2	88.9
570	260.6	252.0	96.7	244.1	93.7	233.8	89.7
598	269.1	255.9	95.1	250.7	93.2	240.4	89.3
626	270.5	259.0	95.7	249.8	92.3	240.0	88.7
654	270.5	259.7	96.0	249.4	92.2	243.6	90.1
682	273.1	263.4	96.4	251.6	92.1	242.4	88.8
710	274.3	267.2	97.4	250.1	91.2	245.8	89.6

^aBolded & italicized values are significantly different from controls at that time point; $p \leq 0.05$. Group mean body weight, organ weight, and percent organ-to-body weight data were tested for statistical significance using Path-Tox[®] software. After testing for an overall trend among test groups by an analysis of variance, Bartlett's test was used to establish the homogeneity of the data. If the data were homogeneous, group differences were evaluated using a modified Dunnett's test. If data were non-homogeneous, group differences were assessed using a modified t-test. Significance levels were set at $p \leq 0.05$.

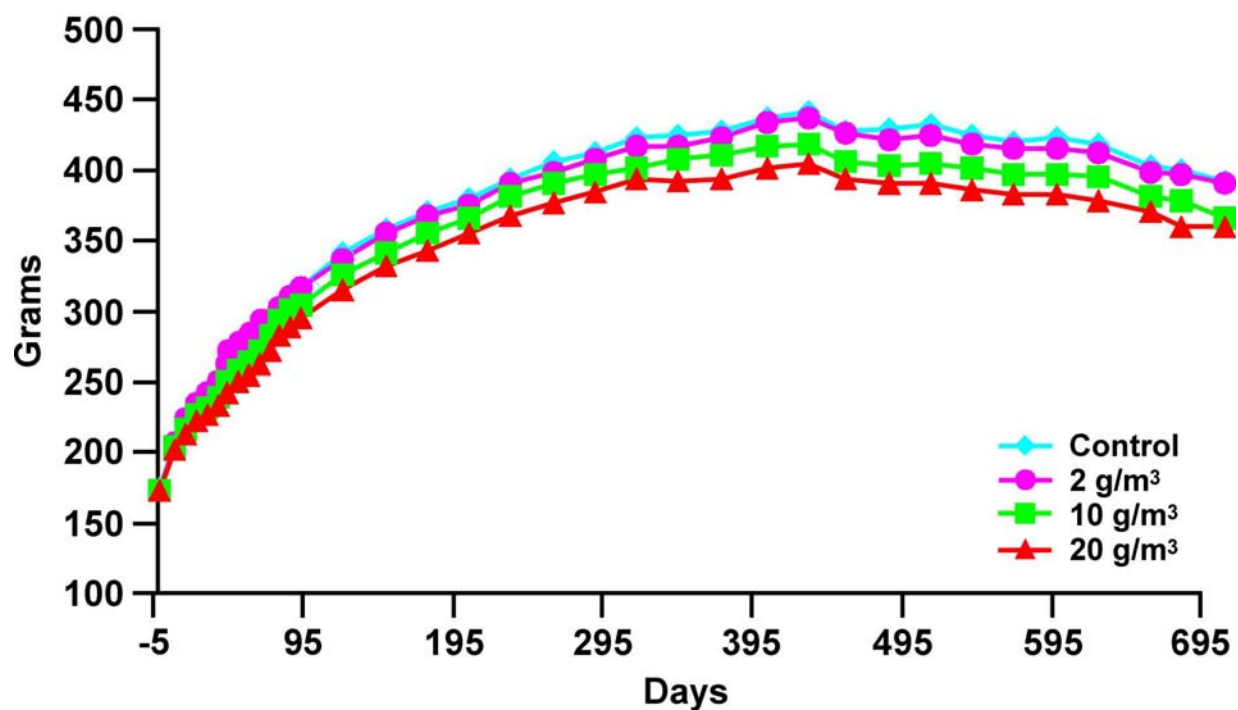


Figure 3-3. Body Weights of Male Rats Exposed to BGVC

5432-2

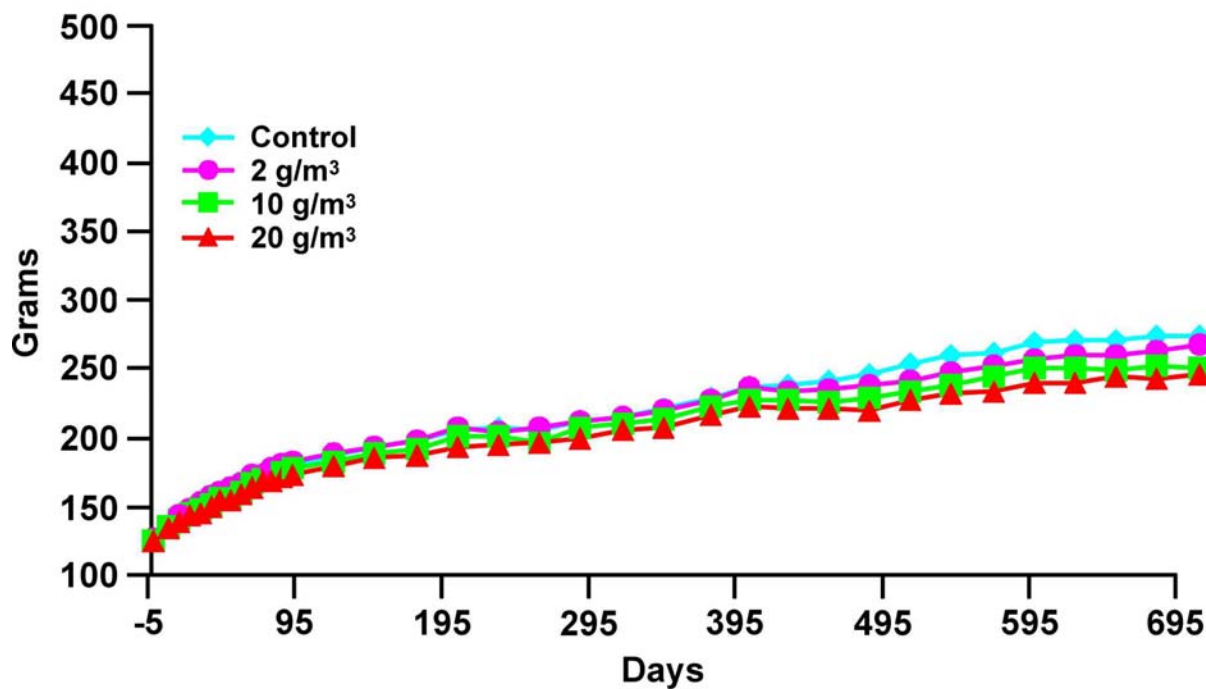


Figure 3-4. Body Weights of Female Rats Exposed to BGVC

5432-1

Table 3-7. Summary Statistics for Terminal Body Weights of Male and Female Rats

	Control	2 g BGVC/m ³	10 g BGVC/m ³	20 g BGVC/m ³
<u>Males</u>				
Mean ± SD	377.5 ± 31.3	388.3 ± 24.0	357.8 ± 36.3	341.9 ± 60.7 ^a
n	18	22	26	17
<u>Females</u>				
Mean ± SD	273.9 ± 20.5	261.5 ± 24.1	247.8 ± 22.4 ^b	251.0 ± 19.4 ^b
n	29	27	27	33

^aMean significantly different from control; data nonhomogeneous by Bartlett's test. Means compared using a Modified t test.

^bMean significantly different from control; data homogeneous by Bartlett's test. Means compared using Dunnett's test of significance.

Table 3-8. Absolute Organ Weight (g ± SD)

Exposure Group	Number in Group	Adrenal Glands	Brain	Epididymis/ Uterus	Heart	Male			Spleen	Testes/ Ovaries
						Liver	Lungs	Kidneys		
1	18	0.069 ± 0.014	2.035 ± 0.047	0.456 ± 0.077	1.175 ± 0.168	3.284 ± 2.189	14.255 ± 2.553	2.191 ± 0.751	3.641 ± 3.883	4.889 ± 2.113
2	22	0.069 ± 0.009	2.135 ± 0.270	0.449 ± 0.129	1.156 ± 0.093	2.761 ± 0.199	13.541 ± 2.077	1.958 ± 0.262	2.529 ± 2.005	6.133 ± 2.135 ^a
3	26	0.076 ± 0.037	2.049 ± 0.062	0.386 ± 0.058 ^b	1.127 ± 0.123	2.834 ± 0.534	14.113 ± 5.676	2.115 ± 0.446	3.945 ± 4.330	5.649 ± 2.678 ^c
4	17	0.078 ± 0.019	2.043 ± 0.067	0.430 ± 0.183	1.153 ± 0.127	2.857 ± 0.256	13.572 ± 2.169	2.389 ± 0.674	4.893 ± 3.963	4.963 ± 2.626
Female										
1	29	0.059 ± 0.011	1.871 ± 0.067	0.774 ± 0.333	0.853 ± 0.057	1.747 ± 0.104	8.493 ± 0.897	1.343 ± 0.125	0.913 ± 0.875	0.713 ± 3.246
2	27	0.071 ± 0.046	1.861 ± 0.044	1.154 ± 1.763	0.868 ± 0.131	1.948 ± 0.780	8.506 ± 1.167	1.366 ± 0.203	1.261 ± 1.314	0.104 ± 0.018
3	27	0.059 ± 0.012	1.846 ± 0.056	0.722 ± 0.193	0.830 ± 0.083	1.853 ± 0.238 ^b	9.400 ± 2.775	1.562 ± 0.740	2.867 ± 4.752 ^b	0.148 ± 0.158
4	33	0.067 ± 0.032	1.861 ± 0.052	0.741 ± 0.285	0.851 ± 0.061	1.840 ± 0.112 ^b	8.429 ± 0.772	1.351 ± 0.130 ^d	1.010 ± 0.811	0.323 ± 0.889

^an = 21.

^bMean significantly different from control. Data are nonhomogeneous by Bartlett's test. Means were compared with Modified t test; p = 0.05.

^cn = 25.

^dn = 32, one lung (L380) not weighed.

Percent Organ-to-Body Weight at Final Sacrifice

In males the mean percent epididymis-to-body weight was significantly lower than the control value for the mid-level group. The percent adrenal-to-body and brain-to-body weight values of high-level males were significantly greater than control values. Among mid-level females, the percent organ-to-body weight values for brain, heart, kidney, liver, lungs, and spleen were significantly greater than the value for controls. Among high level females, the percents organ-to-body weight for adrenals, brain, heart, liver, kidney, and lungs were significantly greater than controls. However, there was not a trend for increasing organ:body weight ratio with increasing exposure level, suggesting the effect was not treatment related. Summary statistics are provided in Table 3-9. Individual animal data are provided in K-2.

Percent Organ-to-Brain Weight at Final Sacrifice

There were few effects on percent organ weights since there were no differences in absolute brain weights between control and treated animals. The patterns of effects were similar to those reported above for the absolute organ weights. For males, the percent epididymis-to-brain weight was significantly lower for the mid-level group than for the controls.

The percent kidney-to-brain weight for mid- and high-level females and the percent spleen-to-brain weight for the mid-level females were significantly greater than the corresponding control values. However, there was not a trend for increasing organ-to-brain weight ratio with increasing exposure level, suggesting the effect was not treatment related. Summary statistics are provided in Table 3-10. Individual animal data are provided in K-3.

Terminal Body Weight and Organ Weights of Euthanized (Moribund) Rats

As the rats reached about 20 months of age (18 months on study), mortality due to the effects of aging increased. Most animals not surviving to the final sacrifice were euthanized and terminal body and organ weights were recorded. Because the animals were euthanized over a period of several months, interpretation of the statistical significance of differences among exposed and control groups is not as straightforward as with data obtained at the final sacrifice. Summary statistics and individual animal terminal body and organ weight data are provided in Appendices K-4 through K-6.

Table 3-9. Percent Organ-to-Body Weight (% ± SD)

Exposure Group	Number in Group	Adrenal Glands	Brain	Epididymis/ Uterus	Heart						Lungs	Spleen	Testes/ Ovaries
						<u>Male</u>							
1	18	0.018 ± 0.005	0.543 ± 0.046	0.122 ± 0.024	0.312 ± 0.038	0.874 ± 0.577	3.810 ± 0.809	0.588 ± 0.237	0.994 ± 1.101	1.281 ± 0.517			
2	22	0.018 ± 0.003	0.551 ± 0.074	0.116 ± 0.039	0.298 ± 0.023	0.712 ± 0.041	3.491 ± 0.519	0.508 ± 0.094	0.665 ± 0.547	1.580 ± 0.542 ^a			
3	26	0.022 ± 0.011	0.578 ± 0.058	0.108 ± 0.014 ^b	0.317 ± 0.042	0.808 ± 0.232	4.001 ± 1.883	0.603 ± 0.178	1.139 ± 1.345	1.556 ± 0.662 ^c			
4	17	0.023 ± 0.006 ^b	0.610 ± 0.079 ^d	0.123 ± 0.029	0.344 ± 0.058	0.850 ± 0.109	4.071 ± 0.926	0.727 ± 0.273	1.560 ± 1.377	1.452 ± 0.728			
						<u>Female</u>							
1	29	0.022 ± 0.004	0.686 ± 0.048	0.287 ± 0.138	0.313 ± 0.025	0.640 ± 0.044	3.105 ± 0.288	0.492 ± 0.056	0.338 ± 0.334	0.254 ± 1.152			
2	27	0.028 ± 0.021	0.718 ± 0.074	0.436 ± 0.623	0.335 ± 0.068	0.752 ± 0.321	3.256 ± 0.393	0.528 ± 0.106	0.497 ± 0.538	0.040 ± 0.006			
3	27	0.024 ± 0.005	0.751 ± 0.072 ^d	0.293 ± 0.081	0.337 ± 0.038 ^b	0.759 ± 0.162 ^b	3.894 ± 1.601 ^b	0.656 ± 0.408 ^b	1.314 ± 2.379 ^b	0.063 ± 0.078			
4	33	0.027 ± 0.014 ^b	0.745 ± 0.057 ^d	0.297 ± 0.116	0.341 ± 0.032 ^b	0.735 ± 0.046 ^b	3.365 ± 0.275 ^b	0.542 ± 0.060 ^{b,e}	0.407 ± 0.331	0.142 ± 0.416			

^an = 21.

^bMean significantly different from control. Data nonhomogeneous by Bartlett's test. Means were compared with Modified t test; p ≤ 0.05.

^cn = 25.

^dMean significantly different from control. Data homogeneous by Bartlett's test. Means were compared with Dunnett's test of significance.

^en = 32, one lung (L380) not weighed.

Table 3-10. Percent Organ-to-Brain Weight (% ± SD)

Exposure Group	Number in Group	Adrenal Glands	Brain	Epididymis/ Uterus	Heart	Kidneys	Liver	Lungs	Spleen	Testes/ Ovaries
<u>Male</u>										
1	18	3.40 ± 0.72	100.00 ± 0.00	22.45 ± 3.89	57.75 ± 8.19	161.54 ± 108.31	700.66 ± 126.57	107.58 ± 36.89	178.11 ± 188.90	239.63 ± 102.91
2	22	3.24 ± 0.48	100.00 ± 0.00	21.26 ± 6.58	54.75 ± 6.59	130.61 ± 13.93	641.59 ± 116.50	92.81 ± 15.43	120.40 ± 98.22	289.52 ± 103.19 ^a
3	26	3.70 ± 1.80	100.00 ± 0.00	18.85 ± 2.83 ^b	55.00 ± 5.90	138.57 ± 28.19	689.96 ± 289.49	103.27 ± 22.15	193.09 ± 216.78	273.90 ± 126.42 ^c
4	17	3.83 ± 0.92	100.00 ± 0.00	20.93 ± 8.25	56.41 ± 5.65	139.89 ± 12.37	665.12 ± 109.59	116.99 ± 33.18	239.78 ± 191.92	243.65 ± 129.07
<u>Female</u>										
1	29	3.17 ± 0.56	100.00 ± 0.00	41.45 ± 18.26	45.60 ± 2.76	93.41 ± 5.17	453.94 ± 45.88	71.73 ± 5.69	48.89 ± 47.27	38.61 ± 176.22
2	27	3.80 ± 2.37	100.00 ± 0.00	61.82 ± 93.72	46.63 ± 7.04	104.71 ± 42.21	457.35 ± 64.13	73.34 ± 10.03	67.07 ± 68.05	5.58 ± 0.97
3	27	3.21 ± 0.69	100.00 ± 0.00	39.17 ± 10.82	45.02 ± 4.81	100.47 ± 13.34 ^b	510.17 ± 155.28	84.72 ± 40.96	155.70 ± 257.16 ^b	8.05 ± 8.76
4	33	3.60 ± 1.67	100.00 ± 0.00	39.91 ± 15.61	45.74 ± 3.39	98.89 ± 5.99 ^b	453.17 ± 41.82	72.64 ± 6.67 ^d	54.43 ± 44.13	17.59 ± 48.58

^an = 21.

^bMean significantly different from control. Data nonhomogeneous by Bartlett's test. Means were compared with Modified t test; p ≤ 0.05.

^cn = 25.

^dn = 32, one lung (L380) not weighed.

The absolute kidney weights of low- and high-level males were significantly greater than control, while the epididymis weight for high-level males was significantly below control. Similarly, the percent kidney-to-body weight was significantly increased for the high-level males, and the percent testes-to-body weight was significantly increased among mid- and high-level males. The percent kidney-to-brain weight was significantly increased for high-level males. The percent epididymis-to-brain weight was significantly decreased in high-level males. There were no effects on terminal body or organ weights in females. Since cause of death of euthanized animals was not determined, the significance of the changes that did not correlate with terminal sacrifice data is unknown.

Hematology

Percentage and estimated absolute numbers of white blood cells are presented in Appendix L. As might be expected, there are numerous, statistically significant changes over time in WBC counts in the control and high-level groups (Table 3-11). These may be regarded as less important than differences between the groups at a given time-point, in that such time-related changes are expected to occur with aging and concomitant development of degenerative, inflammatory, and neoplastic (e.g., mononuclear cell leukemia [MCL]) changes. Statistically significant interactive effects between time and exposure were not assessed.

For females, there are significantly fewer numbers of total WBCs, neutrophils, lymphocytes, eosinophils, and nucleated red blood cells (RBCs) in the high-level group at the 12 month time-point (estimated absolute numbers; $p \leq 0.05$, Kruskal-Wallis test). The significantly decreased numbers at 12 months may not be an indication of myelosuppression in the high-level exposure groups, in that the controls have slightly elevated total WBC and absolute lymphocyte counts from what might be expected in a group of normal female F344 rats of this age. The results in the 12 animals possibly were affected by the tail blood sampling technique, which improved by 18 months. At 12 months we had difficulty sampling blood from the tail; that may have resulted in artifactual results. At 18 months and at the final sacrifice, there are no significant differences between the groups of females in any of the estimated WBC numbers. Overall, the relationship of treatment with the changes observed in females at 12 months is unknown.

Table 3-11. Summary of Hematological Findings in Rats Inhaling BGVC; Median (range)

Cell Type	Males				Females			
	Cells × 10 ³ /μL				Cells × 10 ³ /μL			
	12 Months ^a	18 Months ^b	24 Months ^c	Trend Over Time ^d	12 Months ^a	18 Months ^b	24 Months ^c	Trend Over Time ^d
WBC Estimate								
Control	12.0 (7.5, 22.8)	8.5 (5.0, 25.2)	3.8 (1.8, 450.0)	–	10.8 (6.4, 21.2)	6.4 (2.6, 14.4)	2.3 (0.8, 17.2)	–
High dose	11.6 (4.8, 26.0)	9.3 (3.4, 42.0)	7.2 (2.4, 263.5)	–	9.0 (4.0, 18.8) ^e	6.5 (2.6, 16.8)	2.2 (0.8, 18.2)	–
Absolute Neutrophils								
Control	4.1 (1.9, 14.4)	3.3 (1.0, 12.9)	2.1 (0.0, 6.2)	–	3.0 (1.6, 5.3)	1.7 (0.7, 5.7)	0.9 (0.4, 3.3)	–
High dose	4.9 (1.3, 13.0)	3.2 (1.1, 21.0)	2.2 (0.8, 17.5)	–	2.1 (0.0, 8.2) ^e	2.0 (0.5, 5.7)	0.9 (0.3, 9.1)	–
Absolute Band Neutrophils								
Control	0.00 (0.00, 0.00)	0.00 (0.00, 0.08)	0.00 (0.00, 0.00)	none	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.03)	+
High dose	0.00 (0.00, 0.00)	0.00 (0.00, 0.42)	0.00 (0.00, 0.26)	none	0.00 (0.00, 0.08)	0.00 (0.00, 0.16) ^e	0.00 (0.00, 0.36)	none
Absolute Lymphocytes								
Control	6.6 (4.3, 11.2)	4.6 (2.8, 14.6)	1.1 (0.6, 346.5)	–	7.2 (4.5, 15.1)	4.0 (1.8, 8.2)	1.1 (0.4, 11.2)	–
High dose	6.1 (2.7, 14.3)	5.2 (1.8, 19.7)	4.2 (0.9, 189.6) ^e	none	5.7 (0.0, 11.7) ^e	4.4 (1.5, 11.9)	1.0 (0.4, 6.7)	–
Absolute Monocytes								
Control	0.5 (0.0, 2.0)	0.5 (0.2, 2.3)	0.1 (0.0, 1.6)	–	0.2 (0.0, 1.0)	0.2 (0.0, 0.9)	0.0 (0.0, 0.2)	–
High dose	0.5 (0.0, 1.8)	0.4 (0.0, 1.4)	0.1 (0.0, 7.9)	–	0.2 (0.0, 0.7)	0.1 (0.0, 0.5)	0.0 (0.0, 0.4)	–
Absolute Eosinophils								
Control	0.2 (0.0, 0.9)	0.2 (0.0, 1.1)	0.0 (0.0, 0.5)	–	0.2 (0.0, 0.5)	0.1 (0.0, 0.4)	0.0 (0.0, 0.2)	–
High dose	0.2 (0.0, 1.6)	0.1 (0.0, 0.8)	0.0 (0.0, 1.0)	–	0.1 (0.0, 0.4) ^e	0.1 (0.0, 0.8)	0.0 (0.0, 0.3)	–
Absolute Basophils								
Control	0.00 (0.00, 0.00)	0.00 (0.00, 0.08)	0.00 (0.00, 0.03)	none	0.00 (0.00, 0.08)	0.00 (0.00, 0.11)	0.00 (0.00, 0.03)	none
High dose	0.00 (0.00, 0.00)	0.00 (0.00, 0.06)	0.00 (0.00, 1.02)	none	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.03)	none
Absolute Atypical Lymphs								
Control	0.0 (0.0, 0.2)	0.0 (0.0, 0.8)	0.2 (0.1, 94.5)	+	0.0 (0.0, 0.0)	0.1 (0.0, 0.7)	0.1 (0.0, 2.4)	+
High dose	0.0 (0.0, 0.0)	0.0 (0.0, 2.1)	0.6 (0.1, 105.4) ^e	+	0.0 (0.0, 0.0)	0.1 (0.0, 1.1)	0.1 (0.0, 5.1)	+
Absolute Blasts								
Control	0.00 (0.00, 0.00)	0.00 (0.00, 1.24)	0.00 (0.00, 9.00)	+	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 0.17)	none
High dose	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.00 (0.00, 8.10)	+	0.00 (0.00, 0.00)	0.00 (0.00, 0.16)	0.00 (0.00, 1.09)	none
Nucleated RBC (#/100 WBC)								
Control	0.0 (0.0, 2.0)	1.0 (0.0, 27.0)	1.5 (0.0, 21.0)	+	1 (0, 4)	1 (0, 9)	3 (0, 11)	+
High dose	0.0 (0.0, 2.0) ^e	2.0 (0.0, 15.0)	2.0 (0.0, 22.0)	+	0.0 (0.0, 0.5) ^e	1 (0, 26)	5 (1, 14)	+

^an = 49 for control males and n = 47 for control females; n = 49 for high-level males and n = 40 for high-level females.

^bn = 48 for control males and n = 45 for control females; n = 48 for high-level males and n = 42 for high-level females.

^cn = 18 for control males and n = 28 for control females; n = 17 for high-level males and n = 33 for high-level females.

^d– = significant downward trend; + = significant upward trend.

^eMedian significantly different from control at that time point; Kruskal Wallis test, p ≤ 0.05.

Only the absolute number of lymphocytes is significantly elevated in the high-level male group at the final sacrifice time-point ($p = 0.01$, Kruskal-Wallis test) in male rats. The range of lymphocyte numbers at this time-point, however, is very large (0.6×10^3 to 346.5×10^3 cells/ μL for controls and 0.9×10^3 cells/ μL to 189.6×10^3 cells/ μL for high level) and may be attributable to the incidence of MCL in these aged animals. This is corroborated by the enlarged number of atypical lymphocytes at the final sacrifice time-point. Given that BGVC exposure did not definitively enhance splenic involvement with MCL (below), the relationship of treatment to lymphocyte differences is unknown.

Individual animal data are provided in Appendix L.

Histopathology

Proliferative Changes. There was an increased incidence of renal tubule adenoma and carcinoma in males that peaked at the mid-dose level with incidences of 4/50 and 3/50, respectively. These results, presented in Table 3-12, are consistent with previous studies on wholly vaporized unleaded gasoline (MacFarland et al., 1984). Although these results taken individually are not statistically different from control values in the present study, the combined adenoma + carcinoma incidences are significant ($p = 0.004$, Fisher's Exact [FE] test, Table 3-12, and Appendix O). Pair-wise tests did not demonstrate any significant differences between groups in the combined incidence of renal adenoma + carcinoma ($p > 0.008$), although this incidence in the mid-level exposure group compared to the in the high-level exposure group approached the significance level ($p = 0.01$, FE test with Bonferroni's correction: *c.f.* Appendix O). The reason for the lack of renal neoplasms in the high-level group of male rats is unknown. These neoplasms may be considered to be exposure-related. For compounds inducing renal tumors in male rats through increased alpha 2u-globulin accumulation and subsequent increased renal tubule cell turnover, the human health relevance of the mechanism, and thus, the tumors, is often regarded as suspect (Baetcke et al., 1991; Hard, 1998; Hard and Khan, 2004). Of an ancillary note, however, is that increased severity of the hydrocarbon-induced alpha 2u-globulin nephropathy, which is assessable only at early time-points in an exposure, is not always predictive of increased incidence of renal neoplasia in rats exposed to hydrocarbons for 2 years or more. Rather, the severity of chronic progressive nephropathy may be correlated more

reliably with the incidence of renal neoplasms in carcinogenesis bioassays of light hydrocarbon exposure (Doi et al., 2007).

Table 3-12. Summary of Select Proliferative Lesions in Male and Female Rats

Tissue	Diagnosis	Control (0 g/m ³)	Low (2 g/m ³)	Mid (10 g/m ³)	High (20 g/m ³)
<u>MALES</u>					
Kidney	<i>No. examined</i>	50	50	50	50
	Adenoma, renal tubule	1 (2%)	1 (2%)	4 (8%)	0 (0%)
	Carcinoma, renal tubule	0 (0%)	0 (0%)	3 (6%)	0 (0%)
	Renal tubule adenoma and carcinoma, combined ^a	1 (2%)	1 (2%)	7 (14%)	0 (0%)
Nasal Passages (Note that the same tumor may occur at more than one level.)	<u>Turbinate Level 2</u>				
	<i>No. examined</i>	50	50	50	50
	Carcinoma, squamous cell	0 (0%)	0 (0%)	0 (0%)	1 (2%)
	<u>Turbinate Level 3</u>				
	<i>No. examined</i>	50	50	49	50
	Carcinoma, squamous cell ^b	0 (0%)	0 (0%)	0 (0%)	3 (6%)
	<u>Turbinate Level 4</u>				
	<i>No. examined</i>	50	50	49	50
	Carcinoma, squamous cell	0 (0%)	1 (2%)	0 (0%)	3 (6%)
Testes	<i>No. examined</i>	50	49	50	50
	Mesothelioma, malignant ^c	0 (0%)	0 (0%)	4 (8%)	0 (0%)
	Adenoma, interstitial cell ^d	48 (96%)	46 (94%)	50 (100%)	49 (98%)
Thyroid	<i>No. examined</i>	50	29	27	50
	Hyperplasia, follicular cell ^{e,f}	1 (2%)	0 (0%)	2 (7%)	6 (12%)
	Avg. severity	0.0	0.0	0.1	0.2
	Adenoma, follicular cell	0 (0%)	2 (7%)	0 (0%)	2 (4%)
	Carcinoma, follicular cell ^g	0 (0%)	0 (0%)	2 (7%)	0 (0%)
	Follicular cell adenoma and carcinoma, combined ^h	0 (0%)	2 (7%)	2 (7%)	2 (4%)
Spleen	<i>No. examined</i>	50	34	38	50
	Leukemia, mononuclear cell	32 (64%)	23 (68%)	25 (66%)	32 (64%)

Table 3-12. Summary of Select Proliferative Lesions in Male and Female Rats (Concluded)

Tissue	Diagnosis	Control (0 g/m ³)	Low (2 g/m ³)	Mid (10 g/m ³)	High (20 g/m ³)
<u>FEMALES</u>					
Spleen	<i>No. examined</i>	50	25	32	50
	Leukemia, mononuclear cell ⁱ	13 (26%)	14 (56%)	18 (56%)	15 (30%)

^aIncidences are different than expected by chance ($p = 0.004$, overall FE test), although pair-wise comparisons are not significant ($p > 0.008$, FE tests with Bonferroni's correction; *c.f.*, Appendix O).

^bSignificant trend for increased incidence with increasing exposure level ($p < 0.019$, CA). Note that mid-dose turbinate levels 3 and 4 for one animal (K221) were autolytic, resulting in an n of 49.

^cIncidences are different than expected by chance ($p = 0.014$, overall FE test), although pair-wise comparisons are not significant ($p > 0.008$, FE tests).

^dIncidences are not different than expected by chance ($p = 0.26$, overall FE test).

^eAverage for all animals examined (both affected and unaffected).

^fThere is a significant increasing trend with exposure concentration (CA; $p = 0.018$; Logistic regression $p = 0.040$).

^gIncidences are different than expected by chance ($p = 0.029$, overall FE test), although pair-wise comparisons are not significant ($p > 0.008$, FE tests).

^hIncidences are not different than expected by chance ($p = 0.14$, overall FE test).

ⁱIncidences are different than expected by chance ($p = 0.007$, overall FE test), although pair-wise comparisons are not significant ($p > 0.008$, FE tests).

The incidence of squamous cell carcinoma in the nasal passages (turbinate levels 2-4) was elevated in the high-level group of male rats, with tumor incidence of 1/50, 3/50, and 3/50 for levels 2, 3 and 4, respectively. It was not appropriate to combine carcinoma incidence of nasal turbinate levels for statistical analysis in the high dose group because the lesion spanned two or three levels in the same three animals. The level 3 incidence gave a statistically significant trend for increased incidence with increased exposure concentration ($p = 0.019$, Cochran-Armitage [CA] test). Squamous cell carcinoma was noted in the nasal specimens of only one female control rat of the current study. The squamous cell carcinomas in all of the male nasal specimens had morphologies consistent with origination from the oral mucosa and had varying degrees of invasion into the nasal passages. By comparison, in the concurrent Gasoline MTBE Vapor Condensate study (FY01-013), squamous cell carcinoma of oral origin occurred in one male control, two male GMVC low dose and two male GMVC dose, but in no female rats. While the fact that the nasal tumors were of oral origin might suggest that the inhalation

exposure was not causal, spontaneous oral squamous cell carcinomas in other studies have been nearly as rare as those originating from the nasal mucosa (~ 0.6%; Haseman et al., 1990; NTP, 1999). The historical incidence of squamous cell carcinoma of oral origin in a large cigarette smoke study conducted at LRRI in the 1990s was 0/118 (0%) in males and 3/119 (2.5% in females). Taken together, the data provide some evidence that the development of nasal squamous cell carcinomas in male rats may have resulted from test article exposure.

Similar to the incidence of renal neoplasms, there was an increased incidence of testicular mesothelioma in the mid-level group of male rats (4 of 50 examined; Table 3-12). Fisher's exact test demonstrated that this incidence was greater than expected, but pair-wise testing between the groups and trend tests for exposure concentration-response effects did not indicate statistically significant effects of exposure. The reason for the lack of an exposure concentration-response effect are not known. However, the incidence in the mid-level group is approximately 3-fold greater than the average incidence in male control F344 rats exposed to air in inhalation toxicology studies (28 tumors in 1,055 rats or 2.7%; NTP, 1999) and may be regarded as an effect of exposure to the test substance. Mesothelioma was also evident in sections of a variety of abdominal organs from male rats and was regarded to be the result of metastasis, in that all but one of these (epididymal mesothelioma; one of 28 epididymides examined in the low-level group) occurred in the mid-level group of male rats. Incidences of mesothelioma in the duodenum (2 of 23 examined), jejunum (3 of 23), colon (3 of 24), pancreas (3 of 24), spleen (3 of 38), epididymis (3 of 25), prostate gland (2 of 25), and seminal vesicle (3 of 25) of the mid-level male rats were statistically significant by FE tests. Most mesotheliomas are thought to originate in the tunica vaginalis of the scrotum (Hall, 1990), and those found in this study are consistent with an origination from this investment of the testes. As with this study, there were no mesotheliomas found in the testes or other tissues of the control male animals of the GMVC study (LRRI protocol FY01-013), although there was a statistically non-significant incidence (2 in 50 animals or 4%) of the tumor in the testes of the high-level GMVC-exposed rats and sporadic incidence of the tumor in other tissues of GMVC-exposed male rats. The absence of testicular mesothelioma in control animals of the present study, where 1 or 2 tumors might normally be expected to be found in 50 animals (NTP, 1999), may have influenced the statistical

analysis to indicate a significant exposure-related incidence in the mid-level BGVC-exposed male rats, when no causal increase is actually present.

There was a trend for increased incidence of thyroid follicular cell carcinoma in male animals ($p < 0.05$, FE test over all groups; see Table 3-12). There were two tumors in the 27 mid-dose glands examined and no tumors in any other male control or exposed group. Whether the incidence of thyroid follicular carcinoma was a causal effect of exposure to the test substance or a sporadic occurrence remains a question, especially given that there is no apparent exposure concentration-response effect. Pair-wise testing suggests no statistically significant differences existed among the groups ($p > 0.008$, FE test). However, the incidence (7%) in the mid-level group of males is considerably higher than that reported in control F344 rats exposed to air (average incidence of 1% in 21 studies, range of 0 carcinomas in 52 rats to 1 in 45; NTP, 1999). Thyroid follicular epithelial adenomas were also present in the low-level (2 of 29 examined) and high-level groups of male rats (2 of 50 examined; see Table 3-12), although the incidences were not statistically significant. While the incidence of thyroid follicular adenomas reported here are similar to or slightly higher than those previously reported for control animals, the incidence of thyroid follicular carcinomas and adenomas combined are higher than what might be expected (NTP, 1999). However, there are no statistically significant differences in the combined incidence of thyroid follicular adenomas and carcinomas among the exposure groups (Table 3-12). In the concurrent Gasoline MTBE Vapor Condensate study, there were no significant increases in thyroid follicular cell adenomas, carcinomas, or combined adenomas and carcinomas in males or females. Thyroid follicular carcinoma might be considered to be caused by the exposure despite the lack of an apparent exposure concentration-response effect. Further, follicular epithelial hyperplasia at minimal to mild severity was noted in male rats of the control, mid-, and high-level groups (2–12% incidence) and also displayed an increased incidence with increasing exposure level (CA p -value = 0.018; logistic p -value = 0.040). This also suggested that thyroid follicular proliferative lesions in the male animals were caused by the exposure.

Despite the proliferative lesions in the thyroid glands of male rats, the relevance of the test article exposure to human risk might be questioned, given that mechanisms of chemical thyroid carcinogenesis are believed to be different between humans and rodents (U.S. Environmental

Protection Agency, 1998). Mutation of thyroid follicular cell DNA may lead directly to cancer and is the only mechanism verified to be carcinogenic in humans, but rodents are believed to be more susceptible to carcinogenic processes involving stimulation of thyroid follicular cell growth through disruption of pituitary-thyroid hormonal physiology. Mutagenesis of thyroid follicular cells and hormone disruption were not evaluated in the current study. Other proliferative (and nonproliferative) lesions occurred which were not clearly significant or were judged to have confounding factors which must be considered in interpretation. In males these included mononuclear cell leukemia (MCL) in several tissues with the spleen considered to be the tissue of origin where there was no difference between control and treatment group incidence and mesothelioma affecting the spleen and segments of the gastrointestinal and genitourinary tract. Male rats of the low- and mid-dose levels had a statistically significant higher incidence of keratoacanthomas in the skin (3 tumors in 29 rats and 3 in 26, respectively). However, there is a lack of a dose-response for these incidences, and most likely a result of protocol-driven gross sampling bias in non-target organ tissues where only tissues with gross lesions were evaluated in the low- and mid-dose groups at terminal sacrifice. In females, there was a statistically significantly increased incidence of splenic MCL in the low- and mid-dose groups. This finding was considered not to be treatment-related, but rather attributable to gross sampling bias in the low- and mid-dose groups. Additionally, some findings were decreased in exposed animals compared to controls, these are not generally discussed, as their significance with respect to treatment is unknown.

Histopathology summaries and individual animal data for males and females are provided in Appendices M and N, respectively. Statistical evaluation of lesions is provided in the statistician's report, Appendix O. The pathologist's report is provided in Appendix P.

Nonproliferative Changes. Kidneys of nearly all male rats on study have evidence of chronic progressive nephropathy, and there is no significant difference in the incidence among the groups. However, overall combination of incidence and severity of the lesion is significantly worse in the mid- and high-level exposure groups ($p \leq 0.05$, Kolmogorov-Smirnoff test, Table 3-13). This finding in males is consistent with male-rat specific light hydrocarbon-induced alpha-2u globulin overload nephropathy that has been reported previously in studies with wholly vaporized unleaded gasoline. It should be noted that in F344 male rats, after approximately

1 year of age, alpha-2u globulin overload nephropathy is essentially masked by age-related chronic progressive nephropathy, since both forms of nephropathy share similar if not identical diagnostic hallmarks. Therefore the effects alpha-2u globulin overload nephropathy typically manifests itself in increased severity, rather than incidence, since essentially all males surviving past 1 year of age will develop CPN. Thus, the statistically significant worsening of chronic progressive nephropathy in male rats with higher levels of exposure is consistent with an effect induced by the test substance.

Table 3-13. Incidence and Severity of Chronic Progressive Nephropathy in Male and Female Rats Exposed to BGVC

	Control	Low Level	Mid Level	High Level
<u>Males</u>				
No. examined	50	50	50	50
No. with lesion (%)	49 (98)	49 (98)	50 (100) ^a	50 (100) ^a
No. with minimal grade 1 (%)	6 (12)	4 (8)	3 (6)	2 (4)
No. with mild grade 2 (%)	20 (40)	18 (36)	9 (18)	10 (20)
No. with moderate grade 3 (%)	20 (40)	24 (48)	28 (56)	27 (54)
No. with marked grade 4 (%)	3 (6)	3 (6)	10 (20)	11 (22)
Average severity grade	2.4	2.5	2.9	2.9
<u>Females</u>				
No. examined	50	24	25	50
No. with lesion (%)	27 (54)	12 (50)	16 (64)	32 (64)
No. with minimal grade 1 (%)	21 (42)	3 (12.5)	10 (40)	22 (44)
No. with mild grade 2 (%)	6 (12)	6 (25)	5 (20)	9 (18)
No. with moderate grade 3 (%)	0	2 (8.3)	1 (4)	1 (2)
No. with marked grade 4 (%)	0	1 (4.2)	0	0
Average severity grade	0.7	1.0	0.9	0.9

^aOverall severity/incidence is significantly greater than control using the Kolmogorov-Smirnoff test, $p \leq 0.05$.

The incidence and severity of chronic progressive nephropathy in female rats was much less than in males and was not significantly different from that of the control group of female rats (Table 3-13). Renal findings in females that are part of the constellation of chronic progressive nephropathy, including cortical tubular epithelial regeneration and corticomedullary tubular protein accumulation, had increased incidence with increasing exposure level ($p < 0.05$, CA and logistics tests; Appendix P). Therefore, even though overall severity scores were not different from controls, at least some renal findings in females are consistent with enhancement of chronic progressive nephropathy with increasing exposure level.

Degeneration of the nasal epithelium, characterized primarily by accumulation of globular, homogeneous, brightly eosinophilic material in the cytoplasm of respiratory and/or olfactory epithelial cells, is present at minimal-to-moderate severity in all groups of animals (Tables 3-14 and 3-15). This is a common finding in aged rats, but its severity or incidence can be enhanced by inhalation exposure to irritants or toxicants. The finding is somewhat sporadic, but its incidence in BGVC-exposed male rats is significantly greater in the respiratory epithelium of the nasal passages at turbinate level 2 (approximately at the level of the incisive papilla) with a significant trend for increased incidence with increasing level of exposure ($p < 0.005$, Cochran-Armitage and logistics tests, Table 3-14). The combination of incidence and severity of the lesion at turbinate level 2, however, is not significantly different among the groups. Difference in incidence of the degenerative change in respiratory epithelium at turbinate level 3 (second palatal ridge) of male rats is also statistically significant ($p = 0.007$, Fisher's exact test over all groups), but the CA and logistic trend tests do not demonstrate any significance among the groups. The incidence of the lesion in the mid-level group is significantly greater than in the low-level group (Table 3-14).

The incidence of nasal respiratory epithelial degeneration for female rats is somewhat greater than that of the males suggesting that this finding is more of a female-specific lesion (Table 3-14). Higher incidences with significant trends for increases with increasing exposure level of the respiratory epithelial lesion were noted at both turbinate levels 2 and 3 of the female rats. The incidences at turbinate levels 2 and 3 in the high-level group were significantly greater than the control and low-level groups. The incidence at turbinate level 3 in the mid-level group was significantly greater than in the low-level group. The combination of incidence and severity of the respiratory epithelial lesion was significantly greater only in the female high-level exposure group and only at turbinate level 2. The incidence and/or severity of the lesion in both sexes are likely induced by exposure to the test substance.

Interestingly, BGVC exposure caused a statistically significant exposure-related decrease in the degeneration of olfactory epithelium in turbinate levels 3 and 4 (level of the first molar) in the nasal passages from male rats (Table 3-15). At turbinate level 4, the incidence in the high-level male group was significantly less than in the control and low-level groups. This exposure-response relationship was also present in the females at turbinate level 4 (Table 3-15), where the incidence in the mid- and high-level rats was less than in controls. This suggests that the BGVC exposure was associated with some protection of the olfactory epithelium. Given that olfactory epithelium contains relatively large amounts of inducible toxicant metabolizing enzymes (e.g.,

CYP450, glutathione-S-transferases, carboxyl esterases), there is a possibility that these cells over time became relatively resistant to toxic or irritant effects of BGVC exposure because of metabolizing enzyme induction and enhanced detoxification capacity.

Table 3-14. Incidence and Severity of Respiratory Epithelial Degeneration
in the Nasal Passages from Rats Exposed to BGVC

	Control	Low Level	Mid Level	High Level
<u>Males, Turbinate Level 2</u>				
No. examined	50	50	50	50
No. with lesion (%)	2 (4)	3 (6)	7 (14)	11 (22) ^a
No. with minimal grade 1 (%)	2 (4)	3 (6)	4 (8)	10 (20)
No. with mild grade 2 (%)	0	0	1 (2)	1 (2)
No. with moderate grade 3 (%)	0	0	1 (2)	0
Average severity grade	0	0.1	0.2	0.2
<u>Males, Turbinate Level 3</u>				
No. examined	50	50	49	50
No. with lesion (%)	1 (2)	0	7 (14) ^b	2 (4)
No. with minimal grade 1 (%)	0	0	5 (10)	2 (4)
No. with mild grade 2 (%)	1 (2)	0	2 (4)	0
Average severity grade	0	--	0.2	0
<u>Females, Turbinate Level 2</u>				
No. examined	49	49	50	50
No. with lesion (%)	8 (16)	11 (22)	18 (36)	26 (52) ^{a,c,d}
No. with minimal grade 1 (%)	6 (12)	8 (16)	10 (20)	15 (30)
No. with mild grade 2 (%)	2 (4)	3 (6)	7 (14)	10 (20)
No. with moderate grade 3 (%)	0	0	1 (2)	1 (2)
Average severity grade	0.2	0.3	0.5	0.8
<u>Females, Turbinate Level 3</u>				
No. examined	49	49	50	50
No. with lesion (%)	2 (4)	0	8 (16) ^e	12 (24) ^{a,d}
No. with minimal grade 1 (%)	2 (4)	0	6 (12)	5 (10)
No. with mild grade 2 (%)	0	0	2 (4)	7 (14)
Average severity grade	0	--	0.2	0.4

^aSignificant trend for increased incidence with increasing exposure level ($p < 0.005$, Cochran-Armitage and logistics tests).

^bSignificantly different from the low-level group ($p = 0.006$, pair-wise Fisher's exact test).

^cOverall severity/incidence is significantly greater than control ($p < 0.05$, Kolmogorov-Smirnov test).

^dSignificantly different from the control and low-level groups ($p < 0.008$, Fisher's exact test).

^eSignificantly different from the low-level group ($p < 0.008$, Fisher's exact test).

Table 3-15. Incidence and Severity of Olfactory Epithelial Degeneration
in the Nasal Passages from Rats Exposed to BGVC

	Control	Low Level	Mid Level	High Level
<u>Males, Turbinate Level 3</u>				
No. examined	50	50	49	50
No. with lesion (%)	15 (30)	3 (6)	7 (14) ^a	4 (8) ^b
No. with minimal grade 1 (%)	14 (28)	1 (2)	6 (12)	4 (8)
No. with mild grade 2 (%)	1 (2)	2 (4)	1 (2)	0
Average severity grade	0.3	0.1	0.2	0.1
<u>Males, Turbinate Level 4</u>				
No. examined	50	50	49	50
No. with lesion (%)	18 (36)	10 (20)	6 (12)	1 (2) ^{b,c,d}
No. with minimal grade 1 (%)	17 (34)	10 (20)	2 (4)	1 (2)
No. with mild grade 2 (%)	1 (2)	0	4 (8)	0
Average severity grade	0.4	0.2	0.2	0
<u>Females, Turbinate Level 3</u>				
No. examined	49	49	50	50
No. with lesion (%)	15 (31)	13 (27)	11 (22)	13 (26)
No. with minimal grade 1 (%)	15 (31)	11 (23)	9 (18)	11 (22)
No. with mild grade 2 (%)	0	2 (4)	2 (4)	2 (4)
Average severity grade	0.3	0.3	0.3	0.3
<u>Females, Turbinate Level 4</u>				
No. examined	49	49	50	49
No. with lesion (%)	21 (43)	10 (20)	7 (14) ^e	2 (4) ^{b,c,e}
No. with minimal grade 1 (%)	20 (41)	10(20)	6 (12)	2 (4)
No. with mild grade 2 (%)	1 (2)	0	1 (2)	0
Average severity grade	0.4	0.2	0.2	0

^aSignificantly greater than the low-level group ($p < 0.008$, Fisher's exact test).

^bSignificant trend for decreased incidence with increasing exposure level ($p < 0.005$, Cochran-Armitage and logistics tests).

^cOverall severity/incidence is significantly less than control ($p < 0.05$, Kolmogorov-Smirnoff test).

^dSignificantly less than control and low-level groups ($p < 0.008$, Fisher's exact test).

^eSignificantly less than control ($p < 0.008$, Fisher's exact test).

CONCLUSIONS

The overall purpose of this study was to evaluate the long-term toxicity, including carcinogenicity of Baseline Gasoline Vapor Condensate. The results of this study provide a basis for comparing the results of a companion study in which the vapor condensate contained 20% by weight of the additive methyl tertiary-butyl ether, “211(b) Chronic Carcinogenicity Study Gasoline MTBE Vapor Condensate.”

Chronic BGVC inhalation suppressed body weight in males, and to a greater extent in females, increased the severity of chronic progressive nephropathy in males and caused epithelial degeneration in the nasal passages of both sexes. The degenerative nasal effects were most likely caused by effects of the test material.

Chronic exposure to BGVC did not enhance the development of proliferative lesions (hyperplastic lesions, neoplasms) in female rats. However, there was an increased incidence in mononuclear cell leukemia in low- and mid-level exposed females, most likely due to protocol-driven sampling bias in these groups. Consistent with previous studies and the concurrent GMVC study chronic exposure to BGVC did enhance the development of renal adenomas and carcinomas (combined) in male rats. In contrast with the concurrent GMVC study, BGVC may also have enhanced squamous cell carcinoma in the nasal passages in male rats, testicular mesothelioma. Also, in contrast to the GMVC study, there is some evidence that BGVC exposure caused thyroid follicular cell carcinoma in males.

Consequently, due primarily to treatment-related effects in the male kidney, and to possible effects on testes, and nose, and thyroid, chronic inhalation of Baseline Gasoline Vapor Condensate was determined to be carcinogenic in male rats in this study. Chronic inhalation of Baseline Gasoline Vapor Condensate was determined not to be carcinogenic in female rats in this study.

LOCATION OF SPECIMENS, RAW DATA, AND FINAL REPORT

Specimens were identified by test system, study, nature, and date of collection. All raw data and records and specimens that are required to reconstruct the study will be maintained in the LRRI archives for 10 years. The Sponsor will be notified and will authorize in writing the destruction or transfer of any specimens, raw data, and study records to the Sponsor or to an archive specified by the Sponsor's Contracting Representative.

PROTOCOL DEVIATIONS

Calculation of Nominal Concentration

Section IX.C.6. of the protocol states "Nominal concentration will be determined daily and will reflect the concentration generated for all three exposure systems combined. Therefore, the total mass of test substance generated will be divided by the total flow distributed to all three exposure chambers. The value will be compared to the sum of the mean concentrations achieved in the three chambers throughout that exposure day."

The actual and nominal (anticipated) usage were calculated as follows: The daily nominal usage was calculated by multiplying the average BGVC concentration in each chamber by the total flow through each chamber and then summing the values for all three chambers. This value was compared to the actual BGVC usage determined by taking the difference between the weight of the 20-pound cylinder before and after the exposure. There is no impact because the way nominal concentration was calculated and compared to the actual BGVC usage provides the same answer as the method described in the protocol.

Environmental Conditions

Throughout the BGVC exposure study, environmental conditions in the animal exposure chambers deviated periodically from protocol required ranges, or data were not acquired because of problems with the monitoring system. Most excursions or periods of data loss were of short duration. These deviations are judged to have no significant effect on the outcome of the study. Tables summarizing deviations from protocol-specified environmental conditions are included in the Study File.

Exhaust System Failure During Conditioning/Quarantine Period Affecting Chamber Pressure

On August 2, 2001, a subcontractor was at the Institute to repair a motor that had burned out on the backup exhaust system for the exposure wing. While here, he was asked to change the filters on the main system. To do this, he had to temporarily shut off the main blower. After the filters had been changed, the blower motor could not be restarted. This occurred about 12:30 p.m. The Study Director, Dr. Benson and LRRI Animal Care Operations were notified because the study animals were being quarantined in chambers in that wing. Since air was still being supplied to the chambers, the corks in the doors of the chamber were taken out to allow air flow. During this time the pressure in the chambers changed from negative (approximately -1 inch of water) to positive (about 1 inch of water). The maintenance crew attempted to restart the motor all afternoon. At 4:00 p.m., the exhaust system was still not working, so the exhaust lines were totally disconnected from the chambers housing the study animals. All environmental parameters continued to be monitored by computer as before the exhaust system went down. Surveillance personnel also checked the conditions in the room twice per shift and were directed to open the chamber doors in the event that temperatures exceeded 24°C. A new motor was delivered and installed early August 3, 2001. The exhaust system was up and running by 8:30 a.m.

The impact to the study was small, because animals had air, and were monitored by personnel throughout the time the exhaust system was down.

Temporary Loss of Environmental Monitoring System Operation

On May 20, 2003 a major power outage occurred at 12:20 a.m. Normally, in power outage events, emergency backup diesel generators are turned on within 10–15 seconds. During this particular outage, a breaker was tripped and emergency power was not provided to the environmental monitoring system and the system was on uninterrupted power supply battery power until they were drained. During this outage, the supply air fan stopped operating, but due to the lack of environmental monitoring capability, this was not detected until morning. All systems were restored by approximately 6:30 a.m. The animals appeared healthy. Estimated flow rates through the chamber were approximately 200–300 L/minute. While supply air was not available, the vacuum system was still operational and pulled air from the room into the

chambers. Several actions, described in a memo to the study file, were taken to prevent similar situations in the future.

On August 28, 2002 (14:29) to August 29, 2002 (06:29) no environmental data were acquired or saved to file. This was because on August 28, 2002, the LRRI network domain configuration modifications were performed on the monitoring PC in the study control room. The software program controlling and recording environmental data was shut down and inadvertently not restarted.

Light and Sound Measurements

According to the study protocol, light and sound measurements in the animal exposure room were to be measured quarterly. In 2001, measurements were not made for the July–September quarter. In 2002, no measurement was made between January and March. In 2003, no measurement was made between July and the end of the study in August. The impact on the outcome of the study is considered minimal because light and sound levels were similar among locations and did not vary greatly between measurements that were made.

Gas Chromatographic Analysis

The original version of LRRI SOP ASP-1166.0, “Calibration of the Shimadzu GC-17A/FID for Analyses of Gasoline Vapor Condensate,” indicated GC calibration for monitoring the exposure atmosphere and test article hydrocarbon composition profile be performed daily. However, calibrations were performed only on the days when analyses were conducted. The SOP was modified and signed by management September 25, 2002, changing the requirement for calibration to only days when analyses are performed. The impact of this SOP deviation is minimal because the GC was calibrated on days when analyses were performed.

No chamber composition sample was analyzed during the week of September 10, 2001 due to problems with the gas chromatograph. The impact of this deviation was judged to be minimal.

Cage Board Usage/Availability

In December 2002, we ran out of untreated cage board used to line the excreta pans in the H2000 chambers. Although additional cage board was ordered, it was on “back order” and was

estimated to arrive December 20, 2002. Until we received permission from the Sponsor to switch to treated (with neomycin) cage board, no cage board was used and pans were washed twice daily instead of the usual once per week. Dr. Benson contacted the Sponsor and received permission to use treated cage board on December 13, 2002. Use of untreated cage board continued when it arrived.

There should be no adverse effect on the study associated with not using cage board (with twice daily pan washes to keep ammonia levels down) or associated with use of neomycin-treated cage board. The latter type is used throughout the institute for short-term and long-term inhalation studies.

Collection of Organ Weights at Necropsy

The study protocol states that lung, liver, kidneys, testes, epididymis, ovaries, uterus, spleen, brain, and heart will be weighed at necropsy. It also states that these weights are not to be recorded on animals found dead. Organ weights were recorded, however, for the following rats found dead: J165, J179, J19, K259, K271, and K285. Organ weight summaries have been prepared to exclude natural death animals, so their weights do not skew the group means for the final sacrifice and euthanized animal reports. The lung weight was not obtained on one female, L380, at final sacrifice. The impact of these deviations are minimal.

Assignment of Severity Scores

LRRI SOP PAN-0455.7, "Rodent Necropsy Procedure," Section 6.2, states, "For animals that are entered into the Path/Tox computer system, the lesions are described and graded on a scale of 1 to 4...." There were several instances of severity codes not assigned to lesions that were masses or nodules (examples are K54, L390, L383, L363, I576, K265, K251, and L399). There is no impact on the quality or integrity of the study because other descriptors of the masses are provided.

Statistics

Proposed statistical methods were presented in the protocol. In several cases the actual evaluations differed from those originally proposed. This occurred in three specific cases. First,

the Hoel Walburg test was not used to analyze tumor data although originally listed as a possible statistic in the protocol. Second, hematology results were analyzed as a function of time as well as a function of dose. Finally, in the case of chronic progressive nephropathy, where the incidence of lesions was similar among control and dosed groups, the Kolmogorov-Smirnov test was used to assess differences in severity scores. This evaluation was done within the P module of the Path-Tox[®] software, and not by the Study Statistician.

The actual statistics used have been described in the Statistics section of the report. The fact that the statistics proposed in the protocol were not always those used for data analysis has no major impact on the quality of the study. In most cases the analyses were more thorough and appropriate than those originally proposed. Since BGVC inhalation did not affect animal survival, the Hoel Walburg analysis, that assumes that treatment affects animal survival, was not necessary. Since the hematology data did not have normal distributions, alternative statistical approaches were warranted. Since repeated measurements were made on the same rats over time, evaluation of the effect of time and exposure concentration on the hematology parameters was appropriate. The use of the Kolmogorov-Smirnov statistic in the Path-Tox[®] database was appropriate because nephropathy was an important endpoint in this study. Statistical analysis of the severity of the lesion as a function of exposure concentration in the absence of a concentration-response effect on lesion incidence provided more information than would have been gained by analysis of incidence alone.

Neat Test Article Characterization of 420-pound Tanks

Test article lot number API 99-01, tank BG22, was inadvertently not analyzed prior to use September 24 thru November 18, 2002. However, the tank was analyzed during use for the inhalation exposures and the composition was verified. For this reason, this deviation did not affect the validity of the study.

Personnel

On page 3 of the study protocol, key personnel was listed as Quint H. Powell, MS, as Inhalation Engineer. During the study, Mr. Powell left the Institute and was replaced by Mr. Edward B. Barr, MSEE, an inhalation engineer with over 25 years experience at Lovelace. Betty J. Skipper,

PhD, Professor, Department of Family and Community Medicine (MSC 095040, 1 University of New Mexico, Albuquerque, NM, 87131), was subcontracted to perform the statistics.

There was no impact on the quality of the study as the above listed personnel were replaced by individuals of equal or greater experience.

Tissue Collection

Nasal tissue and pituitary (protocol required tissues) were inadvertently not collected for animal I093 (female control). Tissue was not present at trim, so likely not collected at necropsy tissue harvest. This omission had little effect on the overall outcome of the study.

Hematology

Due to blood sampling difficulties at the 12 month sampling time, blood smears were not obtained for male animals I011 and L321 or for female animals I072, I074, L353, L355, L356, L358, L362, L371, L3673, and L378. At the 18 month time point, no blood smear was collected from males I011 and L321 or from females I072 and I074.

Loss of Supply Air to Exposure System

On November 27, 2001, at approximately 10:30 a.m., supply airflow to the animal chambers dropped, resulting in low exposure chamber flows and pressures. Surveillance personnel were summoned to the room. They monitored the room closely and made certain Facility Engineering and Exposure personnel were aware of the situation. It was determined that the flows and pressures were in a range that would not adversely affect the health of the animals and therefore the animals remained within the chambers during the night.

During this time, chamber pressures did not exceed -3" water (within normal range of LRRI SOP). Chamber flows did not drop below 350 lpm (10 changes per hour), above the minimum stated in EPA OPPTS 870.4200 guidelines, but below those specified in the study protocol. Chamber temperatures remained within protocol-defined limits.

On the following morning, November 28, 2001, the Study Engineer and Facility Engineering personnel examined the problem. Flows to the chamber were adjusted to within normal

operating parameters. Facility Engineering personnel examined the supply air blower and could not find anything wrong with the supply air system.

At this time, the Study Engineer determined that it was ok to begin exposures. Approximately 30 minutes into the exposure, Facility Engineering discovered that the chamber supply air blower was not working properly. Exposures were terminated after 40 minutes of exposure were completed so that repairs could be made. Exposure concentrations achieved during the 40-minute run were 1.53 g/m³, 7.88 g/m³ and 13.62 g/m³ for the 2, 10 and 20 g/m³ chambers, respectively. Flow through the chambers was maintained above 10 air changes per hour and chamber temperatures were within limits while the blower was being repaired. The environment in the exposure chamber was safe for animal occupancy at all times. Repairs to the blower were completed by 12:00 p.m. Exposures were not continued that day. One exposure day was missed but this was not considered significant because there were over 500 exposure days during the study.

Body Weights of Males Not Collected According to Schedule (two deviations)

The protocol states that body weights were to be performed weekly for the first 13 weeks and ten every 4 weeks thereafter. Observations were to be made weekly.

During February 2003, the weigh/observation schedule was modified. Sessions were moved to the weekends (February 9 and 16) from weekdays so that we could collect the 18 month blood samples from control and high-level rats as per study protocol. Therefore the time between sessions was altered from that prescribed in the protocol. The impact on the study was limited, as we did perform weigh/observations, but on different days than per the original schedule. The normal schedule was resumed once the blood collections were complete.

Male body weights were not collected on May 28, 2003 due to a scheduling error. Weights were collected on June 4, 2003. Therefore, the interval was 5 weeks instead of 4. The next scheduled day for weighing was June 25. This resulted in a smaller interval (3 233ks) between weigh sessions.

The impact was that we did not have data assessing the health status of the animals as scheduled. However, weighing occurred during the very next week, and the schedule was followed thereafter.

Quantitation of Exposure Atmosphere

On October 25, 2001 and November 16, 2001, one point checks of the Miran calibration curves were compared to the wrong calibrations curves. The low-level Mirans would not have passed the one-point checks if the current calibration curves had been used.

For both days, MIR 2 would not have passed the one-point check and should have been recalibrated. However, MIR 3 and 4 would have passed and therefore do not deviate from the standard system operation. This error was not noted until well after the exposures had been completed, therefore the MIR 2 was not recalibrated and the existing calibration curve (not updated) was used. The daily run sheets were corrected.

The impact of using the wrong standard curve was minimal because the extent of deviation from the acceptable range was only 3% from each curve (i.e., 0.082 instead for 0.085 for MIR 2 and 1.18 instead of 1.15 on October 25, and November 16, 2001, respectively).

LIST OF ABBREVIATIONS

API	American Petroleum Institute
BGVC	Baseline gasoline vapor condensate
CA	Cochran-Armitage test
CFR	Code of Federal Regulations
CRTC	Chevron Research and Technology Center
EDTA	Ethylenediamine tetra-acetic acid
EMBSI	ExxonMobil Biomedical Sciences, Inc.
FE	Fisher's exact test
FID	Flame ionization detector
GC	Gas chromatograph
GLP	Good Laboratory Practices
KRV/H-1	Kilharn rat virus/H-1 virus
LPM	Liters per minute
LRRI	Lovelace Respiratory Research Institute
MCL	Mononuclear cell leukemia
NBF	Neutral buffered formalin
NFPA	National Fire Protection Association
OPPTS	Office of Prevention, Pesticides, and Toxic Substances (U.S. EPA)
PVM	Pneumonia virus of mice
RBC	Red blood cell
RCV/SDAV	Rat corona virus/sialodacryoadenitis
SOP	Standard operating procedure
SD/Stdev	Standard deviation
SE	Standard error
T90	Time for chamber concentration to reach 90% of equilibrium value
WBC	White blood cell

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