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HAZLETON LABORATORIES AMERICA, INC.

9200 LEESBURG TURNPIKE VIENNA VIRGINIA 22180, U.S.A.

24-MONTH ONCOGENICITY STUDY OF METHYLENE CHLORIDE IN MICE

FINAL REPORT

VOLUME I

Submitted to
National Coffee Association
New York, New York

November 30, 1983

HAZLETON
LABORATORIES AMERICA, INC.

OFFICE OF QUALITY ASSURANCE

Project Title: 24-Month Oncogenicity Study in Mice

Project No.: 2112-106

Quality Assurance inspections of the study and review of the final report of the above referenced project were conducted according to the standard operating procedures of the Office of Quality Assurance and according to the general requirements of the Good Laboratory Practice regulations that were issued on December 22, 1978, by the Food and Drug Administration for compliance on and after June 20, 1979. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

Inspections/Review		Findings Reported	Inspector/Reviewer
Protocol	9/18,19/80	9/24/80	B. Dickinson
Study (1)	12/1-4/80	12/11/80	B. Dickinson
(2)	3/18-20/81	3/26/81	B. Dickinson, E. Prins
(3)	6/2,4,5/81	6/10/81	B. Dickinson
(4)	9/18-25/81	10/7/81	G. Congleton
(5)	12/8-11/81	12/16/81	K. Hogan
(6)	3/1,9-11/82	3/17/82	B. Dickinson
(7)	6/23,24/82	7/1/82	B. Dickinson, S. Hurwitch
(8)	9/30;10/1,4/82	10/7/82	M. Woodmansee
Final Report	6/3,6-10, 13-15/83;	6/20/83	P. Runge
	8/10-12/83; 9/21-24/83	9/28/83	P. Runge

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HAZLETON LABORATORIES AMERICA, INC.

SUBJECT: 24-Month Oncogenicity Study in Mice
Project No. 2112-106

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

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HAZLETON LABORATORIES AMERICA, INC.
9200 LEESBURG TURNPIKE, VIENNA, VIRGINIA 22180, U.S.A.

SPONSOR: National Coffee Association

DATE: November 30, 1983

MATERIAL: Methylene Chloride

SUBJECT: FINAL REPORT
24-Month Oncogenicity Study in Mice
Project No. 12-106

SUMMARY

This report presents the findings of a chronic study designed to evaluate the oncogenic potential of methylene chloride when administered in deionized drinking water to $B_6C_3F_1$ mice.

Four groups of mice (unbalanced group size) received the test material at levels of 60, 125, 185, and 250 mg/kg/day (Groups 3, 4, 5, and 6, respectively). Two groups of mice (Groups 1 and 2) received deionized drinking water only and served as control groups.

Indices monitored for compound effect were survival, body weight changes, water consumption, clinical signs (including palpable tissue masses), total and differential leukocyte counts, and gross and microscopic pathology.

Histomorphologic alterations of the liver were observed in the high-dose males and females and consisted of a marginal increase in the amount of Oil Red O positive material. These findings were considered to be treatment related.

Proliferative hepatocellular lesions were observed in all groups of both sexes including untreated controls. The incidence of these

lesions in treated female groups was comparable to control groups. The incidence of these lesions in treated male groups was slightly higher than control values. This increase, however, was not dose related or statistically significant when compared to concurrent control rates. Furthermore, the incidence of these lesions found in the treated male groups was well within both the historical range of control values compiled at Hazleton Laboratories America and reported in the literature.

No treatment-related effects were noted with regard to any other parameters examined.

Based upon the results of this study, methylene chloride did not induce a carcinogenic response. A "no-effect" level of toxicologic and non-neoplastic histopathologic effects was observed at a dose level of 185 mg/kg/day.

INTRODUCTION

This report presents the findings from a twenty-four-month study designed to evaluate the oncogenic potential of methylene chloride when administered in drinking water to $B_6C_3F_1$ mice. This route was chosen because it most closely mimics human exposure. For work schedule convenience, compound administration began on different dates for each sex with males starting on September 23, and females starting on September 25, 1980. Terminal sacrifice of the males began on September 22, 1982, and was completed on October 6, 1982. Terminal sacrifice of the females began on September 24, 1982, and was completed on October 4, 1982.

CONTROL AND TEST MATERIAL

Municipal tap water deionized to a resistance of 18 megohms/cm^3 (Hydro Service Water Purification System, Rockville, Maryland) served as the vehicle control. The test material, methylene chloride (Lot #8122), a clear liquid, was received in a fifty-five gallon drum[a] on September 22, 1980, from Diamond Shamrock Industries. The total bulk liquid was transferred to one-gallon amber bottles and stored in a refrigerator until use. For dosage calculation purposes, the test sample was considered to be 100% methylene chloride. Samples of the test article (approximately

[a] A preliminary 1 quart sample of the test material was hand-delivered to HLA on Sunday, September 21, 1980, so the study could start on schedule. This small sample was also from Lot #8122. This sample was not used.

100 ml each) taken at six month intervals were forwarded to the sponsor for purity assay. Results are presented in Appendix 12.

TEST ANIMALS

One thousand one hundred thirty-five (731 male and 404 female) $B_6C_3F_1$ mice were received from Charles River Breeding Laboratories, Portage, Michigan, on September 3, 1980. Mice in this shipment were born between July 29, and August 4, 1980, inclusive. Upon receipt and until initiation of study, the animals were held in quarantine. During this time, the animals were examined for general health by a staff veterinarian to assess their fitness to be placed on study. Initial body weights ranged from 15.4 to 26.2 grams for males, and from 15.5 to 22.2 grams for females.

Mice were used in this study because of the historical precedence of using this species in safety evaluation and cancer bioassay studies. The $B_6C_3F_1$ mouse was used because it has been found to be sensitive to the induction of cancer by halogenated compounds.

METHODS

Assignment to Groups

One thousand mice (650 males and 350 females) found to be clinically acceptable were assigned to groups and treatments listed on the following page using a computerized randomization process. This process

involved generating random numbers, assigning them to all available animals, ranking the numbers and assigning the ranked numbers and corresponding animals to treatment groups.

Group Number	No. of Animals		Target Dose
	Males	Females	mg/kg/day
1 (Control)	60	50	0
2 (Control)	65	50	0
3 (Low-Dose)	200	100	60
4 (Mid-Dose 1)	100	50	125
5 (Mid-Dose 2)	100	50	185
6 (High-Dose)	125	50	250

Following randomization, each animal was housed individually and identified by a unique animal number on the animal cage. Animals not used on study were sacrificed and discarded without necropsy.

Animal Husbandry

The mice were individually housed in stainless-steel hanging wire-mesh cages on racks. The cages were color coded by group. The drinking water (from water bottles with stainless-steel ballpoint sipper tubes) containing the appropriate concentration of methylene chloride, and the basal diet of Purina Rodent Laboratory Chow® #5001 (Ralston Purina, St. Louis, Missouri) were available ad libitum. The study was conducted in a laminar airflow room design (Bioclean®, Hazleton Systems, Inc., Aberdeen, Maryland).

The temperature and humidity were monitored and recorded at least twice daily. The mean temperature and relative humidity during the stud were 70.1°F (S.D. 1.93) and 54.6% (S.D. 4.89), respectively. A twelve-hour light/dark cycle was maintained. The Bioclean® filtration system was checked periodically and verified to be functioning properly. Room air was sampled for methylene chloride once during the study (Week 70) by the sponsor. Results are presented in Appendix 13.

A vertical numbering arrangement of cages was used to minimize possible environmental influences arising from spatial arrangement. A diagram of the arrangement is presented in Appendix 8. The location of the racks was systematically rotated in the room every two weeks.

Diet Analyses

Each batch of diet was analyzed on a prospective basis to ascertain that the following specifications were met:

	Minimum	Maximum
Lead		1.2 ppm
Arsenic		1.0 ppm
Cadmium		0.4 ppm
Mercury		1.0 ppm
Selenium	0.05 ppm	0.6 ppm
Total Aflatoxin		.02 ppm

A pesticide screen was also performed. Feed batches which did not meet the specification limits were excluded from use on the study.[a] No feed older than six months was used. Results of these analyses are presented in Appendix 7. The dates for which different feed lots were used were recorded.

Water Supply and Analysis

Deionized water was prepared on site using a Hydro Service Purification System (Rockville, Maryland). A detailed description of the system is given below. Analysis of the municipal water is on file with the Department of Veterinary Medicine of Hazleton Laboratories America, Vienna, Virginia.

The system was a model C2-44 (which used four separate tanks). It was set up with a .5 to 1 micron nominal pore filter for particulates. This filter was followed by a 44B tank which contained pharmaceutical grade activated carbon. The activated carbon was of a size to give maximum surface area for the most efficient organic adsorption. The tank was designed so that incoming water must pass through the entire tank before exiting, thus maintaining maximum contact time to the carbon.

The next two tanks were mixed bed resin tanks. Hydro Service uses Dow-type 1, nuclear grade resin which, when new, was subjected to seven cycles of chemical regeneration and exhaustion. A propietary regeneration process maintained strict quality control of resin preparation to assure maximum performance. During resin regeneration, special attention was given to removal of organic contamination according to ASTM recommendations.

[a] Batches #17 and #18 with lead levels of 1.35 and 1.40 ppm, respectively, were used because an alternative feed supply was not available.

A light between the two deionizer tanks was a service indicator light. When the light was out, Hydro Service was notified. Built-in precautions, however, were taken to insure the final product water quality would not change at that time. These precautions consisted of a polisher tank which continued to deionize the water and the 44B organic absorbent tank which had a normal life much longer than the deionizer.

The final water quality was 18 megohm/cm^3 specific resistance with neutral pH, and was ammonia, chlorine and flourescence free.

Water from the deionizer unit was sampled at least every four weeks[a] and each time the cartridges to the unit were changed. Samples were subjected to bacterial analysis using 5% sheep blood agar, FC broth, MacConkey agar and plate count media. All enteric organisms were identified as to species. All non-fermenting gram-negative rods were identified as to genus. Results of these assays are presented in Appendix 6.

Water samples (approximately 30 ml each) were retained from the new cartridge after installation, and from the old cartridge prior to replacement. These samples were stored under refrigeration.

Compound Preparation and Administration

The test material was added to deionized water on a volume/volume basis. An appropriate amount of methylene chloride was added to a partially prefilled glass carboy and diluted to yield a 1% (10,000 ppm) stock solution. A sample was analyzed to determine if the mixture was within 5% of the desired 1% concentration. If the stock was not within

[a] At several times during the study, microbiological analysis of water was performed at greater than four-week intervals due to scheduling problems.

the 5% range, it was corrected to produce the desired concentration. The corrected stock was analyzed retrospectively.

Volumes of the 1% stock were taken and added to partially pre-filled glass carboys and diluted to yield the desired concentrations of methylene chloride[a]. For the first week, dosage formulation was based on mean body weights recorded four days prior to initiation for males, and three days prior to initiation for females, and water consumption values of 45.4 ml[b]/week (males) and 39.8 ml[b]/week (females). During the first thirteen weeks, dosage formulations were adjusted to the most recently recorded body weight and water consumption data; dosage formulations were altered every four weeks thereafter. The preparation of the stock solutions and dilutions, and the filling of the water bottles occurred one day prior to presentation.[c] Fresh solutions were made and presented twice per week for animals of each sex.[d] No reserve samples of the dosing solutions were retained due to the volatility of the test material in the delivery system.

[a] Based on data from a pilot study (Hazleton Project No. 2112-100) to determine the stability and homogeneity of methylene chloride in the drinking water, it was determined that there was a consistent loss of methylene chloride of approximately 15% from the time of mixing until the water was presented to the animals. For that reason, dose solutions were formulated 15% above the target.

[b] These values were derived from previous studies with $B_6C_3F_1$ mice at this laboratory.

[c] Preparation of the stock solution was rescheduled by a day or two when preparation would ordinarily have fallen on a national holiday.

[d] The week was split into a 3-day followed by a 4-day period for the males, and into a 4-day followed by a 3-day period for the females.

Analysis of Dosing Solutions

Samples of dosing solutions (each sample consisting of three water bottles) were taken and analyzed after the four-day period, each week, at Weeks 1 through 13, and also at Weeks 16, 18, 22, 26, 30, 34, 38, 40, 42, 46, 51, 54, 58, 62, 66, 70, 74, 78, 82, 86, 90, 94, 98, and 102. Representative samples were chosen arbitrarily from bottles which were on the animal cages for that period. The analytical method for determining methylene chloride is presented in Appendix 5.

Observations and Records

All animals were observed twice daily for mortality and signs of moribundity. The second observation on Saturday was made as late in the day as possible. After Week 52, a third observation (during late evening) from Sunday through Thursday, was added.[a] Body weight measurements, clinical observations and digital palpation were performed weekly. Water consumption was measured twice weekly (after three- and four-day periods) and the values combined to yield a seven-day total consumption. Estimates of compound consumption were calculated weekly through Week 13 and thereafter at the same time that four-day samples of the dosing solutions were assayed. Average water consumptions (with standard deviations) were calculated at the same intervals as assays of the four-day samples with the exception of Week 40 when compound consumption was not calculated.

[a] Due to scheduling problems, the third daily observation was omitted during the periods from 10/1/81 to 10/22/81, 3/23/82 to 3/30/82, and 4/19/82 to 5/9/82.

Clinical Pathology

Blood samples were collected by orbital sinus puncture from ten mice[a]/sex/group after fifty-two weeks of treatment and at termination for leukocyte counts and leukocyte differentials. The animals were food-fasted overnight prior to bleeding. References for hematology methods are presented in Appendix 14.

Sacrifice and Necropsy

Animals found dead and those in a moribund condition were necropsied as soon as possible following death or sacrifice. When an animal could not be necropsied within thirty minutes, it was refrigerated. If the necropsy could not be performed within one hour, the abdominal, thoracic, and cranial[b] cavities were opened and the carcass placed in cold 10% neutral buffered formalin (15 volumes of formalin/volume of tissue) and refrigerated.

After 104 weeks of treatment, surviving animals were sacrificed by exsanguination under sodium pentobarbital anesthesia. The schedule presented on the following page was followed for order of sacrifice.

[a] Those with the highest animal numbers.

[b] If an animal was found dead by the technician making the evening observation, the cranial cavity was not opened.

Date	No. of Mice/Group	Sex	Group
09/22/82	9	Male	2, 3, 4, 5, 6, 1
09/23/82	10	Male	1, 6, 5, 4, 3, 2
09/24/82	6	Female	2, 3, 4, 5, 6, 1
09/24/82	6[a]	Male	1, 6
09/24/82	3[a]	Female	1, 2
09/24/82	6[a]	Female	6
09/27/82	10	Male	2, 3, 4, 5, 6, 1
09/28/82	11	Female	1, 6, 5, 4, 3, 2
09/29/82	13	Male	1, 6, 5, 4, 3, 2
09/30/82	14	Female	2, 3, 4, 5, 6, 1
10/01/82	5	Male	1
10/01/82	7	Male	2
10/01/82	22	Male	3, 4, 5, 6
10/04/82	5	Female	2
10/04/82	40	Female	3
10/04/82	10	Female	4
10/04/82	7	Female	5
10/04/82	8	Female	6
10/04/82	7	Male	6, 5, 4, 3
10/05/82	40	Male	3
10/05/82	9	Male	4
10/05/82	10	Male	5
10/05/82	24	Male	6
10/06/82	48	Male	3

[a] These animals were sacrificed on the same day to obtain liver and kidney sections for genetic typing as described under RESULTS: Exceptional Clinical Findings - Convulsions, page 20 of this report.

Complete necropsies were performed on all animals and findings recorded. A pathologist was present during the terminal sacrifice and necropsy. Photographs of tissue masses and/or unusual lesions were taken in situ at the discretion of the pathologist.

Tissue Preservation and Histopathology

Tissues from all animals found dead or sacrificed in a moribund condition during the study and animals sacrificed at termination were

preserved in 10% neutral buffered formalin. Tissues stored for more than twenty-four hours before histological preparation were changed to fresh formalin after twenty-four hours. The following tissues from all Group 1, 2 (control), and 6 (high-dose) animals were examined microscopically after embedding in Paraplast®, sectioning, and staining with hematoxylin and eosin:

Urinary bladder
Prostate
Skin (dorsal lumbar midline)
Mesenteric lymph nodes
Mammary gland
Salivary gland
Stomach
Small intestine (duodenum, jejunum, and ileum)
Colon
Cecum
Rectum
Thyroid with parathyroids
Esophagus
Costochondral junction
Larynx
Lung
Pituitary
Nerve muscle (right thigh)
Sciatic nerve
Ovaries
Uterus
Brain (three sections)
Liver
Spleen
Kidneys
Adrenals
Sternum (with marrow)
Thymus
Trachea
Seminal vesicles
Tongue
Zymbal's glands
Gallbladder
Femur (with joint)
Spinal cord (three levels)
Testes
Eyes
Heart
Dorsal aorta
Pancreas
Gross lesions (including tissue masses and associated regional lymph nodes)

In order to maximize the numbers of parathyroids available for microscopic examination, the thyroid was cut at cranial and caudal poles providing a section containing both lobes of thyroid attached to the trachea, embedded caudal pole down in paraffin, and two slides were taken 50 or 60 microns apart. Every attempt was made to obtain slides of thymus and male mammary

tissue. The thymus, however, is generally not obtainable in geriatric mice due to atrophy, and male mammary tissue is generally difficult to obtain in mice of any age.

Livers, tissue masses, eyes (males only), and suspected neoplasms were examined from animals in Groups 3, 4, and 5. Additional sections of liver from all mice were frozen and stained with Oil Red O stain and examined.

Statistical Analyses

The following data from control and treated groups of the same sex were compared statistically: body weight changes (from initiation through Weeks 6, 13, 26, 52, 78, and 104); mean weekly water consumption (weekly average over the period from Week 1 through Weeks 6, 13, 26, 52, 78, and 104); and leukocyte counts (Week 52 and Week 104). Statistical analyses consisted of a preliminary test for variance homogeneity (Bartlett, 1937) followed by one-way classification analysis of variance (ANOVA; Snedecor and Cochran, 1967). If variances were homogeneous, Fisher's least significant difference (LSD) test (Snedecor and Cochran, 1967) was used to compare the control vs. treatment group means. If the variances proved to be heterogeneous, Wilcoxon's two-sample non-parametric rank-sum test (Snedecor and Cochran, 1967) was used for control vs. treatment group mean comparisons. Simple linear regression analysis and lack of fit test (Draper and Smith, 1966) were performed upon all data against control Groups 1 and 2. separately, if group comparisons showed

Groups 1 and 2 to be significantly different. If Groups 1 and 2 were comparable, linear regression analysis and lack of fit tests were also performed using combined data from Groups 1 and 2. Cumulative survival data (through Week 104) were analyzed by the National Cancer Institute Package (Thomas, Breslow and Gart, 1977).

The following tumor data from control and treated groups were compared statistically: numbers of mice with hepatocellular adenomas, numbers of mice with hepatocellular carcinomas, and numbers of mice with hepatocellular adenomas and/or carcinomas. The analysis consisted of computing the Kaplan-Meier (Kaplan and Meier, 1958) estimated expected numbers at risk in each case and then performing the Cochran-Armitage test (Cochran, 1954; Armitage, 1955) for linear trend as well as asymptotic normal test for control vs. treatment group comparisons. Because the two-sample normal test was used for multiple pairwise comparisons, a Bonferroni correction (Miller, 1980) was made to keep the overall error rate equal to the pre-assigned significance level.[a] All tumor incidences in treatment groups were evaluated against incidence in the individual control groups and incidence in the control group combined so that normal biological variation could be identified.

[a] The Bonferroni correction consists of dividing the significance level by the number of group comparisons and using this result as the significance level for group comparisons. In this case, the overall significance level = .05; therefore, each comparison was evaluated at the .0125 (one-tailed) level.

All analyses were conducted at the 5% significance level. Group comparisons were made at the 5% (two-tailed) significance level except in tumor data, which was evaluated as noted in footnote a. Statistically significant differences, as indicated from the aforementioned tests, are designated in the tables and appendices of this report as listed below.

S+ = Significantly higher than Group 1 control
s+ = Significantly higher than Group 2 control
S- = Significantly lower than Group 1 control
s- = Significantly lower than Group 2 control

The term "significant" used in this report is to mean a statistically significant finding.

Statistical references are presented in Appendix 14.

Specimen, Raw Data, and Final Report Storage

All specimens, raw data, and the final report are stored in the archives of Hazleton Laboratories Ame , Inc.

RESULTS

Mortality

Survival data are presented in Table 1. Graphs of survival data are presented in Figure 1.

Adjusted[a] male survival at study termination (Week 104) was 88.3% for Group 1, 76.6% for Group 2, 81.5% for Group 3, 81.0% for Group 4, 82.8% for Group 5, and 81.5% for Group 6. Statistical analysis revealed no significant linear trend or differences between groups. Adjusted[a] female survival at study termination was 69.4% for Group 1, 78.0% for Group 2, 73.0% for Group 3, 84.0% for Group 4, 76.0% for Group 5, and 91.8% for Group 6. Statistical analysis revealed significant negative linear trend in mortality. Group comparisons showed survival for Group 4 and Group 6 was significantly higher than Group 1. Group 6 survival was also significantly higher than Group 2.

Clinical Signs

Alopecia was the most frequently noted clinical sign throughout the study. Both treated and control groups were affected and no relationship to treatment was evident. Other findings noted much less frequently than alopecia were hunched and/or thin appearance; urine stains; rough haircoats; squinted, lacrimating, or opaque eyes; and swellings or sores affecting various body parts. These findings were noted for both treated

[a] Survival values were adjusted to compensate for animals that died as a result of an accidental death or that were sacrificed at an interim necropsy, i.e., these animals were subtracted from the sample size as well as the number of surviving animals.

FIGURE 1 – ADJUSTED SURVIVAL

MALES 2112-106

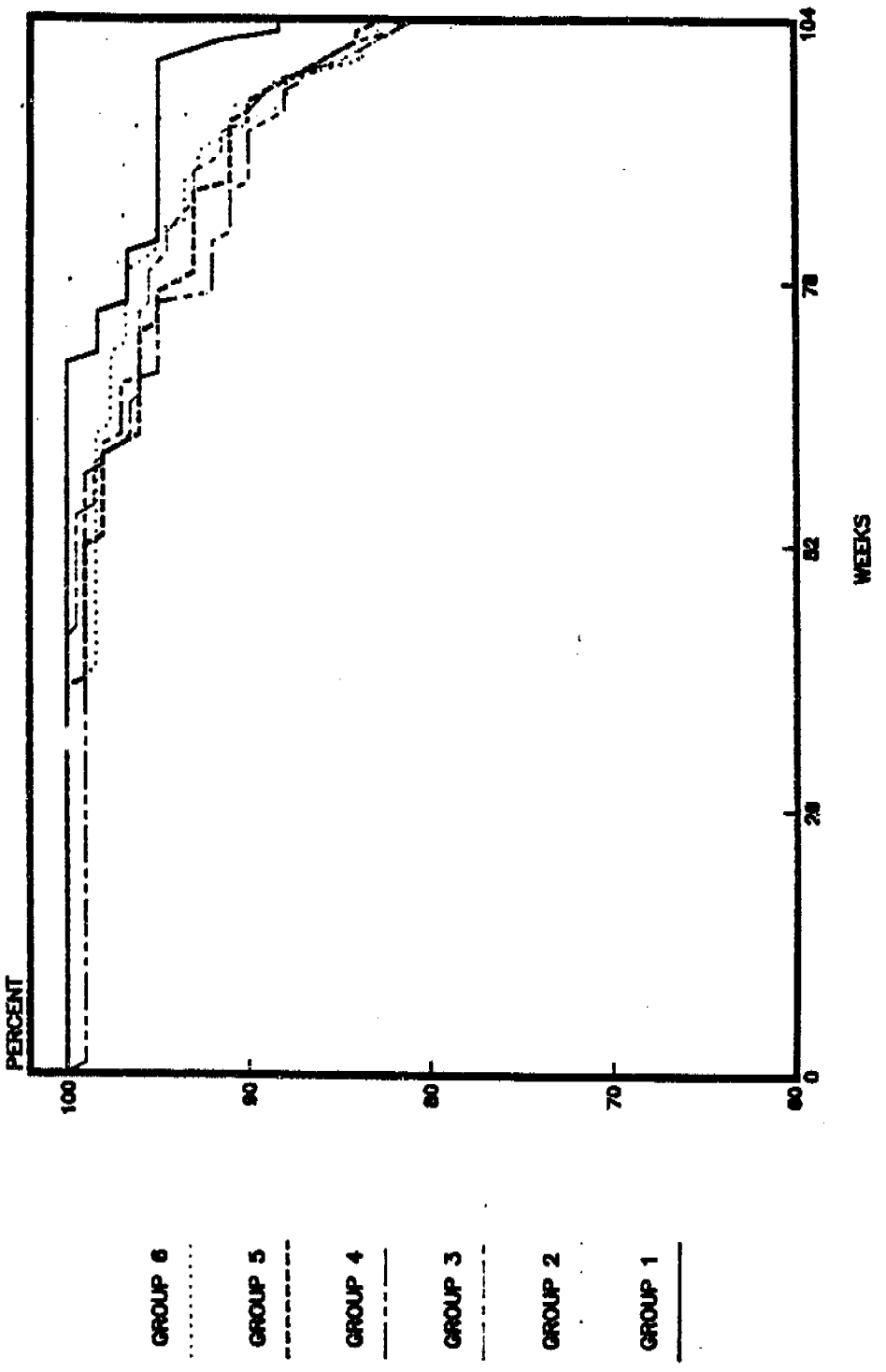
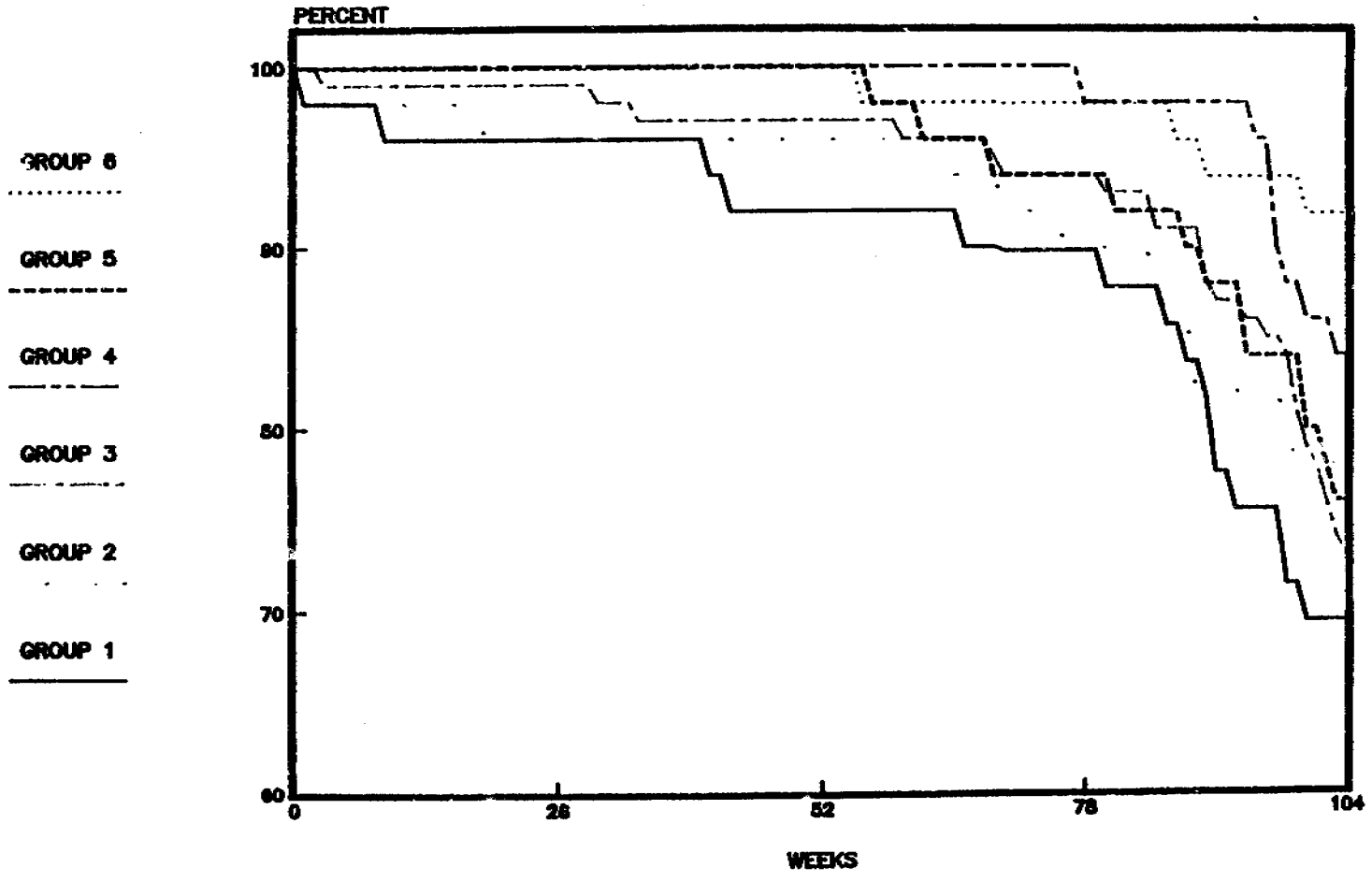


FIGURE 1 – ADJUSTED SURVIVAL
FEMALES 2112–106



and control groups and frequencies showed no apparent dose-response relationship.

Exceptional Clinical Findings - Convulsions

Weekly incidence of convulsions by individual animal number is presented in Appendix 3. Weekly incidence by group is presented numerically in Table 2A, and graphically in Figure 2. Group incidence and frequency summary data are presented in Table 2B.

At Week 37, a convulsion was noted in a Group 6 female, and at Week 39 a similar observation was noted for a Group 3 male. This behavior was characterized by 1) quaking, 2) extension of the head, and 3) lowering of the pinnae of the ears (Peyer reaction). The behavior was either limited to these signs or progressed to full seizure including opisthotonos and prostration. By Week 52 for males and Week 54 for females, convulsions were noted in at least one animal from all groups.[a] A review of the individual data indicated that the number of animals "affected" (i.e., animals which had demonstrated convulsions at least once during the study) increased with time, but this increase did not correlate with any increased mortality rate.[b]

The tabulation of the weekly incidence of convulsions indicated a high degree of variation from week to week in the proportion of animals

[a] Convulsions were only noted during body weight procedures when the animals were placed on the weighing pan.

[b] On December 9, 1981 (Week 64), animals exhibiting representative convulsions were filmed.

demonstrating convulsions in any particular group (see Figure 2 and Table 2A). The proportion of animals surviving to study termination (Week 104) demonstrating convulsions at least once was approximately equal in all dosage groups including control as was the mean number of times convulsions were noted in convulsing mice. These values are presented below.

Males						Females					
1	2	3	4	5	6	1	2	3	4	5	6
Percent with Convulsions at Least Once											
86.8	89.8	80.4	74.1	76.8	77.2	100.0	89.7	90.4	88.1	94.7	91.1
Mean Frequency of Occurrence											
5.4	6.3	4.1	4.3	5.2	5.0	8.6	7.6	8.0	8.0	7.1	8.3

Time adjusted analysis of incidence data (trend and homogeneity analysis of proportions, Thomas, Breslow, and Gart, 1979) revealed no linear trend in data from either sex, but group comparisons showed significantly lower incidence in Groups 3 and 4 male mice when compared to both control groups either separately or combined.

Several diagnostic procedures (hematology and virology studies, and histopathological examination) were implemented in an attempt to explain the convulsions. Two affected animals, Group 5 female No. 93169 and Group 6 female No. 93363, were sacrificed in moribund condition at Weeks 62 and 56, respectively. In addition to all tissues processed for histopathological examination in accordance with the protocol, serial sections of the brain were prepared, stained with hematoxylin and eosin,

and examined by a pathologist. These data are presented in Appendix 9A. No probable cause for the convulsions was found. At Week 70, four animals (Group 2 male No. 92545, Group 6 male No. 93290, Group 1 female No. 92455, and Group 6 female No. 93352, selected based upon the highest frequency of reported convulsions at that time) were sacrificed and necropsied. Blood samples were obtained for hematology and virology profiles and all tissues preserved after necropsy.

Hematology: Hematocrit; hemoglobin; red blood cell, white blood cell, platelet and reticulocyte counts; and differential white blood cell counts were determined. These data are presented in Appendix 1C. The results did not contribute to an explanation of the convulsions.

Virology: Viral antibody titres were determined for PVM, Reo 3, GDV II, K, Sendai, MVM, MHV, and LCM. These data appear in Appendix 2. The results were negative.

Histopathology: Sections of the brain (stained with hematoxylin and eosin) were subjected to histopathological examination. These data are presented in Appendix 10A. The histopathological examination did not explain the cause of the convulsions.

At the sponsor's request, additional diagnostic procedures were implemented to evaluate mice in the study colony for genetic purity. The evaluation was made by Dr. H. Hoffman, of Animal Genetic Systems, Wheaton, Maryland. The procedures involved taking blood samples (during Week 103) and frozen liver and kidney sections (at study termination) from the twenty-four mice presented on the following page.

1) mice with the highest frequency of convulsions;

Group 1 Males	Group 1 Females
92387*	92432*
92393*	92454*
92418*	92460*
Group 6 Males	**Group 6 Females**
93225	93351
93238	93358
93300	93360

2) mice which had never convulsed;

Group 1 Males	Group 2 Females
92372*	92547*
92374*	92549*
92375*	92551*
Group 6 Males	**Group 6 Females**
93196*	93321
93198*	93322
93199*	93324

Bloods: Samples of 0.3 ml were obtained from nonfasted mice by orbital sinus puncture technique. Acid citrate dextrose (ACD), supplied by Dr. Hoffman, was used as the anticoagulant. The samples, labeled only 1 through 24 with no other identification, were placed in a cooler over wet ice and hand delivered to Dr. Hoffman in Wheaton, Maryland.[a]

[a] All animals listed above were bled on August 27, 1982, using EDTA as the anticoagulant (as approved by Dr. Hoffman). This anticoagulant, however, proved to be unsatisfactory to the assay, necessitating a rebleeding. Those fifteen animals whose numbers are followed by * were bled again on September 13, 1982, and the samples were handled as described in the text.

Liver and kidney sections: An approximate 1 cm transverse section from the left lateral lobe of the liver, and one-half of each kidney (right cut cross-sectionally, and left cut longitudinally) were taken from each of the aforementioned mice. Liver and kidney samples were placed in Petri dishes (one for each mouse) labeled only 1 through 24 with no other identification. The Petri dishes were immediately frozen over dry ice, and delivered to Dr. Hoffman. A key to the sample numbers for bloods and tissues is included in the raw data for this study. A copy of Dr. Hoffman's report is presented in Appendix 11. Genetic typing confirmed that these animals were, in fact, $B_6C_3F_1$ mice and, accordingly, failed to reveal any probable cause for the convulsions.

Tissue Masses

A summary of tissue mass incidence is presented in Table 3.

Weekly tissue mass incidence was below 10% for males throughout the study, except for Week 74 when Group 1 incidence was 6/59 or 10.17%. At termination, incidence was less than 3% for all male groups. Tissue mass incidence for females remained low throughout the study.

Body Weights

Group mean body weights are presented in Table 1. Mean body weight data are presented graphically in Figure 3. A summary of statistical results is presented beginning on page 27.

INCIDENCE OF CONVULSIONS

MALES 2112-106

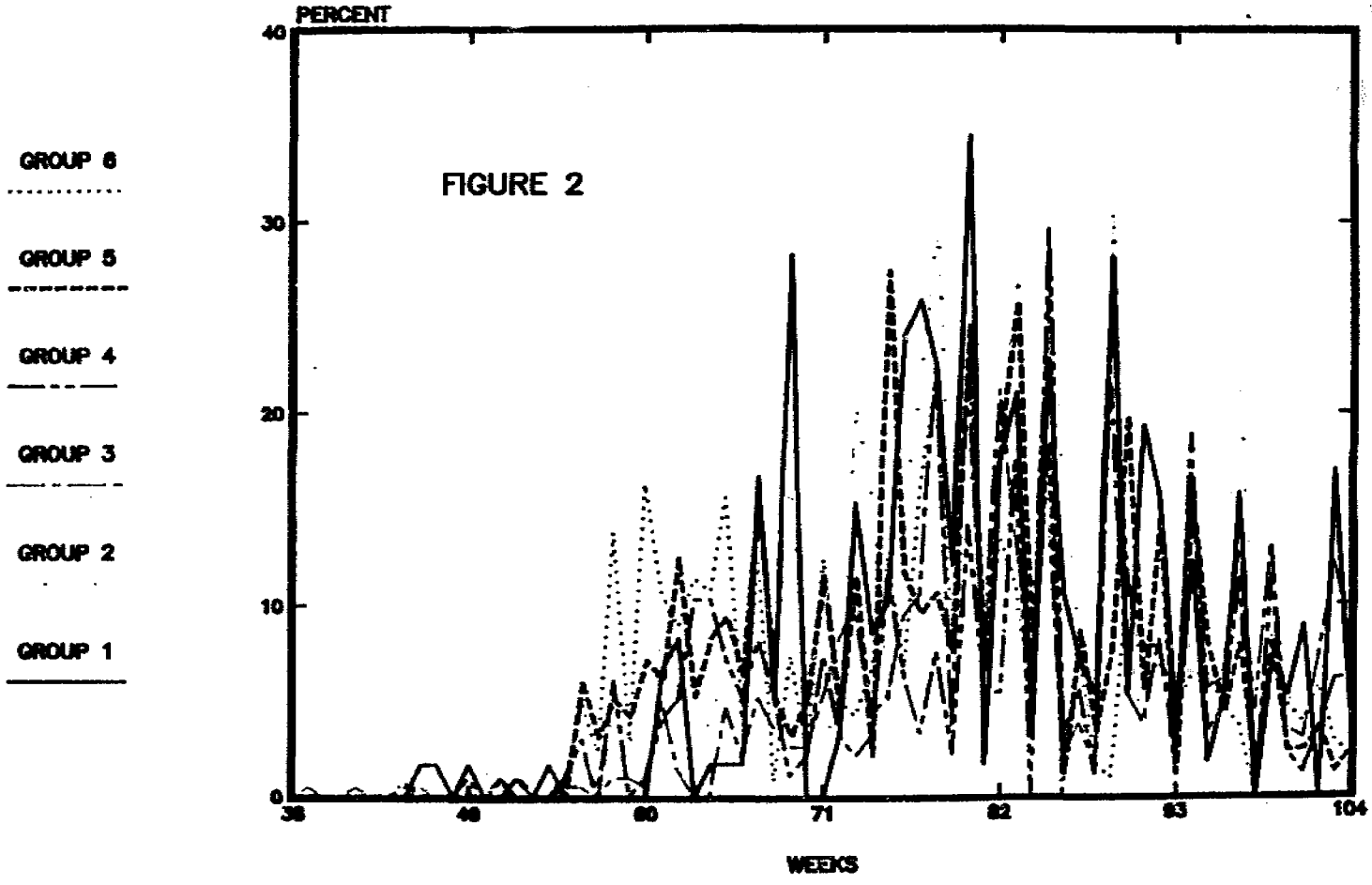


FIGURE 2

INCIDENCE OF CONVULSIONS

FEMALES 2112–106

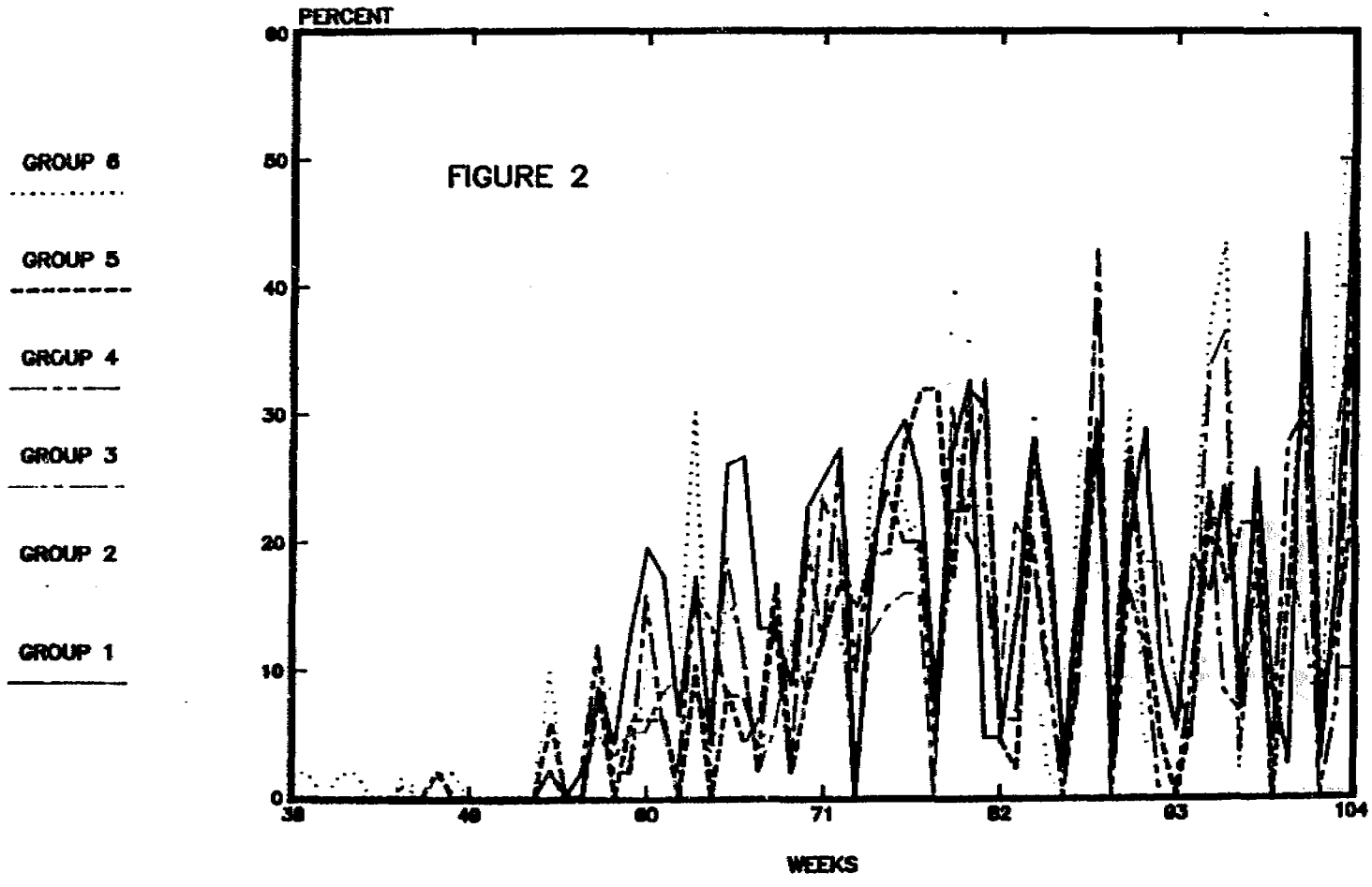


FIGURE 2

Summary of Group Comparisons

Males					Females				
2	3	4	5	6	2	3	4	5	6
Start Through Week 6									
S-,s-			s+		S-			s+	
Start Through Week 13									
		s+	s+	s+		S+,s+	s+	S+,s+	
Start Through Week 26									
		S+,s+					S-		
Start Through Week 52									
No significant differences between treated and control groups.									
Start Through Week 78									
		s+				s+		S-	
Start Through Week 104									
		s+			S+	S+			

Summary of Linear Regression and Lack of Fit Analyses

Groups Included in Analyses	Males	Females
Start Through Week 6		
1, 3-6	Significant regression (p = .000) and lack of fit (p = .017) Slope = 0.0018 SE = 0.0005	Slope = -0.0003 SE = 0.0006

Summary of Linear Regression and Lack of Fit Analyses - Continued

Groups Included in Analyses	Males	Females
	Start Through Week 6 (Continued)	
2, 3-6	Significant regression (p = .001) and lack of fit (p = .010) Slope = 0.0017 SE = 0.0005	Slope = 0.0008 SE = 0.0006
1 & 2 Combined, 3-6	Significant regression (p = .001) and lack of fit (p = .004) Slope = 0.0014 SE = 0.0004	Inappropriate because of significant difference between control groups
	Start Through Week 13	
1, 3-6	Significant regression (p = .000) Slope = 0.0018 SE = 0.0005	Significant lack of fit (p = .046) Slope = -0.0001 SE = 0.0008
2, 3-6	Significant regression (p = .000) Slope = 0.0023 SE = 0.0005	Significant lack of fit (p = .036) Slope = 0.00005 SE = 0.0008
1 & 2 Combined, 3-6	Significant regression (p = .000) Slope = 0.0021 SE = 0.0005	Significant lack of fit (p = .010) Slope = 0.0006 SE = 0.0007
	Start Through Week 26	
1, 3-6	Significant lack of fit (p = .004) Slope = 0.0008 SE = 0.0006	Significant lack of fit (p = .020) Slope = -0.0005 SE = 0.0009

Summary of Linear Regression and Lack of Fit Analyses - Continued

Groups Included in Analyses	Males	Females
	Start Through Week 26 (Continued)	
2, 3-6	Significant lack of fit (p = .014) Slope = 0.0003 SE = 0.0006	Significant lack of fit (p = .030) Slope = 0.0002 SE = 0.0009
1 & 2 Combined, 3-6	Significant lack of fit (p = .007) Slope = 0.0008 SE = 0.0006	Significant lack of fit (p = .023) Slope = -0.0003 SE = 0.0008
	Start Through Week 52	
1, 3-6	Slope = -0.0010 SE = 0.0008	Slope = -0.0008 SE = 0.0013
2, 3-6	Slope = -0.0005 Se = 0.0008	Slope = -0.0006 SE = 0.0013
1 & 2 Combined, 3-6	Slope = -0.0006 SE = 0.0008	Slope = -0.0005 SE = 0.0011
	Start Through Week 78	
1, 3-6	Slope = -0.0004 SE = 0.0010	Significant lack of fit (p = .004) Slope = -0.0018 SE = 0.0014
2, 3-6	Significant lack of fit (p = .040) Slope = 0.00003 SE = 0.0010	Significant lack of fit (p = .001) Slope = -0.0003 SE = 0.0014
1 & 2 Combined, 3-6	Significant lack of fit (p = .042) Slope = 0.0002 SE = 0.0009	Significant lack of fit (p = .001) Slope = -0.0005 SE = 0.0013

Summary of Linear Regression and Lack of Fit Analyses - Continued

Groups Included in Analyses	Males	Females
	Start Through Week 104	
1, 3-6	Slope = 0.0011 SE = 0.0009	Slope = 0.0009 SE = 0.0015
2, 3-6	Significant regression (p = .047) Slope = 0.0019 SE = 0.0009	Slope = -0.0017 SE = 0.0014
1 & 2 Combined, 3-6	Slope = 0.0015 SE = 0.0008	Inappropriate because of significant difference between control groups

Significant regression was noted through Weeks 6 and 13 in the male data. The significant regression through Week 6 was accompanied by significant lack of fit, and significant regression was not noted consistently for the other intervals analyzed. The isolated case of significant regression here is probably not indicative of treatment effect.

Water Consumption

Group mean weekly water consumption values are presented in Table 5A. Group mean four-day water consumption data (with standard deviations) are presented in Table 5B. A summary of statistical analyses is presented on page 33.

FIGURE 3 – MEAN BODY WEIGHTS

MALES 2112–106

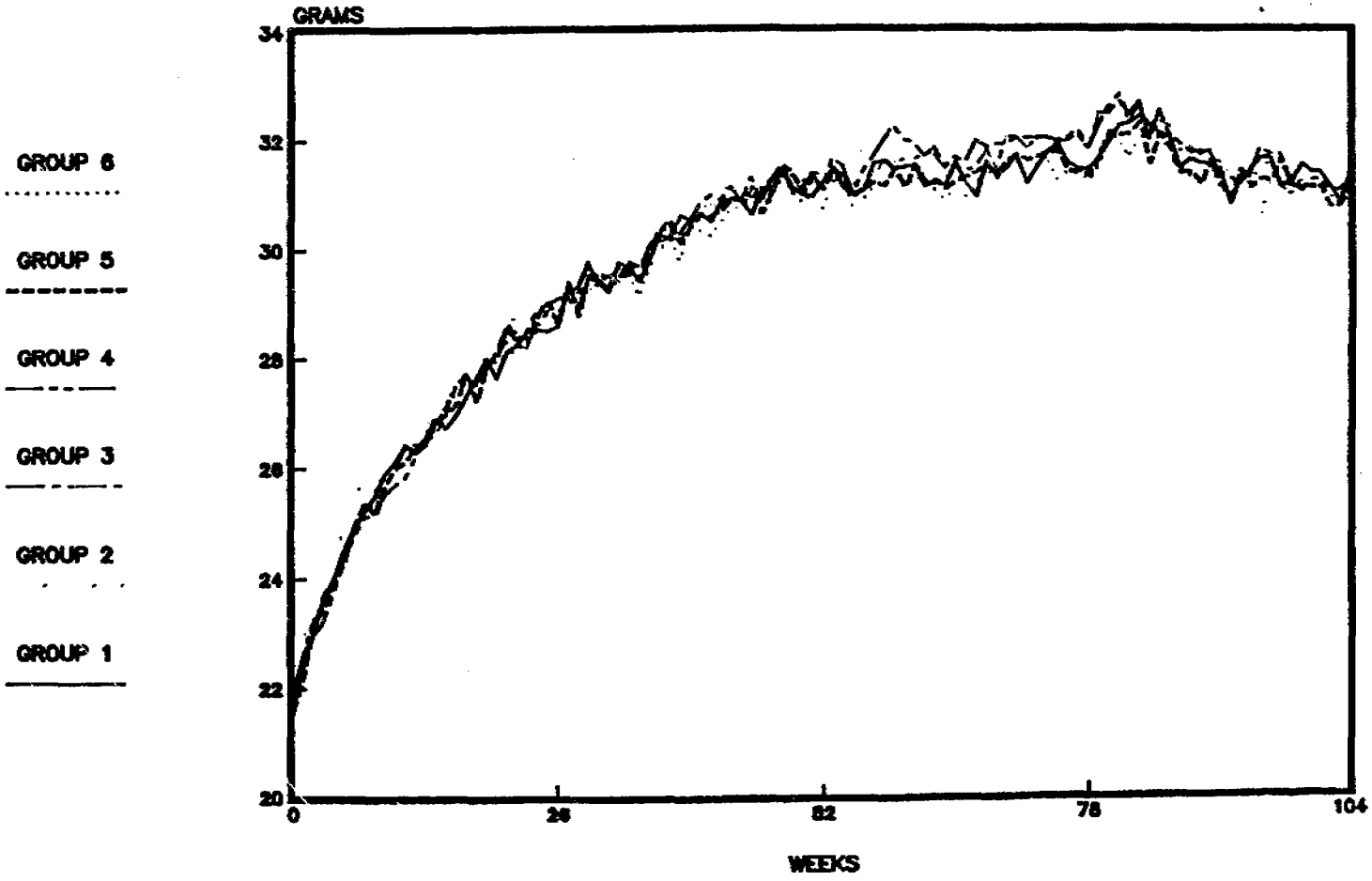
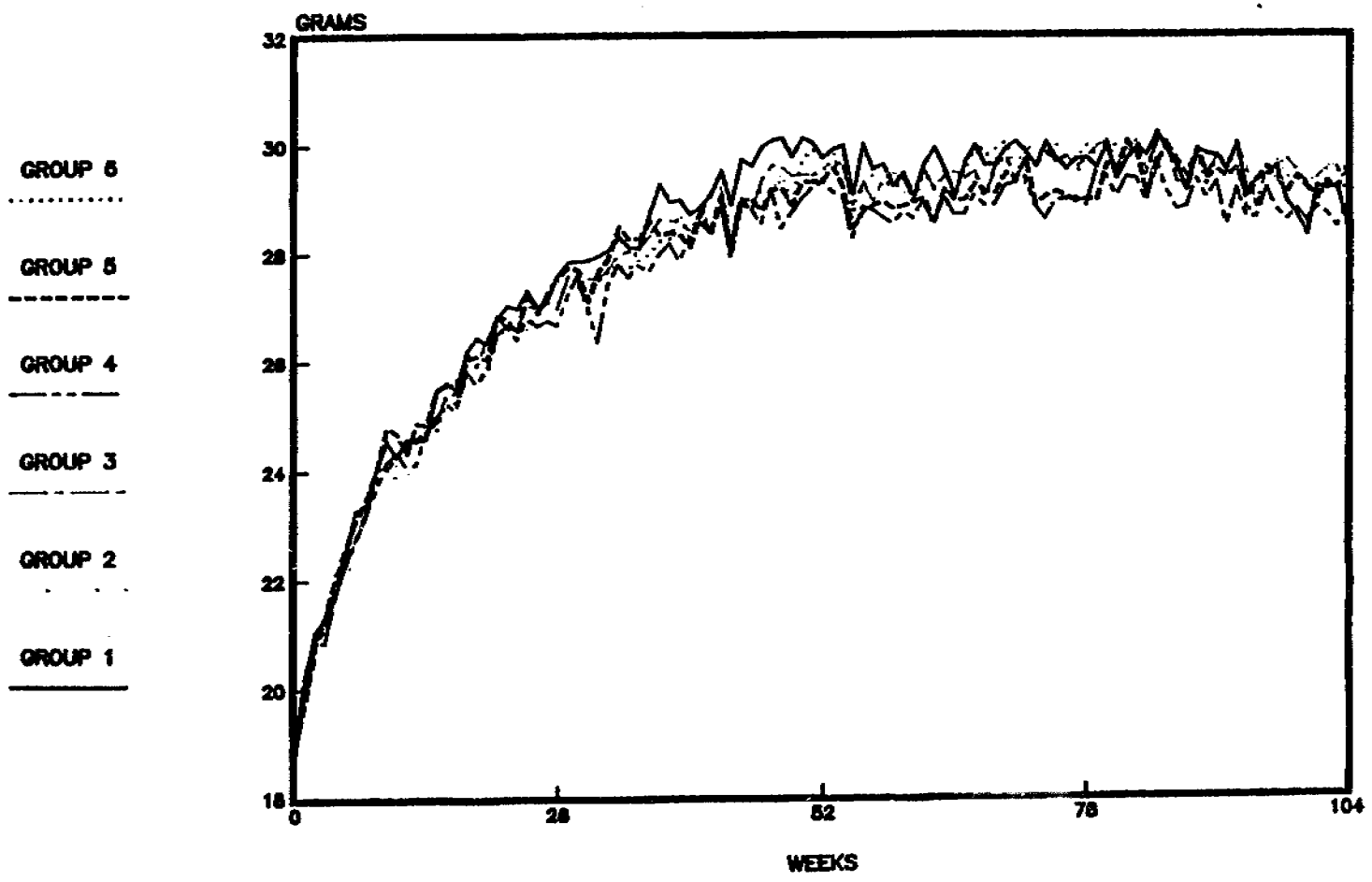


FIGURE 3 – MEAN BODY WEIGHTS

FEMALES 2112–106



Summary of Group Comparisons

Males					Females				
2	3	4	5	6	2	3	4	5	6
Week 1 Through Week									
S+	S+	S+,s-	S+	S+,s-			S+		S+
Week 1 Through Week 13									
S+	S+	S+	S+	s-	S+	S+	S+		
Week 1 Through Week 26									
S+	S+	S+	S+	s-	S+	S+,s+	S+	S+	S+
Week 1 Through Week 52									
S+	S+	S+	S+	s-	S+	S+,s+	S+	S+	S+
Week 1 Through Week 78									
S+	S+,s-	S+	S+,s+	s-	S+	S+,s+	S+	S+	S+
Week 1 Through Week 104									
S+	S+,s-	S+	S+	s-	S+	S+,s+		S+	S+

Regression and lack of fit analyses were not performed using combined data from the control groups except where presented starting on the following page because of statistically significant differences between control groups in all but one case (Week 1 through Week 6 - females).

Summary of Linear Regression and Lack of Fit Analyses

Groups Included in Analyses	Males	Females
	Week 1 Through Week 6	
1, 3-6	Significant lack of fit ($p = .004$) Slope = 0.0079 SE = 0.0043	Slope = 0.0090 SE = 0.0053
2, 3-6	Slope = -0.0066 SE = 0.0038	Slope = -0.0010 SE = 0.0061
1 & 2 Combined, 3-6	Inappropriate because of significant difference between control groups	Slope = 0.0046 SE = 0.0054
	Week 1 Through Week 13	
1, 3-6	Significant lack of fit ($p = .000$) Slope = 0.0052 SE = 0.0037	Significant lack of fit ($p = .003$) Slope = -0.00003 SE = 0.0049
2, 3-6	Significant regression ($p = .017$) and lack of fit ($p = .003$) Slope = -0.0076 SE = 0.0028	Slope = -0.0082 SE = 0.0048
	Week 1 Through Week 26	
1, 3-6	Significant lack of fit ($p = .000$) Slope = 0.0053 SE = 0.0033	Significant lack of fit ($p = .000$) Slope = 0.0011 SE = 0.0035
2, 3-6	Significant regression ($p = .002$) and lack of fit ($p = .001$) Slope = -0.0097 SE = 0.0029	Significant regression ($p = .036$) and lack of fit ($p = .004$) Slope = -0.0072 SE = 0.0033

Summary of Linear Regression and Lack of Fit Analyses - Continued

Groups Included in Analyses	Males	Females
	Week 1 Through Week 52	
1, 3-6	Significant lack of fit (p = .000) Slope = 0.0035 SE = 0.0022	Significant lack of fit (p = .000) Slope = 0.0041 SE = 0.0025
2, 3-6	Significant regression (p = .000) and lack of fit (p = .000) Slope = -0.0086 SE = 0.0018	Significant lack of fit (p = .000) Slope = -0.0044 SE = 0.0023
	Week 1 Through Week 78	
1, 3-6	Significant lack of fit (p = .000) Slope = 0.0026 SE = 0.0020	Significant lack of fit (p = .000) Slope = 0.0032 SE = 0.0020
2, 3-6	Significant regression (p = .000) and lack of fit (p = .000) Slope = -0.0069 SE = 0.0017	Significant lack of fit (p = .001) Slope = -0.0004 SE = 0.0019
	Week 1 Through Week 104	
1, 3-6	Significant lack of fit (p = .000) Slope = 0.0015 SE = 0.0025	Significant lack of fit (p = .000) Slope = 0.0026 SE = 0.0017
2, 3-6	Significant regression (p = .005) and lack of fit (p = .000) Slope = -0.0067 SE = 0.0023	Significant lack of fit (p = .004) Slope = -0.00003 SE = 0.0016

Mean weekly water consumption was consistently higher than Group 1 for male Groups 2, 3, 4, and 5 and lower than Group 2 for male Group 6 through Weeks 6, 13, 26, 52, 78, and 104. Mean weekly water consumption was consistently higher than Group 1 for female Groups 2, 3, 5, and 6 and higher than Group 2 for female Group 3 through Weeks 26, 52, 78, and 104.

While several trends were noted in the water consumption data, they were associated with significant lack of fit in each case. Therefore, the relevancy of the findings to treatment is questionable.

Analysis of Dosing Solutions

Assay results of dosing solutions for methylene chloride are presented in Appendix 4. The average percents of target for all treated groups are presented below.

Group:	3	4	5	6
		Males		
Mean	104.55	101.12	100.83	96.80
S.D.	12.895	10.503	11.719	10.549
N	36	36	36	36
		Females		
Mean	105.33	99.55	98.38	98.10
S.D.	16.004	10.119	11.140	10.974
N	36	5	36	36

Compound Consumption

Estimates of mean daily methylene chloride consumption are presented in Table 6. These data are based on group mean body weights,

mean four-day water consumption (from Table 5B), and levels of methylene chloride determined analytically. The estimated mean daily consumption of methylene chloride during the study is presented below.

Group	Target Level mg/kg/day	Males mg/kg/day ± S.D.	Females mg/kg/day ± S.D.
3	60	60.55 ± 7.680	59.46 ± 8.413
4	125	123.61 ± 14.356	118.19 ± 16.799
5	185	177.45 ± 19.214	172.41 ± 22.950
6	250	234.29 ± 26.495	237.76 ± 29.329

Leukocyte Counts

Individual leukocyte and differential counts are presented in Appendix 1A (Week 52), and Appendix 1B (Week 104). Mean leukocyte counts are presented in Table 4A (Week 52) and Table 4B (Week 104).

White blood cell counts were elevated in Group 6 males and females at Week 52 with males significantly higher than both male control groups and females significantly higher than Group 2 control only. The mean leukocyte count for Group 3 females was significantly lower than the Group 1 control value. Mean values for the other treated groups were comparable to the control groups of the same sex.

Analysis of white blood cell counts at Week 104 revealed no significant differences between groups for either sex.

Examination of erythrocyte morphological data revealed a slight trend toward increased numbers of echinocytes in treated male and female

groups when compared to the control groups at Weeks 52 and 104. This trend is not considered to be biologically significant.

Necropsy Findings

A summary of necropsy findings from deaths and moribund sacrifices throughout the study is presented in Table 7A. Findings from mice sacrificed at termination are summarized and presented in Table 7B.

Findings at necropsy for animals found dead or sacrificed in a moribund condition during the study did not reveal any results which were suggestive of possible treatment effect. At terminal sacrifice, incidence of small masses in the lung of female mice increased with increasing dose level.

Histopathology

Individual histopathology data are presented in Appendices 9A (unscheduled deaths and sacrifices) and 9B (terminal sacrifice). Summaries are presented in Table 8 categorized in the following way:

Table 8A - Deaths and Moribund Sacrifices - Non-Neoplastic Findings
Table 8B - Deaths and Moribund Sacrifices - Neoplasms
Table 8C - Terminal Sacrifice - Non-Neoplastic Findings
Table 8D - Terminal Sacrifice - Neoplasms
Table 8E - All Deaths and Sacrifices (all mice on study) - Non-Neoplastic Findings
Table 8F - All Deaths and Sacrifices - Neoplasms
Table 8G - All Deaths and Sacrifices - Expanded Summary of Non-Neoplastic Liver Findings

A variety of spontaneous lesions were observed in a number of tissues examined from control and treated groups of both sexes and were

without relationship to treatment (e.g., including the lung lesions noted at necropsy in the treated female groups). Higher incidences of liver and Harderian Gland lesions were noted and warranted further review and explanation.

The incidence of proliferative hepatocellular lesions in female test groups was essentially comparable to control values. The incidence was somewhat higher than control in male groups treated with methylene chloride. These data in male mice are presented below.

Number of Male Mice with Proliferative Hepatocellular Lesions

Group:	1	2	3	4	5	6
Number Examined:	60	65	200	100	99	125
Liver						
(Multi)Focal Hyperplasia[a]	4	6	14	4	10	13
Percent Incidence	7	9	7	4	10	10
Hepatocellular Adenoma	6	4	20	14	14	15
Percent Incidence	10	6	10	14	14	12
Hepatocellular Carcinoma	5	9	33	18	17	23
Percent Incidence	8	14	17	18	17	18
Adenoma and/or Carcinoma	11	13	51	30	31	35
Percent Incidence	18	20	26	30	31	28

[a] Some animals with hyperplasia also had hepatocellular neoplasms.

The incidence of focal or multifocal hepatocellular hyperplasia (foci of cellular alteration), was essentially comparable in all groups.

The incidence of hepatocellular adenoma in Groups 3 through 6 was only marginally elevated when compared to the combined control incidences. A relationship to treatment cannot be determined from these data.

The incidence of hepatocellular carcinoma in all treated male groups was slightly increased when compared to the combined control incidence. This incidence (17-18%), however, falls well within the range (5-34%, Average - 16%) previously observed in this strain and age of mouse at this laboratory. Furthermore, this incidence was not substantially different from the incidence of 13.7% described by Ward et al. (1979) in a total of 2543 male $B_6C_3F_1$ mice. Unfortunately, the range for the various groups that he examined was not presented.

In most instances when comparing liver tumor incidences among treated and control mice, it is appropriate to combine adenomas and carcinomas. There is good evidence for progression from benign to malignant stages and the pathological criteria for separating these stages are somewhat controversial. When animals bearing hepatocellular adenoma and/or carcinoma were combined, the incidence in treated male groups (26-31%) was well within the range reported for this strain of mouse in the National Toxicology Program (7-58%) and was below the mean level (32.1%) reported (Tarone, et. al., 1981). Thus, although there was a slight increase in liver neoplasms in treated male groups which was without relationship to increasing dose concentrations, it is difficult to attribute this to the test compound.

Frozen sections of liver stained by Oil Red O method showed a marginal increase in the amount of Oil Red O positive material in males and females receiving high doses of methylene chloride. More male mice in the intermediate dosage groups had slight amounts of Oil Red O material in the livers compared to controls, but this was not dose-related and is felt to be due to the subjectivity of histological evaluation and stain variation. These data are summarized below. There was no other histologic evidence of toxicity.

Number of Mice with Oil Red O Positive Material in the Liver

	Males						Females					
Group:	1	2	3	4	5	6	1	2	3	4	5	6
No. Examined:	60	65	200	100	99	125	50	50	99	50	50	50
Amount												
None	2	-	-	-	-	1	1	2	-	-	-	-
Minimal	50	60	166	61	82	28	26	32	74	41	34	9
Slight	8	4	34	37	17	90	23	16	25	9	15	32
Moderate	-	1	-	2	-	6	-	-	-	-	1	9
Percent												
None	3	0	0	0	0	1	6	4	0	0	0	0
Minimal	83	92	83	61	83	22	50	64	75	82	68	18
Slight	13	6	17	37	17	72	44	32	25	18	30	64
Moderate	0	2	0	2	0	5	0	0	0	0	2	18

Sections of eye which almost invariably included the Harderian Gland revealed that increased numbers of Harderian Gland neoplasms were observed in males in Groups 4 and 6 (9% versus 5.6% in combined controls), while other intermediate groups were essentially similar to controls. This slight increase is considered to be the result of normal biological variation. Moreover, Ward recently reported a National Toxicology Program

where all glands were examined histologically in which the incidence was 12% in male mice of this strain (Ward, 1983). These data are presented below.

Number of Male Mice with Harderian Gland Neoplasms

Group:	1	2	3	4	5	6
Number Examined:	60	65	200	100	99	121
Harderian Gland						
Adenoma	3	2	13	9	5	11
Carcinoma	0	2	0	0	0	0
Percent						
Adenoma	5	3	7	9	5	9
Carcinoma	0	3	0	0	0	0
Combined	5	6	7	9	5	9

Conclusion: The incidence of proliferative hepatocellular lesions in male mice was somewhat increased in treated groups. This elevation was slight, not dose related, and within the expected range of biological variation. Livers of male and female mice receiving high doses of methylene chloride contained greater amounts of Oil Red O material and this is considered to be treatment related. The increased incidence of Harderian Gland neoplasms in the mid-dose 1 and high-dose males is felt to be related to biological variation.

Statistical Evaluation of Histopathologic Findings: A summary of statistical analyses is presented on the following page. The analyses were performed on each control group separately and on the control groups combined. Text Table A presents the Kaplan-Meier estimates of numbers of

mice actually at risk. The results of trend analysis and group comparisons are presented in Text Table B.

As indicated in Text Table B, there were no statistically significant linear trends at the 5% significance level for any of the comparisons (the lowest significance being noted at p = 0.058 when numbers of mice with adenoma and/or carcinoma from treated groups were compared to data from the combined control groups). With Bonferroni correction of significance level, the only statistically significant group comparison noted was that between control Group 1 vs. the high-dose group, with p = 0.0114 (which was less than the Bonferroni corrected level of 0.0125), in the case of carcinoma alone. For the same case, when the high-dose group was compared against control Group 2 and the combined control groups, the p-values were 0.225 and 0.044, respectively.

Text Table A
Kaplan-Meier Estimates of Liver Tumors

Dose Level (mg/kg):	C_1 (0)	C_2 (0)	C_1+C_2 (0)	60	125	185	250
Carcinoma							
$\hat{P}$ =	.089	.165	.127	.187	.205	.191	.213
SEp =	.038	.050	.032	.029	.043	.042	.039
No. with tumor =	5	9	14	33	18	17	23
No. at risk =	56.1798	54.5455	110.2362	176.4706	87.8049	89.0052	107.9812
Adenoma							
$\hat{P}$ =	.113	.074	.094	.121	.171	.164	.144
SEp =	.044	.036	.028	.025	.042	.040	.034
No. with tumor =	6	4	10	20	14	14	15
No. at risk =	53.0973	54.0541	106.383	165.2893	81.8713	85.3659	104.1667
Carcinoma and/or Adenoma							
$\hat{P}$ =	.199	.231	.215	.291	.343	.346	.322
SEp =	.054	.056	.039	.034	.051	.050	.045
No. with tumor =	11	13	24	51	30	31	35
No. at risk =	55.27638	55.2771	111.62791	175.2577	87.4636	89.5954	108.6957

Text Table B
Results of Test for Linear Trend and Group Comparisons with
Kaplan-Meier Estimated Male Liver Tumor Data

Tumor Type (dose in mg/kg)	Cochran-Armitage Test for Linear Trend						Control vs. Treatment Group Comparisons by Asymptotic Normal Test		
	Trend			Departure			Groups Compared (dose level in mg/kg)		
	X^2	df	P	X^2	df	P		z	P
Adenoma									
vs. Control 1 (0)	0.742	1	.389	1.138	3	.768	0 vs. 60	0.158	.437
							0 vs. 125	0.954	.170
							0 vs. 185	0.858	.196
							0 vs. 250	0.558	.289
vs. Control 2 (0)	1.670	1	.196	1.882	3	.597	0 vs. 60	1.072	.142
							0 vs. 125	1.754	.0398
							0 vs. 185	1.672	.047
							0 vs. 250	1.414	.079
vs. Controls 1+2 (0)	1.864	1	.172	1.552	3	.670	0 vs. 60	0.719	.236
							0 vs. 125	1.525	.064
							0 vs. 185	1.434	.076
							0 vs. 250	1.135	.128

Text Table 8 - Continued
Results of Test for Linear Trend and Group Comparisons with
Kaplan-Meier Estimated Male Liver Tumor Data

Tumor Type (dose in mg/kg)	Cochran-Armitage Test for Linear Trend						Control vs. Treatment Group Comparisons by Asymptotic Normal Test		
	Trend			Departure			Groups Compared (dose level in mg/kg)		
	X^2	df	P	X^2	df	P		Z	P
Carcinoma									
vs. Control 1 (0)	2.052	1	.152	2.192	3	.534	0 vs. 60	2.050	.020
							0 vs. 125	2.021	.022
							0 vs. 185	1.801	.036
							0 vs. 250	2.277	.0114[a]
vs. Control 2 (0)	0.477	1	.490	0.194	3	.979	0 vs. 60	0.381	.352
							0 vs. 125	0.607	.272
							0 vs. 185	0.398	.345
							0 vs. 250	0.757	.225
vs. Controls 1+2 (0)	2.102	1	.147	1.190	3	.756	0 vs. 60	1.389	.082
							0 vs. 125	1.455	.073
							0 vs. 185	1.212	.113
							0 vs. 250	1.705	.044

[a] Statistically significantly different from control group after Bonferroni correction.

Text Table B - Continued
Results of Test for Linear Trend and Group Comparisons with
Kaplan-Meier Estimated Male Liver Tumor Data

Tumor Type (dose in mg/kg)	Cochran-Armitage Test for Linear Trend						Control vs. Treatment Group Comparisons by Asymptotic Normal Test		
	Trend			Departure			Groups Compared (dose level in mg/kg)	Z	P
	X^2	df	P	X^2	df	P			
Carcinoma and/or Adenoma									
vs. Control 1 (0)	2.342	1	.126	2.194	3	.533	0 vs. 60	1.442	.075
							0 vs. 125	1.939	.026
							0 vs. 185	1.998	.023
							0 vs. 250	1.750	.040
vs. Control 2 (0)	1.533	1	.216	1.271	3	.736	0 vs. 60	0.940	.174
							0 vs. 125	1.508	.066
							0 vs. 185	1.563	.059
							0 vs. 250	1.295	.098
vs. Controls 1+2 (0)	3.590	1	.058	2.252	3	.522	0 vs. 60	1.469	.071
							0 vs. 125	1.994	.023
							0 vs. 185	2.066	.019
							0 vs. 250	1.797	.036

OVERALL CONCLUSION

No treatment-related effects were noted with regard to mortality (males), clinical signs, body weight changes, water consumption, and gross necropsy findings. Analysis of female mortality data actually revealed a significant negative linear trend with several instances of significantly higher survival in the treated groups.

While not considered to be compound related, from 74.1 to 100% of the mice in all groups of each sex exhibited well-defined convulsions during the study. This observation was first noted in females during Week 37 and for males during Week 39 of study and the frequency of occurrence increased with time. Efforts to establish a basis for this response were initiated, and included hematology and virology studies, histopathologic examination of serial brain sections, and evaluation of genetic purity. None of these specialized tests, however, helped in establishing a cause for the convulsions in the affected mice. While the incidence and appearance of convulsions in this study were thought to be unusual, the convulsions appeared in mice from all groups including the control groups, and thus were not treatment related.

A statistically significant increase in mean leukocyte count was noted for the high-dose males and females following 52 weeks of treatment, but not following 104 weeks of treatment. While the increase in leukocyte count at fifty-two weeks may bear some relationship to treatment, the

response is considered transitory in nature with little long-term biological significance.

Treatment-related histomorphologic alterations of the liver were observed and consisted of a marginal increase in the amount of Oil Red O positive material in the high-dose males and females. No other treatment-related non-neoplastic histomorphologic findings were observed.

No treatment-related increase in neoplastic histomorphologic findings was observed. While a slight increase in proliferative hepatocellular lesions (adenoma and carcinoma) was noted in the treated male groups, this response was well within the historical range of control values compiled at Hazleton Laboratories and reported in the literature. Statistical analyses of these results failed to yield positive significance for trend, and, except for one case, positive significance for group comparisons. After careful consideration of all findings, no persuasive argument for ascribing the results to a treatment-related carcinogenic response could be reached.

Based upon the results of this study, methylene chloride did not induce a carcinogenic response. A "no-effect" level of toxicologic and non-neoplastic histopathologic effects was observed at a dose level of 185 mg/kg/day.

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Table 1
Mean Body Weights and Standard Deviations
24-Month Oncogenicity Study of Methylene Chloride in Mice

CERTIFICATE OF AUTHENTICITY

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