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8EHQ-1198-13083

November 18, 1998

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Office of Toxic Substances
U.S. Environmental Protection Agency
401 M Street, S.W.
Washington, D.C. 20460
ATTN: 8(e) Coordinator

RE: Follow Up Submission to TSCA 8 (e) Notice 8EHQ 0794-13080

Dear Sir or Madam:

This letter is a follow-up to previously submitted preliminary study results communicated in our correspondence of April 12, 1995 (8EHQ 0495-13083), April 23, 1996 (8EHQ 0496-13083), and November 5, 1996 (8EHQ 1196-13080) providing information to U.S. EPA under Section 8(e) of the Toxic Substances Control Act. As stated in our previous correspondence, Aristech Chemical Corporation is providing a complete copy of the final study report. Aristech previously submitted this report to U.S. EPA on April 29, 1998. This report is entitled "Oncogenicity Study in Mice with Di(isononyl) phthalate including ancillary hepatocellular proliferation and biochemical analyses."

If you have any further questions on this matter, please contact me.

Sincerely,

John R. Bankston II

John R. Bankston II
Manager,
Product Regulation and Stewardship

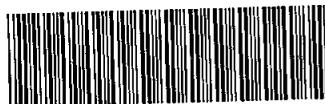
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Enclosure

cc: J. J. Pottmeyer, III
Cover Letter Only



8EHQ-94-13083



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8EHQ-1198-13080

Final Report

Oncogenicity Study in Mice with Di(isononyl)phthalate including
ancillary hepatocellular proliferation and biochemical analyses

PREPARED FOR:

Aristech Chemical Corporation

COVANCE STUDY NUMBER:

2598-105

VOLUME:

Volume 1 of 6





Sponsor:

Aristech Chemical Corporation
600 Grant Street, Room 1140
Pittsburgh, Pennsylvania 15230-0250

FINAL REPORT

Study Title:

Oncogenicity Study in Mice with Di(isononyl)phthalate including ancillary hepatocellular proliferation and biochemical analyses

Author:

Michael R. Moore, Ph.D., D.A.B.T.

Study Completion Date:

January 29, 1998

Performing Laboratory:

Covance Laboratories Incorporated
9200 Leesburg Pike
Vienna, Virginia 22182-1699

Laboratory Study Identification:

Covance 2598-105

Volume 1 of 6

Page 1 of 3259

COMPLIANCE STATEMENT

Oncogenicity Study in Mice with Di(isononyl)phthalate including ancillary hepatocellular proliferation and biochemical analyses

This study, as performed by Covance Laboratories Inc. (Covance), was conducted in compliance with the Good Laboratory Practice Standards as set forth in Title 40 of the U.S. Code of Federal Regulations Part 792, issued November 29, 1983 (effective December 29, 1983), and with any applicable amendments. Deviations from the protocol and/or GLPs are listed in Appendix 14. There were no deviations from the aforementioned regulations which affected the quality or integrity of the study or the interpretation of the results in the report.

Study Director:

Michael R. Moore

Michael R. Moore, Ph.D., D.A.B.T.
Department of Toxicology

January 29, 1998

Date

QUALITY ASSURANCE STATEMENT
Oncogenicity Study in Mice with Di(isononyl)phthalate including
ancillary hepatocellular proliferation and biochemical analyses

Quality Assurance inspections and reviews of this study were conducted according to the standard operating procedures of the Quality Assurance Unit and according to the Good Laboratory Practice Standards as set forth in Title 40 of the U.S. Code of Federal Regulations Part 792, issued November 29, 1983 (effective December 29, 1983), and with any applicable amendments. These inspections and reviews were performed and findings were reported to the Study Director and management as follows:

Dates of Inspection/Review	Dates Findings Reported	Inspector/Reviewer
Protocol Review: 9/17/93	9/17/93	K. Butler
Inspection and/or Data Review:		
9/27;10/18,22/93	10/25/93	K. Butler
12/14/93	12/15/93	K. Butler
3/7,14,15,21/94	3/21/94	K. Butler
6/22,23,27-30/94	6/30/94	D. Wilson
9/20-22/94	9/22/94	L. Cassell
12/5,6,8,9,19/94	12/19/94	L. Cassell
3/8-10,13,15,16/95	3/16/95	L. Cassell
3/28,29/95	3/29/95	L. Cassell
5/24/95	5/24/95	B. Mullett
6/8,9,12/95	6/12/95	L. Cassell
9/28/95	9/28/95	L. Cassell
10/9,11-13,16,23/95	10/23/95	D. Wilson
2/16/96	2/16/96	C. Orantes
Report and Data Review:		
8/28-9/6/95	9/6/95	S. Ballenger
9/21-24,26,27;10/1-10/96	10/11/96	K. Maloid/ M. Grissinger
8/21/97	8/21/97	S. Ballenger
1/23,24,26,27/98	1/27/98	K. Maloid

Kim Maloid
 Kim Maloid
 Quality Assurance Unit

1/29/98
 Date Released

STUDY IDENTIFICATION

Oncogenicity Study in Mice with Di(isononyl)phthalate including ancillary hepatocellular proliferation and biochemical analyses

Covance Study No.: 2598-105

Test Material: Di(isononyl)phthalate, designated DINP

Sponsor's Representative: John H. Butala, D.A.B.T.
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Gibsonia, Pennsylvania 15044
(412) 443-0097

Sponsor: Aristech Chemical Corporation
600 Grant Street, Room 1140
Pittsburgh, Pennsylvania 15230-0250

Study Director: Michael R. Moore, Ph.D., D.A.B.T.
Covance Laboratories Inc.^a
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Vienna, Virginia 22182-1699
(703) 893-5400

Study Timetable

Study Initiation:	August 6, 1993
Initiation of Dosing:	September 27, 1993
Completion of Interim Necropsy:	March 31, 1995
Completion of Terminal Necropsy:	October 4, 1995

^a As of April 1, 1995, the company name, Hazleton Washington, Inc., was legally changed to Corning Hazleton Incorporated. The company name Corning Hazleton Inc. (CHV) was legally changed to Covance Laboratories Inc. on January 2, 1997. All three designations for the company (HWA, CHV, and Covance) may be used in this report.

STUDY PERSONNEL

Oncogenicity Study in Mice with Di(isononyl)phthalate including ancillary hepatocellular proliferation and biochemical analyses

Study Director:	Michael R. Moore, Ph.D., D.A.B.T.
Toxicologist:	Marcia Rodwin, B.A.
Genetic Toxicologist:	Maria A. Cifone, Ph.D.
Study Coordinator:	Krista A. Salley, B.S., LATg
Veterinarian:	Robert L. Ridgway, D.V.M., Diplomate, American College of Laboratory Animal Medicine
Clinical Pathologist:	Renée C. Pearson, M.S., D.V.M., Diplomate, American College of Veterinary Pathologists
Pathologist:	Richard W. Voelker, D.V.M., Ph.D., Diplomate, American College of Veterinary Pathologists
Biostatistician:	Ajit K. Thakur, Ph.D.
Analytical Chemistry Supervisor:	Mark Smyth, B.S.
Analytical Chemistry Principal Investigator:	Li Tian, Ph.D.
Formulations Supervisor:	Danny L. Thomas, B.S., LAT
Laboratory Supervisor:	Nancy M. Centanni, M.S., LATg
Laboratory Group Leader:	Valerie G. Hardee, B.S., LAT
Laboratory Head Technician:	Sylvester Ikpi, M.S., LAT

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SUMMARY

Di(isononyl)phthalate (DINP) was administered daily in the diet to B6C3F1 mice for at least 104 weeks at concentrations of 0, 500, 1,500, 4,000, and 8,000 ppm (Groups 1, 2, 3, 4, and 5, respectively). Mice in Group 6 were administered DINP at a dietary concentration of 8,000 ppm for 78 weeks followed by a 26-week recovery period, during which they were administered the basal diet alone. Parameters evaluated were mortality; clinical observations; body weight, food consumption, and compound consumption data; clinical pathology indices; organ weight data; and necropsy and histopathology findings. Analyses were also conducted at Week 79 and study termination to evaluate chemically induced cell proliferation and peroxisome proliferation in the livers of mice of Group 5 (8,000 ppm) compared to the Group 1 controls (0 ppm).

For Weeks 1-104, the average daily consumed dose in Groups 2, 3, 4, and 5 (based on target dietary concentrations of DINP) was 90.3, 275.6, 741.8, and 1560.2 mg/kg in males and 112.0, 335.6, 910.3, and 1,887.6 mg/kg in females, respectively. For Weeks 1-78 in Group 6 (recovery group), the average daily consumed dose of DINP was 1,377.4 mg/kg in males and 1,581.0 mg/kg in females. Therefore, in each of the DINP dose groups, the actual daily dose consumed by females was 15 to 24% greater than that consumed by males.

The no-observable-effect-level (NOEL) and no-observable-adverse-effect level (NOAEL) for systemic toxicity was 1,500 ppm (Group 3) in males and 500 ppm (Group 2) in females. A DINP dietary concentration of 1,500 ppm produced a statistically significant increase in the combined incidence of hepatocellular adenomas and carcinomas only in females. A DINP dietary concentration of 4,000 ppm induced a significantly increased incidence of liver neoplasms in males and females. A DINP dietary concentration of 8,000 ppm induced a significantly increased incidence of liver neoplasms in both sexes, as well as gross and microscopic evidence

of nonneoplastic effects in the liver and kidneys. In Group 6, administration of a DINP dietary concentration of 8,000 ppm for 78 weeks followed by a 26-week recovery period indicated that DINP effects in the liver, including the induction of neoplasms, and in the kidney were at least partially reversible upon cessation of DINP exposure.

In the 1,500 ppm females (Group 3), the incidence (10/60) of hepatocellular adenoma and carcinoma (combined) was statistically significant compared to the incidence (3/70) in Group 1, although there was no statistical differences between Groups 3 and 1 for incidence comparisons of hepatocellular adenoma only or hepatocellular carcinoma only.

In the 4,000 ppm group (Group 4), there was no treatment-related effect on survival. An increased incidence in males of swelling of the ventral-abdominal region was the only apparent treatment-related clinical abnormality. As a result of DINP administration, mean total body weight gain (Weeks 1-104) was 22% lower in the males and 18% lower in the females compared to control mean values. Liver weights (absolute and relative) exhibited statistically significant increases in males, but not females. However, livers in males and females were remarkably free of any treatment-related nonneoplastic morphologic abnormalities. The incidence of liver masses, and thus the incidence of hepatocellular adenomas and carcinomas, was increased in males compared to that of the controls. In Groups 1 and 4, the percent of mice (all deaths) found to have hepatocellular neoplasms upon histopathologic examination was 23 and 47 in the males and 4 and 18 in the females, respectively. The incidence (all deaths) of hepatocellular carcinomas, but not adenomas, was significantly increased in each sex compared to the respective controls in Group 1. Significant decreases in mean kidney weights (absolute and relative) were noted in males, but there was no correlating histopathological finding. Since there was no histopathologic evidence of a treatment-related effect on spermatogenic activity, the significant decreases in absolute and

relative-to-brain weights for the testis/epididymis were an indirect effect resulting from the treatment-significant depression of body weight gain.

In the 8,000 ppm group (Group 5), a treatment-related, statistically significant decrease in survival occurred in males (63% at Week 104 compared to 87% in the control males). Treatment-related clinical abnormalities were an increased incidence of signs of poor health and/or antemortem condition (hunched posture, hypoactivity, few feces, and urine stains) in males and a notable increased incidence of swelling in the ventral-abdominal region of males and females. As a result of DINP administration, mean total body weight gain (Weeks 1-104) was 40% lower in the males and 20% lower in the females compared to control mean values. A probable reflection of treatment-related physiological stress, leukocyte, lymphocyte, and/or segmented neutrophil counts was lower (often significantly) with repeated frequency in the males and less so in the females. Treatment-related serum and urine chemistry findings at Weeks 26, 52, 78, and 104 indicated the liver and kidney to be target organs of DINP toxicity. At necropsy, dark and/or enlarged livers were frequently noted in males and females. Liver weights (absolute and relative) exhibited statistically significant increases in both sexes. In mice killed at study termination, mean absolute liver weights were 33 and 35% greater in males and females, respectively, compared to the same sex control values. Treatment-related nonneoplastic morphologic abnormalities in the livers of both sexes were diffuse hepatocellular enlargement, increased cytoplasmic eosinophilia, and pigment. Ancillary study results indicated that the liver enlargement was due to peroxisome proliferation and not due to a generalized hepatocellular proliferation. The incidence of liver masses, and thus the incidence of hepatocellular adenomas and carcinomas, was increased in males and females compared to the incidences in the concurrent control group. Hepatocellular neoplasms were diagnosed in 44% of the males and 46% of the females, with statistically significant

increases in both sexes for the total incidences (all deaths) of hepatocellular adenoma, carcinoma, and adenoma/carcinoma combined. Significant decreases in mean kidney weights (absolute and relative) were noted in the males, but not the females; however, the only treatment-related histopathological finding in the kidney was an increase in the incidence and severity of chronic progressive nephropathy in the females, but not the males. Since there was no histopathologic evidence of a treatment-related effect on spermatogenic activity, the significant decreases in mean absolute and relative-to-brain weights for the testis/epididymis were an indirect effect resulting from the treatment-significant depression of body weight gain.

In Group 6 (8,000 ppm for 78 weeks followed by a 26-week recovery period), it appeared that some treatment-related findings were reversible or did not progress after cessation of DINP exposure. Survival in the males decreased only 4%, compared to a 20% decline in the Group 5 males, during Weeks 79-104. Also during Weeks 79-104, the males and females exhibited a group mean body weight gain, while all other groups, including the control, exhibited a group mean body weight loss. Liver weight data indicated a reversal of treatment-related liver enlargement in the males; the incidence of liver masses was slightly decreased in the males and females compared to that of Group 5. In mice killed at study termination, hepatocellular neoplasms were detected in 39% of the males and 37% of the females, compared to 56% of the males and 50% of the females of Group 5. The most remarkable evidence of reversal in the liver was the absence of diffuse hepatocellular enlargement, increased cytoplasmic eosinophilia, and liver pigment in Group 6 mice killed at study termination, indicating complete reversal of these nonneoplastic treatment-related effects after cessation of DINP exposure. Kidney weight data for the males indicated that the decrease in kidney weights observed in Group 5 males was at least partially reversible upon cessation of DINP exposure. Although the incidence and severity of chronic progressive nephropathy were increased

in Group 5 females, the incidence and severity in Group 6 females were comparable to those of the control (Group 1 females), suggesting nephropathy was reversible, or more likely, that exacerbation of this age-associated lesion halted upon cessation of DINP exposure.

INTRODUCTION

This study was designed to evaluate the oncogenic potential of DINP when administered in the diet to mice for at least 104 weeks and to provide information on its ability to cause hepatocellular proliferation and peroxisomal proliferation. Dosing began on September 27 (males) and 28 (females), 1993, and interim and terminal sacrifices were completed on March 31, 1995, and October 4, 1995, respectively. The Group 6 mice were taken off the test diet after Week 78; they continued on basal diet for the remainder of the study.

The protocol was designed in accordance with the applicable guidelines of the U.S. Environmental Protection Agency (Toxic Substances Control Act, 40 CFR Part 798.3300) and conducted in compliance with the U.S. Environmental Protection Agency Good Laboratory Practice Standards (Toxic Substances Control Act, 40 CFR Part 792).

The protocol, in-life phase, and the final report were audited by the Quality Assurance Unit in accordance with Covance Standard Operating Procedures.

The protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Covance and is presented in Appendix 13. Deviations from the protocol and/or GLPs are presented in Appendix 14.

TEST MATERIAL AND BASAL/CONTROL DIET

The test material, Di(isononyl)phthalate, designated DINP, lot No. QCL 9004-273, was received from the Sponsor in two shipments (January 13, 1993 [used pretreatment through Week 81] and March 21, 1994 [used Weeks 82-105]) and stored at room temperature. It was described as a clear liquid with an assumed purity of 100% (first shipment) and a clear, colorless liquid with a reported purity of greater than 99% (second shipment). Information of methods of synthesis and stability,

composition, or other characteristics which define the test material are on file with the Sponsor.

Purina® Certified Rodent Chow® #5002 (PMI® Certified Rodent Diet® #5002) was used as the basal/control diet.

Reserve samples (1 g) of each shipment of the test material (taken prior to use) and the control diet were taken and stored at room temperature. The samples were archived at room temperature and retained at Covance. In addition, a 5-g sample of the neat test material was sent to a Sponsor-designee at the Eastman Kodak Company on January 31, 1994, as per Sponsor request.

TEST ANIMALS AND HUSBANDRY

A total of 910 (454 males/456 females) approximately 4-week-old B6C3F1/Cr1BR mice was received on September 14, 1993, from Charles River Laboratories, Inc., Portage, Michigan. They were assigned temporary numbers, acclimated to laboratory conditions for at least 2 weeks, and released for study use by a staff veterinarian.

Caging Conditions - Upon receipt, animals of the same sex were housed two/cage in suspended stainless-steel, wire-mesh cages (24.4 x 10.5 x 13.2 cm [d x w x h]). After randomization, they were individually housed.

Feed and Water - Purina® Certified Rodent Chow® #5002 (PMI® Certified Rodent Diet® #5002) and tap water, via an automatic watering system, were available ad libitum during the acclimation and study periods, unless otherwise noted. The feed was analyzed by the manufacturer for concentrations of specified heavy metals and nutrients, aflatoxin, chlorinated hydrocarbons, and organophosphates. Water samples are routinely analyzed for specified microorganisms, pesticides, heavy metals, alkalinity, and halogens. The water meets the criteria specified for human drinking water in Fairfax County, Virginia. Results of the feed

and water analyses are reviewed by the Department of Laboratory Animal Medicine for compliance to specified limits and are on file at Covance.

No contaminants were known to be present in the diet or water at levels which might interfere with this study.

Environmental Conditions - Every attempt was made to maintain temperatures at 64.4 to 78.8°F with a relative humidity of $55 \pm 15\%$. Ten or greater air changes/hour and a 12-hour light/12-hour dark cycle (lights on at 0600 to 1800 hours) were maintained.

Justification of Species - The mouse was selected for use on this study because mice historically have been used in safety evaluation studies and are recommended by the appropriate regulatory agencies.

METHODS

Group Assignment and Dietary Levels

Animals were initially accepted into the randomization pool based upon physical examinations; any animals with findings were eliminated from the randomization pool. The animals were randomized within 1 week of initiation of dosing. A total of 810 animals (405 animals/sex) was assigned to study using a computerized weight-randomization program, which first eliminated the animals with extreme body weights, then selected the random assignment that produced homogeneity of variance and means by Bartlett's Test (1937) and One-Way Analysis of Variance (ANOVA). At randomization, the weight variation of the animals selected did not exceed ± 2 standard deviations of the mean body weight for each sex, and the mean body weight for each group of each sex was not statistically different.

The animals were assigned to groups as follows:

Group No.	Group	Dietary Levels ppm	Week		Total Number of Mice M/F
			79 M/F ^{a,b}	105/106 M/F ^{a,b}	
1	Control	0	15/15	55/55	70/70
2	Low	500	15/15	55/55	70/70
3	Mid-Low	1,500	15/15	55/55	70/70
4	Mid-High	4,000	15/15	55/55	70/70
5	High	8,000	15/15	55/55	70/70
6	Recovery-High ^c	8,000 (Weeks 1-78)	0	55/55	55/55

^a Hepatocellular proliferation rates and biochemical analyses (protein concentration, cyanide-insensitive palmitoyl-CoA oxidation, and DNA concentration) were measured during Weeks 79 and 105/106.

^b Five animals/sex/Group 1 and 5 were implanted with osmotic minipumps; they were sacrificed and the tissues collected and appropriately processed for hepatocellular proliferation and biochemical analyses. Five animals/sex/Group 2, 3, 4, and 6 (Week 105/106 only for Group 6) were implanted with osmotic minipumps; they were sacrificed and tissues collected and appropriately processed for hepatocellular proliferation and biochemical analyses. (The hepatocellular tissues were processed to the block stage.) The remaining animals within each group at Week 79 were sacrificed for histomorphological evaluation.

^c Animals assigned to Group 6 were taken off the test diet after 78 weeks of treatment and fed basal diet for the remainder of the study.

During the randomization process, each study animal was assigned a unique number and individually housed. A microidentification device implanted subcutaneously was used to permanently identify each animal.

At initiation of dosing, the animals were approximately 6 weeks of age with body weights ranging from 12.0 to 22.8 g for the males and 12.5 to 20.3 g for the females.

Animals not used on study were removed from the study room.

Compound Formulation and Administration

DINP was assumed to be a 100% active compound for the purpose of dosage calculations. For each dietary level, the test material was weighed on an appropriate balance (mg or kg); the weighed test material was placed into a pocket that was made in approximately 200 g of feed contained in a glass beaker. The test material/feed was mixed in a Waring blender for approximately 2 to 3 minutes to ensure an apparent homogeneous mixture. Based on dietary level, multiple premixes were required

(approximately 30 g test material/premix). The premixes were added to approximately 5 kg of additional feed, mixed in a Hobart mixer for 15 minutes (except Group 2 which required 20 minutes), then transferred to a Patterson-Kelley twin-shell mixer (fitted with an intensifier bar) that contained the remaining amount of basal feed. The diets were mixed for 1 minute/kg (except Group 2 which was mixed for 2 minutes in the twin-shell mixer). Fresh diets were prepared once each week and stored in glass containers fitted with Teflon® lids. No plastic came in contact with the test material. The formulated and basal diets were stored refrigerated (2-8°C).

DINP was administered in the diet, 7 days per week, for up to 78 weeks for the Group 6 animals and for at least 104 weeks to the animals in Groups 2-5. The Group 6 animals received only the basal diet (Purina® Certified Rodent Chow® #5002) after Week 78. The control animals were fed the basal diet in the same manner as the test animals for 105 weeks. All animals received the appropriate diet until the day prior to necropsy. Fresh diet was presented to the animals weekly.

The dietary route of administration was chosen because oral exposure is the customary procedure for evaluating the oncogenicity of plasticizers.

Dose preparation samples (approximately 50 g) for possible analyses of the weekly mix for each dietary level were taken in duplicate, placed into glass containers with Teflon® lids, and stored frozen. The samples not used for analyses were retained at Covance.

Analysis of Prepared Formulations

Homogeneity - Homogeneity of the test material in the dietary mixtures was determined prior to the initiation of treatment for the low- and high-dose levels (Groups 2, 5 (15 kg batch), and 6 (12 kg batch)). Analyses of homogeneity were conducted on samples obtained from the top, middle, and bottom of the test diets. Sampling was conducted in

duplicate. The mix was considered homogeneous if the coefficient of variance for the six samples was $\leq 10\%$.

Stability - Stability of the test material at room temperature for 7 and 14 days was established.

Routine Concentration - The concentration of the test article at all dietary levels was determined from the samples obtained at Weeks 1, 13, 26, 52, 78, and 104.

Analytical Method - The analytical method used to assay the level of test article in the diet involved extraction of the test article from the feed mixture and analysis using reverse-phase, high-performance liquid chromatography. The method is fully outlined in Appendix 1.

Observations and Records

Mortality and Clinical Observations - The mice were observed for mortality and moribundity twice daily. A careful cageside observation for obvious indications of toxic effects was performed once daily.

Physical Examinations - Detailed clinical observations were performed once each week. Special attention was paid to mass development. The following information on each grossly visible or palpable mass was recorded:

time of onset
location
size (small < 1 cm, large > 1 cm)
appearance
progression

Body Weight and Food Consumption - Body weights were recorded at randomization, prior to treatment, and weekly for Weeks 1-17 and once every 4 weeks thereafter. An additional weight was obtained from all groups on the first day of Week 79. Food consumption was measured and recorded weekly for Weeks 1-16 and once every 4 weeks thereafter. Food consumption was also determined at Week 78. When obvious spillage or

wastage of food was recorded for an animal during the detailed physical examination, the estimate of the food consumed by the animal was excluded from the group mean calculation for that particular interval.

Clinical Pathology

Prior to each clinical sampling, all designated animals were placed in urine collection racks and fasted overnight with water available. During Weeks 26, 52, 78, and 104, samples for hematology evaluations (first surviving 10 animals/sex/group) and serum chemistry and urinalyses tests (the next surviving 10 animals/sex/group) were obtained.^a (If possible, samples were collected from the same animals at each respective interval.) Samples were obtained via orbital sinus venipuncture from animals that were anesthetized with CO₂O₂. The following clinical pathology parameters were determined:

Hematology

absolute reticulocyte count	mean cell hemoglobin
corrected leukocyte count	mean cell hemoglobin concentration
erythrocyte count	mean cell volume
hematocrit	platelet count
hemoglobin	reticulocyte count
leukocyte differential and cellular morphology ^b	myeloid/erythroid ratios (at necropsy)
leukocyte count	

^a At Week 26, hematology and urinalysis evaluations were performed on the first 10 surviving animals from each group and serum chemistry evaluations were performed on the next 10 surviving animals from each group.

^b The leukocyte differential and cellular morphology analyses were performed for Groups 1 and 5 only.

Serum Chemistry - (in descending order of priority)

aspartate aminotransferase	calcium
alanine aminotransferase	potassium
gamma glutamyltransferase	inorganic phosphorus
creatinine	glucose
urea nitrogen	total protein
total bilirubin	albumin
sodium	globulin
chloride	

Urine Chemistry

total volume	osmolality
calcium	phosphorus
chloride	potassium
creatinine	sodium
creatinine clearance	

Urinalysis

appearance/color	occult blood
specific gravity (calculated)	microscopic sediment
protein	pH
glucose	turbidity
ketones	urobilinogen
bilirubin	

Clinical hematology analyses were performed using a Coulter Counter® Model S-Plus IV. Serum and urine chemistry analyses were performed using a Hitachi® 737/704 Chemistry Analyzer. Differential leukocyte and cellular morphology quantitations were manually determined by microscopic examination of peripheral blood smears. Myeloid/erythroid ratios were manually determined from bone marrow smears. Semiquantitative urinalysis determinations were performed using Ames™ Multistix® or equivalent. Specific gravity was determined by a refractometer.

Terminal Studies

Sacrifice and Gross Pathology - All animals found dead or sacrificed in extremis (using sodium pentobarbital and exsanguination) during the study were subjected to a gross postmortem examination. After at least 78 or 104 weeks of treatment, animals designated for histomorphological analysis of the tissues were weighed the day of scheduled necropsy, given an intraperitoneal injection of sodium pentobarbital, and exsanguinated. Bone marrow smears were obtained from the femur in order to evaluate myeloid/erythroid ratios. Necropsies were performed on all animals by appropriately trained personnel using procedures approved by board-certified pathologists, and all findings were recorded. A pathologist was readily available for consultation. Necropsies included examination of the following:

all orifices	external surface of the brain (at necropsy)
carcass	external and cut surfaces of the spinal cord (at tissue trimming)
cervical tissues and organs	nasal cavity and paranasal sinuses
cranial cavity	thoracic, abdominal, and pelvic cavities/viscera
cut surfaces of the brain (at tissue trimming)	
external surface of the body	

Organ Weights - At the interim and terminal sacrifices, the following organs (when present) were weighed after careful dissection and trimming of fat and other contiguous tissue:

brain with stem	testes with epididymides (paired weight)
spleen	lung
kidneys (paired weight)	uterus
liver/gallbladder (drained)	

Organ-to-terminal-body-weight (using the fasted terminal body weight) and organ-to-brain-weight ratios were calculated.

Tissue Preservation - The following tissues (when present) from each animal were preserved in 10% neutral-buffered formalin:

adrenal glands	pancreas
all gross lesions (including tissue masses)	peripheral nerve (sciatic)
aorta (thoracic)	pituitary
brain with brainstem	prostate
esophagus	salivary glands (mandibular)
exorbital lacrimal glands ^a	seminal vesicles
eyes with optic nerves ^a	skeletal muscle (thigh) ^a
femur (bone marrow smear) ^b	small intestine (duodenum, jejunum, ileum)
femur with marrow and joint ^a	spinal cord (cervical, lumbar, thoracic)
gallbladder	spleen
heart	stomach (cardia, fundus, pylorus)
kidneys	testes with epididymides
large intestine (colon, cecum, rectum)	thymic region
liver	thyroid with parathyroid
lung	trachea
lymph nodes (mesenteric)	urinary bladder
mammary region	uterus with vagina and cervix
ovaries	

Histopathology - All tissues (except those indicated for possible future examination) from the high-dose (Group 5) and control (Group 1) animals sacrificed after 78 or 104 weeks of treatment and tissues from all animals that died or were sacrificed in a moribund condition during the study were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically. Gross lesions were examined microscopically from all animals.

The liver, testes with epididymides (males), uterus (females), spleen, and kidneys from animals scheduled for sacrifice after treatment

^a These tissues were preserved for possible future examination if indicated by signs of toxicity or target organ involvement.

^b Smear was fixed in methanol and appropriately stained for analysis of the myeloid/erythroid ratio. The bone used to obtain the smear was not the same femur used for histopathology.

for at least 78 or 104 weeks (excluding animals designated for analysis of hepatocellular proliferation, but including those that died or were sacrificed in a moribund condition during the study) were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically.

Postmortem Procedures - Hepatocellular Proliferation/Biochemical Analyses

Animal Selection - Five mice/sex in Groups 1-5 were designated for hepatocellular proliferation studies at following 78 weeks and five mice/sex in Groups 1-6 were designated for hepatocellular proliferation studies following 104 weeks. The animals selected for the hepatocellular proliferation studies were those with the highest animal identification number assignments within the respective group. At the appropriate harvest time, the liver weight was recorded and the following tissues from each cell proliferation animal (last five numbered survivors per group) were preserved in 10% neutral-buffered formalin:

liver (standard tissue samples from the left lateral, median, and right lateral lobes)
duodenum
gross lesions

The remainder of the liver was immediately frozen at approximately -70°C and stored frozen for biochemical analysis.

Hepatocellular Proliferation and Biochemical Analyses - The frozen tissue samples from the three liver lobes (right and left lateral, and median) were evaluated for hepatocellular proliferation and analysis of protein concentration, cyanide-insensitive palmitoyl-CoA oxidation, and DNA concentration from Group 1 and 5 animals sacrificed during Weeks 79 and 105/106. The procedures are detailed in Attachment No. 3 of Appendix 13.

Histopathology - Hematoxylin-and-eosin-stained sections of liver tissue samples from animals scheduled for evaluation of hepatocellular proliferation were processed for routine histomorphological evaluation.

Statistical Analyses

Cumulative survival data were analyzed using the National Cancer Institute Package. Trend analysis of survival was evaluated at the 5% one-tailed probability level.

Statistical analyses of labeling index, palmitoyl-CoA oxidase activity, DNA and protein were performed using analysis of variance techniques for each sacrifice interval. Comparisons of the control group (Group 1) vs. the DINP-treated groups were performed using Dunnett's t-Test.

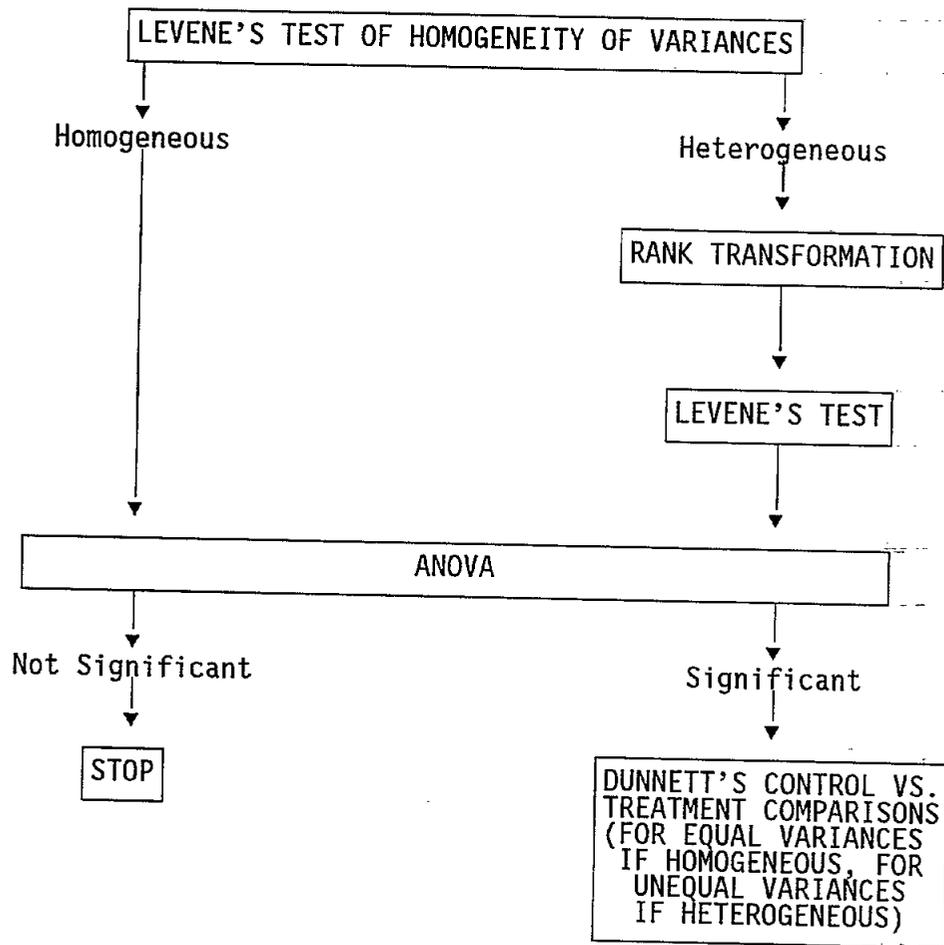
Weekly/monthly body weight, body weight change, and food consumption (Groups 1-6), mean total body weight change (Weeks 1-78, 78-104, and 1-104) and total food consumption (Weeks 1-78, 78-104, and 1-104), clinical pathology data (except hemolysis and cellular morphology gradings and routine urinalysis data), fasted terminal body weights, and organ weight data of the control group were compared statistically to the data from the same sex of the treated groups. Statistical analyses, with the exception of the cumulative survival data, were performed as diagrammed in Figure 1.

If variances of untransformed data were heterogeneous, analyses were performed on rank-transformed data. Group comparisons were performed at the 5% two-tailed probability level.

Histopathologically verified hepatocellular neoplasms (adenomas and carcinomas) were analyzed by Dinse-Lagakos logistic prevalence methods for trend and heterogeneity for control versus treatment comparisons.

Statistical significance is designated throughout the text of this report by the term *significant*. Data transformations are presented in the appropriate tables.

Figure 1
Flowchart of ANOVA and Related Methods



Specimen, Raw Data, and Final Report Storage

All raw data, documentation, records, protocol, any specimens, test material reserve samples, and the final report generated as a result of this study will be archived according to GLP requirements in the storage facilities of Covance for a period of 1 year following submission of the final report to the Sponsor. One year after submission of the final report, all of the aforementioned materials will be sent to the Sponsor or the Sponsor may elect to have the work product retained in the storage facilities of Covance for an additional period of time.

RESULTS

Ancillary Studies

Results of the hepatocellular proliferation and biochemical analyses are discussed in the Genetic Toxicology Report. Data from these analyses are presented in Text Table 1 of the Genetic Toxicology Report.

Cell Proliferation Results - After 78 and 104 weeks of DINP administration, there was no evidence of sustained cell proliferation in the livers of male or female mice from Group 5 (8,000 ppm) compared to livers from the control group (Group 1, 0 ppm). DINP administration did not induce significant increases in the mean labeling index for livers of Group 5 mice compared to Group 1 mice. After 78 weeks of treatment, the mean labeling index in liver for Groups 1 and 5 was 1.24 and 0.59% in the males and 0.30 and 0.12% in the females, respectively. After 104 weeks of treatment, the mean labeling index in liver for Groups 1 and 5 was 0.59 and 1.90% in the males and 0.41 and 1.44% in the females, respectively.

Biochemical Analyses Results - Significant increases in mean palmitoyl-CoA oxidase activity (a monitor of the level of peroxisome proliferation) were evident after 78 and 104 weeks in the livers of Group 5 males and females compared to the Group 1 controls. After 78 weeks of treatment, the mean liver palmitoyl-CoA oxidase activity in Groups 1 and 5 was 3.75 and 36.07 nmols NADH/minute/mg of protein in the males and 2.25 and 37.09 nmols NADH/minute/mg of protein in the females, respectively. After 104 weeks of treatment, the mean liver palmitoyl-CoA oxidase activity in Groups 1 and 5 was 6.70 and 53.79 nmols NADH/minute/mg of protein in the males and 6.54 and 52.82 nmols NADH/minute/mg of protein in the females, respectively.

Analyses of liver DNA concentration did not detect any biologically relevant differences in mean values between the Group 1 and 5 males or females after 78 or 104 weeks of DINP administration. Conversely, liver mean protein concentration was significantly increased

in the Group 5 mice compared to Group 1 at both analysis intervals. After 78 weeks of treatment, the protein concentration in Groups 1 and 5 was 132.7 and 156.2 mg/g liver in the males and 126.0 and 153.0 mg/g liver in the females, respectively; after 104 weeks of treatment, the protein concentration in Groups 1 and 5 was 151.5 and 179.8 mg/g liver in the males and 126.8 and 149.2 mg/g liver in the females, respectively.

Analytical Chemistry

Results of analyses for homogeneity, stability, and routine concentration are presented in Table 1.

Homogeneity analyses indicated that dietary formulations prepared at test material concentrations of 500 and 10,000 ppm were homogeneous (relative standard deviation values of less than 3%) at batch sizes of 15 kg (prepared weekly for Weeks 1-79) and 12 kg (prepared weekly after Week 79).

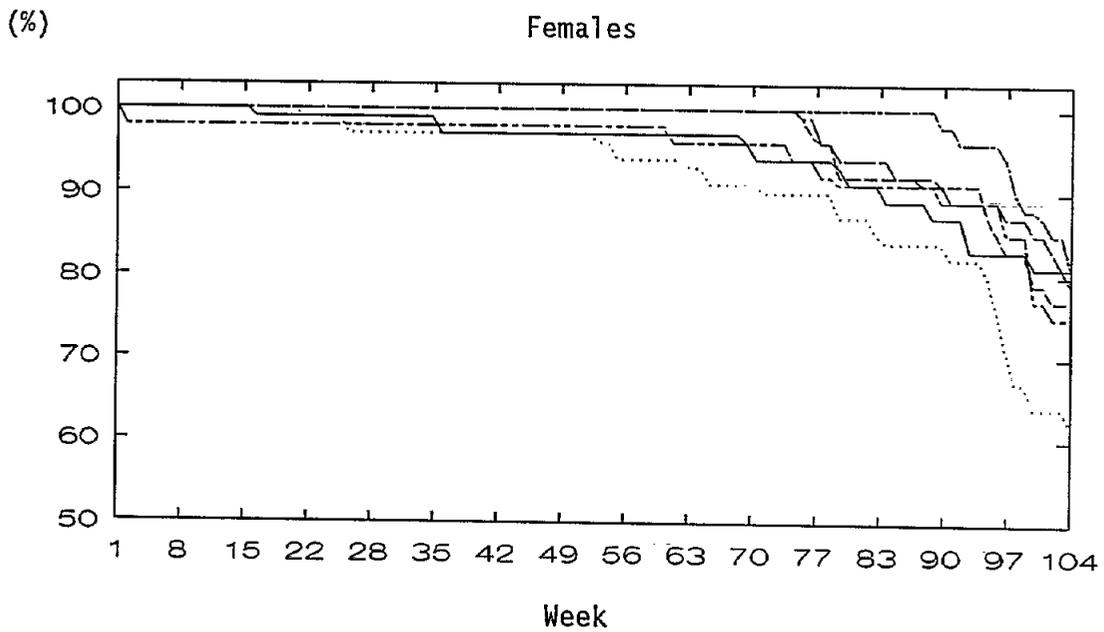
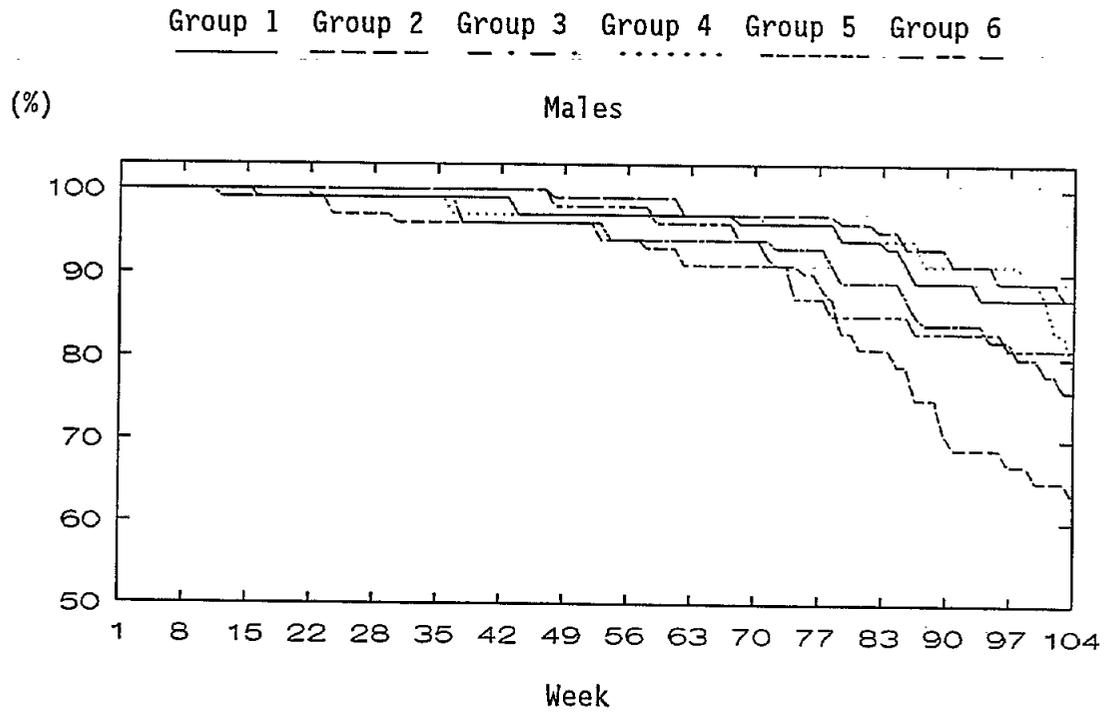
Results of stability analyses indicated that the 500 and 10,000 ppm dietary concentrations were stable for up to 7 days at room temperature and that the 500 and 8,000 ppm dietary concentrations were stable for up to 14 days at room temperature. All 7- and 14-day values were within 12% of initial concentration.

Results of routine concentration analyses indicated that the dietary concentrations were within an acceptable range (92 to 112%) of target concentrations.

In-life Observations

Mortality - Cumulative adjusted survival through Week 104 is presented in Table 2 and depicted graphically in Figure 2; individual animal disposition is presented in Appendix 2.

Figure 2
Adjusted Percent Survival



The greatest number of deaths occurred in Group 5 males (23) and Group 4 females (21). Rates for adjusted survival (excludes the Week 79 interim sacrifice, accidental deaths, and animals removed from study) at the Week 79 in Groups 1-6 were 94, 96, 89, 94, 83, and 85% for the males and 93, 94, 100, 87, 92, and 91% for the females, respectively. Adjusted survival rates at Week 104 in Groups 1-6 were 87, 87, 76, 79, 63, and 81%, for the males and 81, 79, 81, 62, 77, and 75% for the females, respectively. For Weeks 1-104, the adjusted mean survival rate was significantly decreased for the Group 5 males compared to concurrent control value.

Clinical Observations - A summary of daily cageside and weekly physical observations is presented in Table 3. Individual observations are presented in Appendix 3.

Clinical observations noted during the course of the study that exhibited a treatment-related increased incidence were signs of poor health and/or antemortem condition (hunched posture - highest incidence in Group 5 animals, hypoactivity - highest incidence in Group 5 males and Group 4 females, few feces - highest incidence in Group 5 females, and urine stains - predominantly in Group 5 males) and swelling in the ventral-abdominal region, in which the notably increased incidence in the Group 4, 5, and 6 males and Group 5 and 6 females appeared to correlate with the incidence of animals found to have liver masses at necropsy.

All other observed findings occurred sporadically and/or were of the type commonly seen in this species at this laboratory.

Body Weight and Food Consumption - Mean body weight data are presented in Table 4A and depicted graphically in Figure 3; body weight change data are presented in Table 4B. Individual body weight and body weight change data are presented in Appendices 4A and 4B, respectively. Mean food consumption data are presented in Table 5A and depicted graphically in Figure 4; mean total food consumption data are presented in Table 5B. Individual data are presented in Appendix 5.

Figure 3
Mean Body Weights

Group 1 Group 2 Group 3 Group 4 Group 5 Group 6

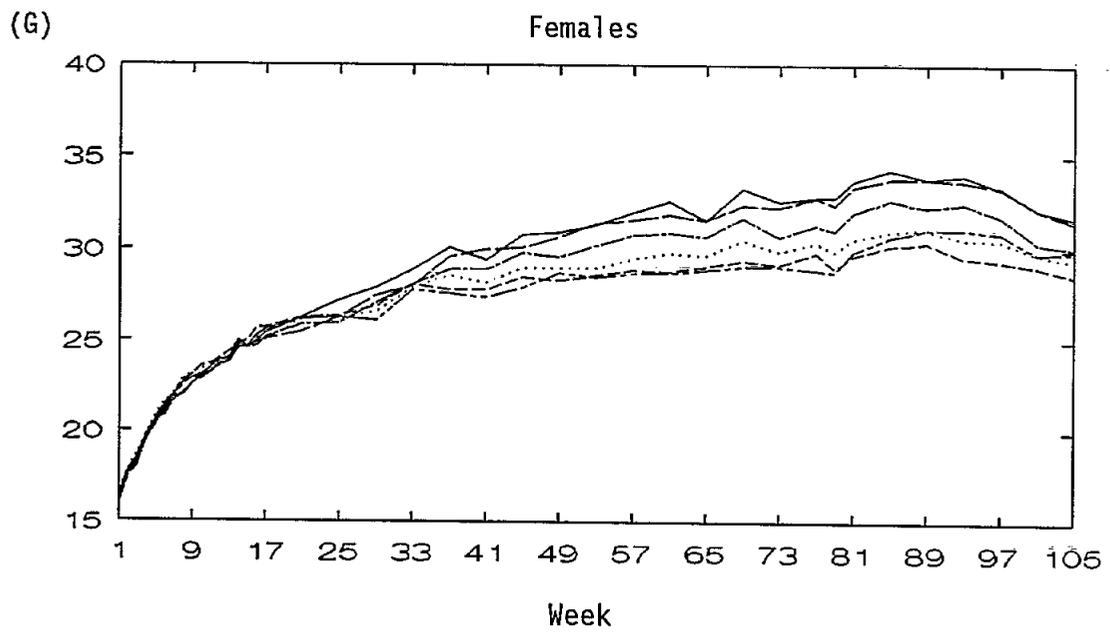
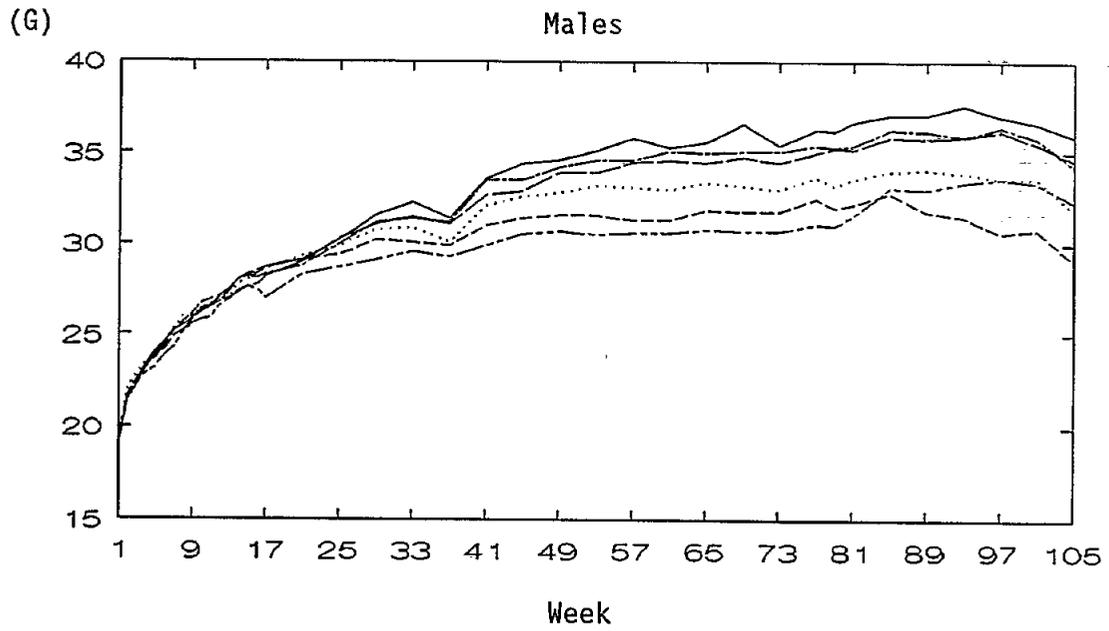
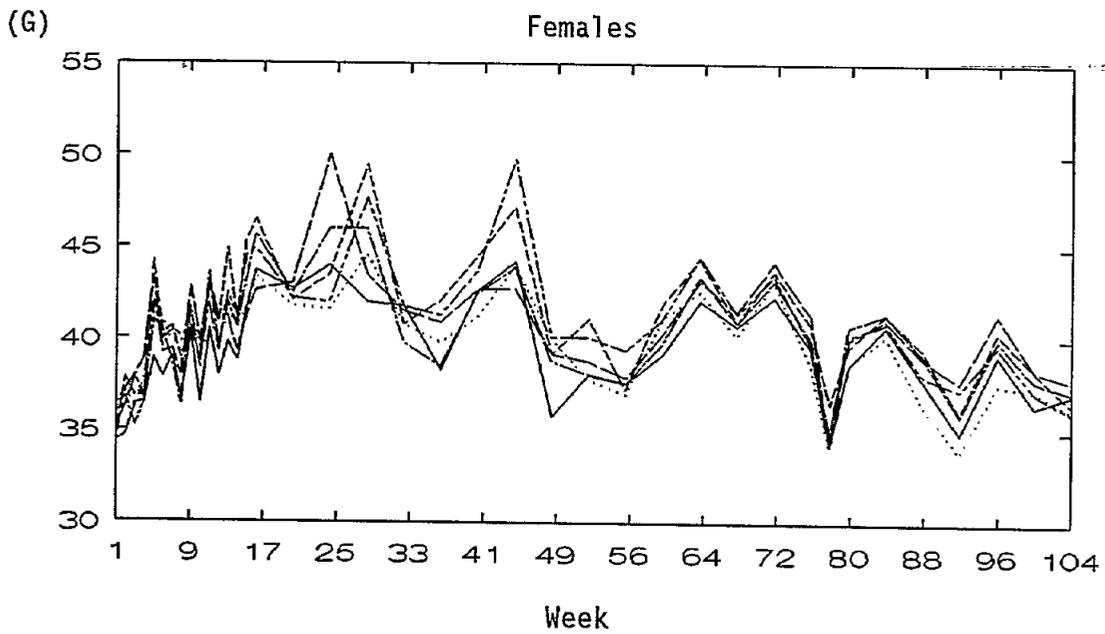
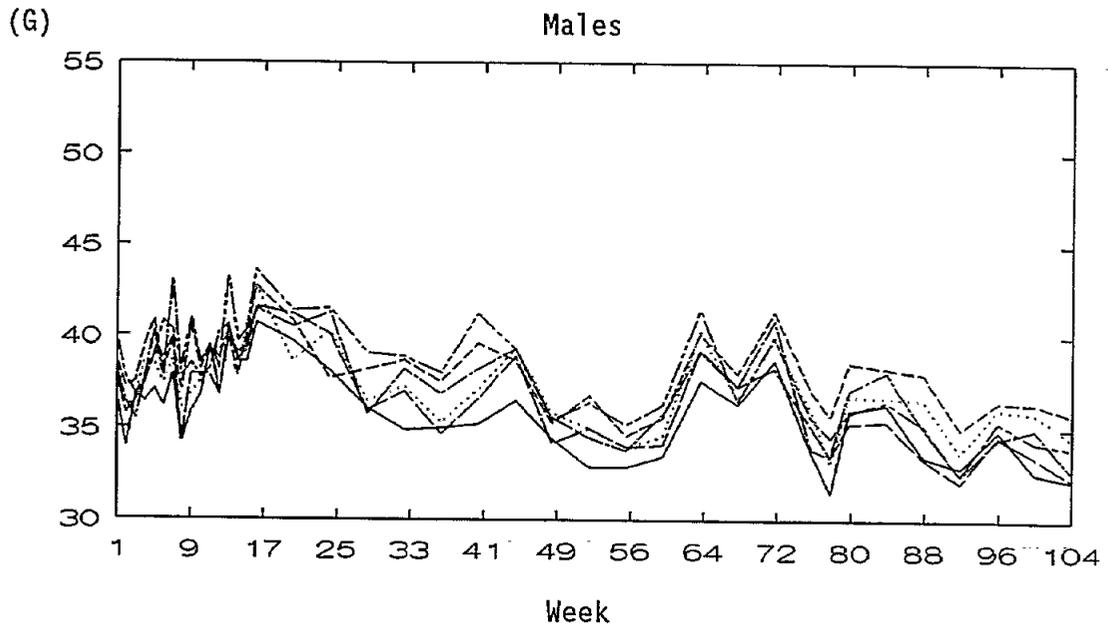


Figure 4
Mean Food Consumption

Group 1 Group 2 Group 3 Group 4 Group 5 Group 6



Significantly decreased body weight means occurred most frequently in the Group 4, 5, and 6 males and females. In the male mice, significantly lower (than control values) body weight means were noted for Group 2 at Weeks 14, 15, 45, 57, 69, and 81; Group 3 at Weeks 69 and 93; Group 4 at Weeks 29-105; Group 5 at Weeks 29-105; and Group 6 at Weeks 5-7, 10-11, and 15-105. In the female mice, significant decreases in mean body weight were noted for Group 2 at Weeks 8, 21, and 25; Group 3 at Weeks 8, 25, 37, 49, 61, and 69-101; Group 4 at Weeks 29, 37, and 45-105; Group 5 at 16 and 37-105; and Group 6 at Weeks 29 and 37-105.

In the male mice, the mean total body weight gains for Weeks 1-78 were statistically decreased for Groups 4, 5, and 6; for Weeks 79-104 total body weight gains were decreased for Groups 4 and 5 but increased for Group 6. In the female mice, mean total body weight gains for Weeks 1-78 were decreased in Groups 3-6; Group 6 mean total weight gain was increased for Weeks 79-104.

The overall mean total body weight change values for Weeks 1-104 were statistically decreased for the Group 4 and 5 animals and Group 6 males compared to that of the concurrent control group as a result of treatment.

There were no significantly decreased food consumption mean values in any of the DINP dose groups compared to the control values. Conversely, significantly increased food consumption mean values were noted in the males for Group 2 at Weeks 2, 6, 44, 52, 64, and 78; Group 3 at Weeks 2, 5, 7, 8, 9, 12, 24, 32, 40, 44, 60, 72, and 78; Group 4 at Weeks 1, 2, 4, 12, 32, 40, 44, 52, 64, 76, 88, 100, and 104; Group 5 at Weeks 1, 2, 4-6, 8, 9, 12, 32-44, 52-64, 72-80, 88, 92, 100, and 104; and Group 6 at Weeks 1, 2, 6-9, 13, 24-44, 52-64, and 78. In the females, significantly increased food consumption mean values were noted for Group 2 at Weeks 5, 10-12, 24, 36, 48, and 92; Group 3 at Weeks 2, 6, 11, 28, and 48; Group 4 at Weeks 2, 6, 8, 10, 14, and 48; Group 5 at Weeks 2,

5, 6, 12-16, 28, 36, 44-52, 60, 64, and 78; and Group 6 at Weeks 5, 8, 14, 15, 28, 36, 44, 48, 64, and 92.

Total mean food consumption values (Weeks 1-78, 78-104, and 1-104) were generally comparable between groups, with the exception of the significantly increased values noted at Weeks 78-104 for Group 4 and 5 males.

Compound Consumption - Mean compound consumption is presented in Table 6; individual data are presented in Appendix 6.

For Weeks 1-104, the average daily consumed dose (based on target dietary concentrations of DINP in Groups 2, 3, 4, and 5 was 90.3, 275.6, 741.8, and 1560.2 mg/kg in the males and 112.0, 335.6, 910.3, and 1887.6 mg/kg in the females, respectively. For Weeks 1-78 in Group 6 (recovery group), the average daily consumed dose of DINP was 1377.4 mg/kg in the males and 1581.0 mg/kg in the females.

Clinical Pathology

Mean hematology values are presented in Table 7 and individual data are presented in Appendix 7. Mean serum and urine chemistry values are presented in Table 8 and individual data are presented in Appendix 8. Individual routine urinalysis data are presented in Appendix 9. Reference ranges are presented in Appendix 12. Findings are further discussed in the Clinical Pathology Report.

Hematology - Evaluation of the hematology data revealed the statistically significant findings detailed in Text Table 1.

Text Table 1
Statistically Significant Findings
Hematology Data

Parameter	Week	Group:	Males					Females				
			2	3	4	5	6	2	3	4	5	6
Mean Cell Volume	52							↓		↓		
Mean Cell Hemoglobin	52							↓		↓		↓
Leukocyte Count	52					↓	↓					
	78					↓					↓	↓
Corrected Leukocyte Count	26					↓						
	52					↓						
	78					↓					↓	
Lymphocytes	26					↓						
	52					↓						
	78					↓					↓	
Eosin	52					↓						
Monocytes	78										↓	
Segmented Neutrophils	26					↓						
	78										↓	

Key: ↓ = Significantly decreased, $p \leq 0.05$.

The lower leukocyte, lymphocyte, and/or segmented neutrophil counts in Group 5 and 6 males and females are attributed (either directly or indirectly) to the administration of the test material. Cellular morphology findings were generally unremarkable and comparable between Groups 1 and 5 at Weeks 26, 52, and 78 and Groups 1-6 at Week 104. No significant differences were noted in the myeloid/erythroid ratios between Groups 1-5 at Week 79 and Groups 1-6 at Week 105.

Clinical Chemistry - Evaluation of the serum and urine chemistry data revealed the statistically significant findings detailed in Text Tables 2 and 3, respectively.

Text Table 2
Statistically Significant Findings
Serum Chemistry Data

Parameter	Week	Group:	Males					Females				
			2	3	4	5	6	2	3	4	5	6
Creatinine	26					↑						
	78				↑	↑	↑					
	104				↑	↑						
Urea Nitrogen	26											↑
Aspartate Aminotransferase	52											↑
	78						↑	↑				
Total Protein	26						↑					
	104						↑					
Alanine Aminotransferase	78											↑
Albumin	26						↑	↑				
Globulin	104						↑	↑				
Calcium	26											
	52											
Total Bilirubin	52			↓								↓
Potassium	52				↓	↓	↓					
Chloride	52											↑

Key: ↓ = Significantly decreased, $p \leq 0.05$.
↑ = Significantly increased, $p \leq 0.05$.

Text Table 3
Statistically Significant Findings
Urine Chemistry Data

Parameter	Week	Group:	Males					Females				
			2	3	4	5	6	2	3	4	5	6
Urine Sodium	52								↓		↓	↓
	78			↓	↓	↓	↓			↓	↓	↓
	104										↓	
Urine Potassium	52					↓	↓					
	78					↓	↓					
	104										↓	
Urine Chloride	26			↑								
	52					↓	↓		↓		↓	↓
	78			↓	↓	↓	↓		↓		↓	↓
	104										↓	
Urine Calcium	26									↑		
	104									↓	↓	
Urine Osmolality	78			↓	↓	↓	↓					↓
	104											↓
Urine Creatinine	78					↓	↓					
	104									↓	↓	

Key: ↓ = Significantly decreased, $p \leq 0.05$.
↑ = Significantly increased, $p \leq 0.05$.

The higher total protein, albumin, and globulin in Group 5 and 6 males; higher aspartate aminotransferase and alanine aminotransferase in Group 5 males and Group 6 males and females; and higher urine volume and lower urine osmolality (with lower concentrations of sodium, potassium, and chloride) primarily in Groups 5 and 6 (male and females) are attributed directly or indirectly to the administration of DINP. Mean creatinine clearance values were generally comparable between groups.

Urinalysis - The urinalysis findings were generally unremarkable and comparable between the groups at Weeks 26, 52, 78, and 104, with the exception that the incidence of ketones was increased in Group 3 and 4 females at Weeks 26 and 104, Group 5 and 6 females at Weeks 26, 52, and 78 and Group 5 females at Week 104.

Terminal Studies

Gross Pathology - Gross pathology findings are summarized in Tables 9A, 9B, 9C, and 9D for unscheduled deaths, interim sacrifice, terminal sacrifice, and all deaths, respectively. Individual gross pathology findings are presented in Appendix 10.

At the Week 79 sacrifice, most notable was an increased incidence of the following necropsy findings: liver - mass in Group 3, 4, and 5 males and Group 5 females; enlarged liver in Group 5 females; and distended urinary bladder in Group 5 males.

At necropsy for study termination, the most frequently observed gross findings were lung - mass (primarily in male mice, all groups); liver - mass, which occurred in greatest numbers in mice of Groups 4, 5, and 6; enlarged spleen (predominantly in the females, all groups); and granular pitted/rough kidneys in Group 5 females. Distended urinary bladder was again noted most frequently in Group 4 and 5 males.

The liver masses noted grossly corresponded to hepatocellular neoplasms or involvement by lymphoma or histiocytic sarcoma. Enlarged appearance of the spleen most frequently was due to increased

extramedullary hematopoiesis or to involvement by hemangioma and/or hemangiosarcoma or hematopoietic neoplasia. The granular pitted/rough appearance of the kidneys in Group 5 females corresponded to the increased incidence/severity of nephropathy as a treatment-related finding. Although a high incidence (21/70) of distended urinary bladder was noted in the Group 5 males, there was no corresponding increased incidence in any kidney histopathological finding. In males, there was no evidence of a treatment-related increase in the incidence or severity of nephropathy.

Other observations noted at necropsy were incidental to treatment or were of the type commonly seen in this age and strain of laboratory animal.

Organ Weights - Mean fasted terminal body weights and organ weight data are presented in Tables 10A and 10B for interim and terminal sacrifices, respectively. Individual data are presented in Appendix 10.

Mean terminal body weights were significantly lower in the Group 4 and 5 males at interim sacrifice and in the Group 4, 5, and 6 males at terminal sacrifice. Mean terminal body weights were lower (not statistically significant) in the Group 4 and 5 females at both the interim and terminal sacrifices.

At the interim sacrifice, there were treatment-related effects in the kidney (males) and liver (males and females) weights. Mean absolute kidney weights were significantly decreased in Group 3, 4, and 5 males and mean kidney-to-body- and kidney-to-brain-weight ratios were significantly decreased for Group 4 and 5 males. Absolute and relative liver weight means were increased in Group 4 males and females (liver weight relative to body weight was significantly increased in Group 4 males) and significantly increased in Group 5 males and females. Other statistically significant mean organ weight changes were increased brain-to-body-weight ratio in Group 4 and 5 males; decreased absolute and relative uterus weight in Groups 2 and 3; and decreased absolute uterus and uterus-to-brain-weight in Group 5.

At terminal sacrifice, treatment-related effects were again evident in the kidney (males) and liver (males and females) weights. For kidney in Group 3, 4, 5, and 6 males, absolute and relative to brain weight means were significantly decreased; relative to body weight means were also decreased (significantly only in Group 4). For liver in males of Groups 4, 5, and 6, mean absolute and relative weights were significantly increased (except for absolute and relative to brain weight in Group 6). For liver in females of Groups 4, 5, and 6, mean absolute and relative weights were increased, but the differences were not significant compared to the control female values.

Other statistically significant mean organ weight changes were decreased testis weights (absolute and relative to brain weight) in Groups 4, 5, and 6, with no histomorphologic correlate; increased lung-to-body-weight ratio for Group 5 and 6 males; and increased brain-to-body-weight ratio for Group 4, 5, and 6 males.

Histopathology - Microscopic findings are summarized in Tables 11A, 11B, 11C, and 11D for unscheduled deaths, interim sacrifice, terminal sacrifice, and all deaths, respectively. Expanded incidence summaries for liver and kidney are presented in Appendix 12. Individual histopathology findings are presented in Appendix 10. The findings are further discussed in the Pathology Report.

Microscopic evaluation revealed test article-related changes in the liver of mice of both sexes and in the kidneys of female mice. An increased incidence of hepatocellular neoplasia was present in Group 5 males and females in addition to nonneoplastic changes consisting of increased cytoplasmic eosinophilia, diffuse hepatocellular enlargement, and pigment. In mice of Group 6 following the recovery period, the nonneoplastic changes were not present, although the incidence of hepatocellular neoplasia was increased in female mice (37%, 13/35) compared to that of the control (7%, 3/42) and lower dose groups. The total incidence of hepatocellular neoplasia in Group 4 males (47%, 28/60)

was also greater than that observed in the control males (23%, 16/70). Test article-related change in the kidneys of female mice of Group 5 deaths consisted of increased incidence/severity of nephropathy.

Other spontaneous disease lesions, including neoplasms, were of the expected types and severity for mice of this age and strain and were not affected by test article administration.

Statistical analyses indicated there were no significant increases in the total incidence (all deaths combined) of hepatocellular adenoma in any of the DINP-treated male groups versus the control males; and only the incidences of 18/70 and 8/50 in the Group 5 and 6 females, respectively, were significantly increased compared to the control females. For hepatocellular carcinoma, only the incidences of 17/60 and 20/70 in the Group 4 and 5 males, respectively, and of 7/60, 18/70, and 13/50 in the Group 4, 5, and 6 females, respectively, were significantly increased compared to the respective same sex of the Group 1 controls. In the Group 3 females, although the incidences of hepatocellular adenoma or carcinoma were not statistically different compared to the incidences in Group 1, the incidence (10/60) for hepatocellular adenoma/carcinoma combined was significantly greater than the combined incidence (3/70) in the Group 1 controls. Corresponding to the statistically significant increases of liver neoplasms in the mid-high and high dose groups, significant positive trends were indicated for hepatocellular carcinomas and hepatocellular adenoma/carcinoma (combined) in males, and for hepatocellular adenomas and carcinomas, as well as hepatocellular adenoma/carcinoma (combined) in females.

DISCUSSION AND CONCLUSION

DINP was administered daily in the diet to B6C3F1 mice for at least 104 weeks at concentrations of 0, 500, 1,500, 4,000, and 8,000 ppm (Groups 1, 2, 3, 4, and 5, respectively). Mice in Group 6 were administered DINP at a dietary concentration of 8,000 ppm for 78 weeks followed by a 26-week recovery period, during which they were administered the basal diet alone.

For Weeks 1-104, the average daily consumed dose in Groups 2, 3, 4, and 5 (based on target dietary concentrations of DINP) was 90.3, 275.6, 741.8, and 1560.2 mg/kg in the males and 112.0, 335.6, 910.3, and 1,887.6 mg/kg in the females, respectively. For Weeks 1-78 in Group 6 (recovery group), the average daily consumed dose of DINP was 1,377.4 mg/kg in the males and 1,581.0 mg/kg in the females. Therefore, in each of the DINP dose groups, the actual daily dose consumed by the females was 15 to 24% greater than that consumed by the males.

The high-dose (8,000 ppm) caused a significant decrease in survival, but only in males. By Week 79, a decrease in survival due to DINP administration was already evident in the Group 5 and 6 males. At the beginning of Week 79, survival was 83% in the Group 5 males, 85% in the Group 6 males, at least 89% in all other male groups, and at least 87% in all female groups. After Week 79, survival continued to decline in the Group 5 males, but a similar decline did not occur in the Group 6 males, which for the recovery phase (cessation of DINP administration) began at Week 79. At Week 104, survival in Groups 1, 2, 3, 4, 5, and 6 was 87, 87, 76, 79, 63, and 81% in the males and 81, 79, 81, 62, 77, and 75% in the females, respectively.

Treatment-related clinical abnormalities observed during daily cageside and weekly physical examinations were an increased incidence of signs of poor health and/or antemortem condition (hunched posture, hypoactivity, few feces, and urine stains) primarily in the Group 5

animals; and a notable increased incidence of swelling in the ventral-abdominal region for the Group 4, 5, and 6 males and Group 5 and 6 females, which appeared to correlate with the incidence of animals found to have liver masses at necropsy.

Treatment-related depressions of body weight gain occurred in males and females of Group 4 (4,000 ppm), as well as Groups 5 and 6 (8,000 ppm). However, a rebound in body weight gain occurred in Group 6 mice during the recovery phase (Weeks 79-104). During Weeks 79-104, mice in Group 6 exhibited a positive group mean body weight gain, while all other groups, including the controls, exhibited a negative group mean body weight gain. Compared to the control mean values, mean total body weight gain in Groups 4, 5, and 6 during Weeks 1-78 was 17, 23, and 29% lower in the males and 20, 22, and 23% lower in the females, respectively. However, mean total body weight gain in Groups 4, 5, and 6 for Weeks 1-104 was 22, 40, and 20% lower in the males and 18, 20, and 11% lower in the females, respectively, compared to control values.

There were no treatment-related decreases in food consumption. Conversely, sporadic occurrences of statistically increased mean weekly food consumption values were detected in DINP male and female dose groups throughout the study. However, mean total food consumption values in male and female DINP dose groups for Weeks 1-78, 78-104, and 1-104 were each within 6% of the comparable mean value of the same sex controls. Therefore, there were no biologically significant differences in food consumption between any of the DINP dose groups and the controls.

Treatment-related clinical pathology findings were associated with the dose-related depression of body weight gain observed in Groups 4, 5, and 6 or with treatment-related histomorphologic alterations observed in liver and kidneys of Groups 4, 5, and 6. Evaluation of hematology parameters at Weeks 26, 52, 78, and 104 in 10 mice/sex/group produced no evidence of a treatment-related decrease in erythrocyte mass. However, as a probable reflection of treatment-related physiological stress,

leukocyte, lymphocyte, and/or segmented neutrophil counts were lower (often significantly) with repeated frequency in the Group 5 and 6 males and less so in the Group 5 and 6 females. At all evaluation intervals, cellular morphology findings and myeloid/erythroid ratios were comparable between the controls and all DINP dose groups.

Treatment-related serum and urine chemistry findings at Weeks 26, 52, 78, and 104 in Groups 5 and 6 indicated that the liver and kidney were target organs of DINP toxicity. Indicating liver toxicity, mean values for serum alanine aminotransferase and aspartate aminotransferase were generally higher (frequently significantly) in the Group 5 and 6 males and occasionally higher, with less consistency, in the Group 5 and 6 females. Mean values for serum total protein, albumin, and globulin were also generally higher (frequently significantly) in males, but not in the females, of Groups 5 and 6. Significant increases in mean urine volume and corresponding significant decreases in mean urine osmolality, with significantly lower mean concentrations for urine sodium, potassium, chloride, and creatinine, were frequently noted in males and females of Groups 5 and 6. Mean creatinine clearance values were generally comparable between groups. The urine chemistry data suggest there was no treatment-related change in glomerular filtration rate, but there may have been a treatment-related alteration in the concentrating ability of the renal tubule epithelium, possibly due to a treatment-related exacerbation (evident histologically in the Group 5 females) of chronic progressive nephropathy.

Treatment-related findings in the necropsy and organ weight data also indicated that the liver and kidneys were target organs for DINP toxicity. At necropsy, dark and/or enlarged livers were noted most frequently in Group 5 males and females (especially in unscheduled deaths and animals killed at study termination). Liver weight data indicated liver enlargement in the 4,000 ppm (Group 4) males, as well as the 8,000 ppm males and females. At Week 79 in the Group 4 males, only the

mean liver-to-body-weight ratio was significantly increased (partially due to a significantly decreased mean terminal body weight); however, the mean absolute liver weight and mean liver-to-brain-weight ratio of the Group 4 males were 22 and 23% greater, respectively, compared to the appropriate mean value of the control males. In the 8,000 ppm (Group 5) males and females, mean absolute and relative liver weights were significantly increased at Week 79, with the mean absolute liver weight being 39 and 38% greater in the Group 5 males and females, respectively, compared to the appropriate mean value of the same sex controls. In mice killed at study termination, mean liver weights (absolute and relative) were significantly increased in the Group 4 and 5 males, but statistically significant increases were not present in the Group 4 or 5 females. Compared to the appropriate mean value of the same sex controls, mean absolute liver weights were 13 and 33% greater, respectively, for the Group 4 and 5 males and 18 and 35% greater, respectively, for the Group 4 and 5 females. A reversal of liver enlargement was evident in males, but not in females. In contrast to Group 5 males, only the mean liver-to-body-weight ratio was significantly increased in the Group 6 (recovery group) males, and the increased ratio was partially due to the significantly decreased mean terminal body weight of the Group 6 males. Mean absolute liver weight in the Group 6 males was only 16% greater compared to the mean value of the control males. In the Group 6 females, mean liver weights (absolute and relative) were nonsignificantly increased; the mean absolute liver weight of this group was 34% greater compared to the control female mean value and was therefore comparable to the mean value of the Group 5 females.

A treatment-related increase in detectable liver masses was clearly evident in the Group 4 (4,000 ppm) males and Group 5 (8,000 ppm) males and females. For all deaths, the incidence of mice with liver masses was 14/70 (20%), 31/70 (44%), and 25/70 (36%) in the Group 1, 4, and 5 males and 2/70 (3%) and 26/70 (37%) in the Group 1 and 5 females,

respectively. There was slight evidence that the incidence of liver masses was decreased in Group 6 (recovery group) compared to that of Group 5. In mice killed at study termination, the incidence of liver masses was 11/46 (24%), 13/32 (41%), and 16/43 (37%) in the Group 1, 5, and 6 males and 1/42 (2%), 13/40 (32%), and 11/40 (28%) in the Group 1, 5, and 6 females, respectively.

An increased incidence of granular/pitted/rough appearance of the kidneys in Group 5 females killed at study termination was the only necropsy finding for kidney suggesting possible treatment-related effects. However, significant decreases in mean kidney weights were noted in males of Groups 3, 4, 5, and 6. At Week 79, absolute mean kidney weights were significantly decreased in Group 3, 4, and 5 males; kidney-to-body- and kidney-to-brain-weight ratios were significantly decreased for Group 4 and 5 males. In mice killed at study termination, significantly decreased kidney weights (absolute and relative to brain weight) were again observed in males of Groups 3, 4, and 5, as well as Group 6. In Group 3, 4, 5, and 6 males killed at study termination, the mean absolute kidney weight was 11, 24, 27, and 17% lower, respectively, compared to the mean value of the control males. The difference in mean absolute kidney weight of Group 6 (recovery group) compared to Group 5 indicates that treatment-related kidney enlargement was reversible upon cessation of DINP exposure.

Histopathology findings confirmed that the liver was a target organ of DINP toxicity. At Week 79, histopathological examination indicated treatment-related liver effects in the 8,000 ppm (Group 5) males and females. Correlating with the significantly increased mean absolute liver weights, diffuse hepatocellular enlargement was detected only in Group 5 and was evident in 11/15 males and 13/15 females. Ancillary study results indicated that the liver enlargement in Group 5 was due to peroxisome proliferation and not due to a generalized hepatocellular proliferation. In the Group 5 males and females, palmitoyl-CoA oxidase activity was significantly increased, and the hepatocyte labeling index

was not increased compared to that of the same sex controls. Increased cytoplasmic eosinophilia and pigment were each present in livers from all of the Group 5 males and females, but were not detected in livers from any of the control males or females. Although mean liver weights were increased at Week 79 in the Group 4 males, there was no correlating histopathologic abnormality (including no evidence of diffuse hepatocellular enlargement). For the Week 79 sacrifice, intergroup differences in incidence of hepatocellular neoplasms were not conclusive.

At study termination, histopathological examination again indicated nonneoplastic treatment-related liver effects only in the 8,000 ppm (Group 5) males and females. Correlating with the significantly increased mean absolute liver weights, diffuse hepatocellular enlargement was detected only in Group 5 and was evident in 32/32 males and 40/40 females. Ancillary study results again indicated that the liver enlargement in Group 5 was due to peroxisome proliferation and not due to a generalized hepatocellular proliferation. In the Group 5 males and females, palmitoyl-CoA oxidase activity was significantly increased, and the hepatocyte labeling index was not increased compared to that of the same sex controls. Increased cytoplasmic eosinophilia and pigment were each present in livers from the majority of the Group 5 males and females, but were not detected in livers from any of the control males or females. In contrast to Group 5, males and females in Group 4 (4,000 ppm), as well as in Group 6 (recovery group), were remarkably free of any nonneoplastic treatment-related histologic findings in the liver. Although mean liver weights were significantly increased at study termination in the Group 4 males, there was no correlating histopathologic abnormality (including no evidence of diffuse hepatocellular enlargement). The data indicate that diffuse hepatocellular enlargement, increased cytoplasmic eosinophilia, and liver pigment did not occur at the 4,000 ppm dose level, and complete reversal of these effects occurred within 26 weeks after cessation of DINP exposure in Group 6.

In mice killed at study termination, the incidence of liver neoplasms was increased in Group 4, 5, and 6 males and Group 5 and 6 females compared to the control incidence. However, there was an indication that the incidence of liver neoplasms was decreased in the Group 6 (recovery group) males and females compared to the Group 5 incidence. In Groups 1, 2, 3, 4, 5, and 6, the percent of mice killed at study termination with hepatocellular neoplasms was 30, 20, 27, 46, 56, and 39% in the males and 7, 11, 14, 17, 50, and 37% in the females, respectively. For all deaths combined, the incidence of liver neoplasms mirrored the findings at study termination, with notably increased incidences occurring in the Group 4, 5, and 6 males and Group 5 and 6 females. In Groups 1, 2, 3, 4, 5, and 6, the percent of mice (all deaths) found to have hepatocellular neoplasms upon histopathologic examination was 23, 22, 30, 47, 44, and 38 in the males; and 4, 8, 17, 18, 46, and 36 in the females, respectively. Statistical analyses of liver neoplasms for total incidence (all deaths combined) indicated significant increases only for hepatocellular adenoma in Group 5 and 6 females (18/70 and 8/50, respectively) compared to Group 1 females (2/70); for hepatocellular carcinoma, only in the Group 4 and 5 males (17/60 and 20/70, respectively) compared to Group 1 males (10/70); and for hepatocellular carcinoma, only in the Group 4, 5, and 6 females (7/60, 18/70, and 13/50, respectively) compared to Group 1 females (1/70). In the Group 3 females, although the incidences of hepatocellular adenoma or carcinoma were not statistically different compared to the incidences in Group 1, the incidence (10/60) for hepatocellular adenoma/carcinoma combined was significantly greater than the combined incidence (3/70) in the Group 1 controls. Corresponding to the statistically significant increases of liver neoplasms in the mid-high and high dose groups, significant positive trends were indicated for hepatocellular carcinomas and hepatocellular adenoma/carcinoma (combined) in males, and for hepatocellular adenomas and carcinomas, as well as hepatocellular adenoma/carcinoma (combined) in females.

The only treatment-related histopathological finding in the kidney was an increase in the incidence and severity of chronic progressive nephropathy in the Group 5 (8,000 ppm) females, but not in the males, compared to the control group. Although significant decreases in mean kidney weights (absolute and relative) were noted in males (but not in the females) of Groups 3, 4, 5, and 6, there was no correlating histopathological finding. In Groups 1, 4, 5, and 6, the incidence and mean severity of chronic progressive nephropathy in females (all deaths) examined histopathologically were 40/60 (mean severity = 0.8), 39/60 (mean severity = 0.8), 61/62 (mean severity = 1.8), and 39/50 (mean severity = 0.9), respectively. The decreased incidence and severity of chronic progressive nephropathy in Group 6 females compared to Group 5 suggest that nephropathy was reversible, or more likely, that exacerbation of this age-associated lesion halted upon cessation of DINP exposure in Group 6 females at Week 79.

Other than liver and kidney, there was no histopathologic evidence of a treatment-related effect in any other tissues. In Group 4, 5, and 6 males killed at study termination, mean absolute and relative to brain weights for testis/epididymis, as well as the mean terminal body weight were significantly decreased compared to the control male values, but organ-to-body-weight ratios were similar. Additionally, there was no histopathologic evidence of a treatment-related effect on spermatogenic activity in the Group 4, 5, or 6 males. Therefore, the decreased testis/epididymis weights were simply a secondary effect to the treatment-related decrease in body weight gain.

In conclusion, for B6C3F1 mice, the no-observable-effect-level (NOEL) and no-observable-adverse-effect level (NOAEL) for systemic toxicity of DINP was 1,500 ppm in males and 500 ppm in females. A DINP dietary concentration of 1,500 ppm produced a statistically significant increase in the combined incidence of hepatocellular adenomas and carcinomas in females. A DINP dietary concentration of 4,000 ppm induced

a significantly increased incidence of liver neoplasms in males and females. A DINP dietary concentration of 8,000 ppm induced a significantly increased incidence of liver neoplasms in both sexes, as well as gross and microscopic evidence of nonneoplastic effects in the liver and kidneys of both sexes. In Group 6, administration of a DINP dietary concentration of 8,000 ppm for 78 weeks followed by a 26-week recovery period indicated that effects in the liver, including the induction of neoplasms, and in the kidney were at least partially reversible upon cessation of DINP exposure.

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CLINICAL PATHOLOGY REPORT

Summary

The test material, Di(isononyl)phthalate, was administered to B6C3F1 mice at dietary levels of 0, 500, 1,500, 4,000, 8,000, and 8,000 ppm (Groups 1-6, respectively) for at least 104 weeks, with the exception of recovery Group 6 which was taken off the test diet beginning at Week 79. Hematology, serum and urine chemistry, and urinalysis tests were performed on samples collected at Weeks 26, 52, 78, and 104. Differential leukocyte counts and cellular morphology evaluations were performed for Groups 1 and 5 at Weeks 26, 52, and 78 and Groups 1-6 at Week 104. Bone marrow smears were prepared from Groups 1-5 (10/sex/group) at Week 79 and from Groups 1-6 (all animals) at Week 105 for the determination of myeloid/erythroid ratios. The significant differences observed in the clinical laboratory data that are attributed directly or indirectly to the administration of the test material were mild and generally occurred inconsistently; they included lower leukocyte, lymphocyte, and/or segmented neutrophil counts in Group 5 and 6 males and females; higher total protein, albumin, and globulin in Group 5 and 6 males; higher aspartate aminotransferase and alanine aminotransferase in Group 5 males and Group 6 males and females; and higher urine volume and lower urine osmolality (with lower concentrations of sodium, potassium, and chloride) primarily in Groups 5 and 6 (males and females).

Results and Discussion

Hematology - Significantly, but mildly, lower mean values were observed for total leukocyte count in Group 5 and 6 males at Week 52 and Group 5 animals and Group 6 females at Week 78 and for corrected leukocyte count due to significantly lower mean lymphocyte and segmented neutrophil counts in Group 5 males at Week 26 and Group 5 females at Week 78 and lymphocyte count in Group 5 males at Weeks 52 and 78. The slightly lower

Lymphocyte counts are consistent with stress and may be an indirect effect of the administration of the test material. It should be noted, however, that no significant differences were observed in the hematology data between Groups 1-6 at Week 104.

The significant differences in the remaining hematology data are considered incidental to the administration of the test material due to the low magnitude and lack of biologic importance of the changes, the inconsistent occurrence of the changes over time, and/or the lack of a dose response; they will not be discussed further in this report. Cellular morphology findings were generally unremarkable and comparable between Groups 1 and 5 at Weeks 26, 52, and 78 and Groups 1-6 at Week 104. No significant differences were noted in the myeloid/erythroid ratios between Groups 1-5 at Week 79 and Groups 1-6 at Week 105.

Serum and Urine Biochemistry - Glucose, total protein, albumin, and globulin data were not obtained for any male at Week 78 due to an insufficient volume of serum. The mean values for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were generally higher in Group 5 and 6 males relative to controls at Weeks 26, 52, 78, and 104, significantly for AST in Group 6 males at Week 52 and Group 5 and 6 males at Week 78. The mean ALT and AST values in the treated female groups were variable and not clearly dose dependent; the mean ALT value was significantly higher in Group 6 females at Week 78. The changes in the aforementioned values correlate with the increased liver weights noted in Group 5 mice and Group 6 males and the histologic findings observed in the Group 5 and 6 mice.

Higher mean values were observed for total protein and albumin in Group 5 and 6 males at Weeks 26 and 104, significantly for total protein in Group 5 males at Weeks 26 and 104, albumin in Group 5 and 6 males at Week 26, and globulin in Group 5 and 6 males at Week 104. The cause of these elevations is not apparent from the data examined (clinical observations, body weights and body weight changes, food consumption data,

necropsy findings, organ weights, and remaining clinical laboratory data). The most common cause of a slight increase in albumin is hemoconcentration due to mild dehydration; however, dehydration was not a prominent clinical observation in these groups.

The significant differences in the remaining serum chemistry analytes are considered incidental to the administration of the test material due to the low magnitude of the change, the lack of a dose response, and/or the inconsistent occurrence of the change over time; they will not be discussed further in this report.

The significant changes observed in the urine chemistry data generally reflect the fact that the urine was more dilute in the affected groups relative to concurrent controls. The mean urine volumes were significantly higher in Group 3 females at Week 52, Group 5 and 6 males and females at Weeks 52 and 78, and Group 4 and 6 males and Group 5 males and females at Week 104. The mean urine osmolality values observed for the aforementioned groups were lower relative to concurrent controls and significantly lower for Group 3-6 males at Week 78 and Group 5 females at Week 104. The significantly lower mean concentrations noted for sodium, potassium, chloride, and creatinine generally correlated with higher urine volume and lower osmolality of the affected groups and the changes were typically more pronounced in Groups 5 and 6; they will not be discussed further in this report. The aforementioned changes indicate an alteration in the concentrating ability of the renal tubular epithelium (especially in light of the dehydration noted clinically in the males) and are attributed to the test material; they correlate with the increased incidence/severity of chronic progressive nephropathy observed histologically in the Group 5 females.

The mean creatinine clearance value was significantly higher in Group 6 females at Week 52; no significant differences were observed between Group 1-6 males at Week 52 or the males and females at Weeks 78 and 104. These findings suggest that, despite the fact that the dilute