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RE: Follow Up Submission to TSCA 3 (e) Notice 8EHQ 0794-13083

Dear Sir or Madam:

As requested in your correspondence of November 17, 1994 acknowledging receipt of information submitted by Aristech Chemical Corporation under Section 8 (e) of the Toxic Substances Control Act, we are providing a complete copy of the final study report. This report is entitled "Oncogenicity Study in Rats 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

Enclosure

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



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# Final Report

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

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PREPARED FOR:  
Aristech Chemical Corporation

COVANCE STUDY NUMBER:  
2598-104

VOLUME:  
1 of 5



Sponsor:

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

FINAL REPORT

Study Title:

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

Author:

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

Study Completion Date:

May 13, 1998

Performing Laboratory:

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Vienna, Virginia 22182-1699

Laboratory Study Identification:

Covance 2598-104

Sponsor Study Identification:

Aristech Chemical Corporation

Volume 1 of 5

Page 1 of 3198

**COMPLIANCE STATEMENT**  
Oncogenicity Study in Rats with Di(isononyl)phthalate including  
ancillary hepatocellular proliferation and biochemical analyses

This study, as performed by Covance Laboratories Inc., 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 are listed in Appendix 11. 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

May 13, 1998  
Date

**QUALITY ASSURANCE STATEMENT**  
**Oncogenicity Study in Rats with Di(isononyl)phthalate including**  
**ancillary hepatoceiular 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 regulations of the Environmental Protection Agency (EPA - TSCA), 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, and 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: 4/21/92	4/21/92	B. Wingard
Inspection and/or Data Review:		
7/8/92	7/8/92	B. Mullett
9/25/92	9/25/92	L. Cassell
11/20/92	11/20/92	P. Tillotson
3/10/93	3/10/93	K. Butler
4/7/93	4/7/93	S. Ballenger
5/18/93	5/18/93	S. Ballenger
8/13/93	8/13/93	M.J. Robertson
11/30/93	11/30/93	D. Wilson
3/4/94	3/4/94	D. Wilson
6/3/94	6/3/94	J. Milazzo
8/9/94	8/9/94	B. Mullett
12/7/94	12/7/94	B. Mullett
Report and Data Review:		
3/15/95	3/15/95	J. Bailey
5/12/98	5/12/98	B. Mullett

*Brad Mullett*  
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 Quality Assurance Unit

*5/13/98*  
 Date Released

Covance 2598-104  
Aristech Chemical Corporation

**STUDY IDENTIFICATION**  
Oncogenicity Study in Rats with Di(isononyl)phthalate including  
ancillary hepatocellular proliferation and biochemical analyses

**Covance Study No.:** 2598-104

**Test Material:** Di(isononyl)phthalate, designated DINP

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**Study Timetable**

Study Initiation:	March 25, 1992
Initiation of Dosing:	May 19/20, 1992
Completion of	
First Interim Necropsy:	May 22/23, 1992
Second Interim Necropsy:	June 1/2, 1992
Third Interim Necropsy:	August 17, 1992
Fourth Interim Necropsy:	November 18/19, 1993
Completion of Terminal Necropsy:	May 25, 1994

<sup>a</sup> The company name Hazleton Washington, Inc. (HWA) was changed to Corning Hazleton Inc. (CHV), and subsequently, on January 2, 1997, to Covance Laboratories Inc. All of the above-mentioned designations for the company name, as well as abbreviations of HWA, CHV, and Covance, may be used in this report.

STUDY PERSONNEL

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

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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
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Laboratory Supervisor:	Howard D. Thornett, B.S., LATg
Laboratory Group Leader:	Charles F. Hatcher, B.S., LAT
Laboratory Head Technician:	Sylvester Ikpi, M.S., LAT

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Aristech Chemical Corporation

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## SUMMARY

Di(isononyl)phthalate was administered daily to F-344 rats in the diet for at least 104 weeks at dietary concentrations of 0, 500, 1500, 6,000, and 12,000 ppm (Groups 1, 2, 3, 4, and 5, respectively). Rats in Group 6 were administered at dietary concentration of 12,000 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 parameters; organ weight data; and necropsy and histopathology findings. Ancillary analyses were also conducted at Weeks 1, 2, 13, 79, and 104 to evaluate chemically-induced cell proliferation and peroxisome proliferation in the livers of the appropriate dose groups. As a positive control group for the ancillary analyses, Group 7, consisting of 15 male rats, was administered WY 14,643 at a dietary concentration of 1000 ppm for at least 13 weeks.

For Weeks 1 through 104, the average daily consumed dose (based on target dietary concentrations of Di(isononyl)phthalate in Groups 2, 3, 4, and 5, was 29.2, 88.3, 358.7, and 733.2 mg/kg/day, respectively, in males; and 36.4, 108.6, 442.2, and 885.4, respectively, in females. For Weeks 1-76 in Group 6 (recovery group), the average daily consumed dose of Di(isononyl)phthalate was 637.3 mg/kg/day in males, and 773.6 mg/kg/day in females. Therefore, in each of the Di(isononyl)phthalate dose groups, the actual daily dose consumed by females was 21 to 25% greater than that consumed by males.

The no-observable-effect-level (NOEL) for systemic toxicity and carcinogenic potential was 1,500 ppm (Group 3) in males and females. The dietary concentration of 6,000 ppm (Group 4) induced gross and microscopic evidence of liver and kidney toxicity, and an increased incidence of mononuclear cell leukemia in males and females. The dietary concentration of 12,000 ppm (Group 5) also induced gross and microscopic evidence of

liver and kidney toxicity, as well as an increased incidence of hepatocellular neoplasms in males and females, renal carcinomas in males, and an increased incidence of mononuclear cell leukemia in both sexes.

In the 6,000 ppm group (Group 4), survival at Week 104 in males and females was 66% and 71%, respectively, compared to 74% and 76% in the control males and females, respectively. Treatment-related clinical abnormalities were increased incidence of urine stains in both sexes, and increased incidence of ante-mortem condition (hunched posture, entire body pale, thin, hypoactive, few or no feces). Mean total body weight gain (Weeks 1 to 104) was 2.7% lower in the males, and 6.5% lower in the females, compared to control mean values. Associated with the depression of body weight gain, erythrocyte mass (erythrocyte count, hemoglobin, and hematocrit) was decreased in both sexes compared to the controls. Serum urea nitrogen was elevated (approximately 15% greater than control mean values) in both sexes at Weeks 26, 52, 78, and 104. Mean values for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were increased in both sexes at Weeks 52, 78, and 104. Enlarged and/or granular/pitted/rough livers were noted at necropsy in males and females; and statistically significant increases in mean liver weights (absolute and relative) occurred in both sexes at Weeks 1, 2, 13, 79, and study termination. However, there was no histopathological correlate to the increased liver weight. Livers from Group 4 rats killed at Week 79 and at study termination were remarkably free of treatment-related histologic findings, with no histologic evidence of hepatocellular enlargement. In the Group 4 rats (6000 ppm) kidneys were frequently noted as dark at necropsy, and statistically significant increases in mean kidney weights (absolute and relative) occurred in both sexes at Week 79, and study termination. Treatment-related histopathologic lesions in the kidney at Week 79 and study termination, were an increase in the incidence and severity of mineralization of the renal papilla in males, but not females; and increased incidence and severity of tubule cell pigment in both sexes.

The incidence of mononuclear cell leukemia was 49% and 45% in males and females, respectively, compared to 34% and 26% in the control males and females, respectively. Correlating with the increased incidence of leukemia, statistically significant increases in mean spleen weights (absolute and relative) occurred in males and females at study termination.

In the 12,000 ppm group (Group 5), survival at Week 104 was 54% (significantly decreased) in males and 66% in females compared to 74% and 76% in the control males and females, respectively. Treatment-related clinical abnormalities were increased incidence of urine stains in both sexes, and increased incidence of ante-mortem condition (hunched posture, entire body pale, thin, hypoactive, few or no feces). Mean total body weight gain (Weeks 1 to 104) was 10.2% lower in the males, and 14.9% lower in the females, compared to control mean values. Associated with the depression of body weight gain, erythrocyte mass (erythrocyte count, hemoglobin, and hematocrit) was decreased (significantly in the erythrocyte mass) in both sexes compared to the controls, and more severely decreased compared to Group 4. Serum urea nitrogen was significantly elevated in both sexes at Weeks 26, 52, 78 and in the males only at Week 104 (females were elevated but not significantly). Mean values for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly decreased at Week 26 for females but were significantly increased for males at Week 52 (all mean values were increased in both sexes at Weeks 52, 78, and 104). Enlarged and/or granular/pitted/rough livers were noted at necropsy in males and females; and statistically significant increases in mean liver weights (absolute and relative) occurred in both sexes at Weeks 1, 2, 13, 79, and study termination. Mean liver weights (absolute and relative) in both sexes were also increased compared to Group 4. Histologic and biochemical analyses indicated the presence of hepatocellular proliferation only during Week 1, and thereafter, only the mean value for palmitoyl-CoA

oxidase activity, an indicator of peroxisome proliferation, was significantly increased in the livers of the 12,000 ppm males and females. Other histological findings in the livers of the Group 5 (12,000 ppm) males and females were diffuse hepatocellular enlargement (first detected at Week 2); increased cytoplasmic eosinophilia (first detected at Week 13); increased pigment in Kupffer cell/bile canaliculi (first detected at Week 79), and increased incidence of neoplasms (first detected at Week 79). Treatment-related histopathologic lesions in the kidney at Week 79 and study termination, were an increase in the incidence and severity of mineralization of the renal papilla in males, but not females; and increased incidence and severity of tubule cell pigment in both sexes. Malignant tubule cell carcinoma was detected in each of two males. The incidence of mononuclear cell leukemia was 46% and 46% in males and females, respectively, compared to 34% and 26% in the control males and females, respectively. Correlating with the increased incidence of leukemia, statistically significant increases in mean spleen weights (absolute and relative) occurred in males and females at study termination.

In Group 6 (12,000 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 Di(isononyl)phthalate exposure. Compared to Group 5, depression of mean total body weight gain was less severe. Although mean values for erythrocyte mass in the recovery group were lower compared to the controls, in contrast to Group 5, the differences were not significant at Week 104. After the 26-week recovery period, the mean value for urea nitrogen (Week 104) in the Group 6 males remained elevated (approximately 47% greater than the control male value), and was comparable to the mean value for the non-recovery Group 5 males; whereas the mean value for urea nitrogen (Week 104) in the Group 6 females was comparable to the mean value of the control females. After the 26-week recovery period, mean values for AST

and ALT (Week 104) in the Group 6 males and females did not exhibit a reversible effect and remained increased compared to control values, with the increased values being comparable to the Group 5 values. Treatment-related liver enlargement appeared to be reversible, in that mean liver weights (absolute and relative) at study termination in the recovery group were comparable to control mean values. Treatment-related histological effects in the liver also appeared to be reversible in that diffuse hepatocellular enlargement and increased cytoplasmic eosinophilia were not evident in Group 6 animals, and the incidence of pigment in Kupffer cell/bile canaliculi was comparable to the control group incidence. Most notable in livers of the recovery animals (Group 6) was the absence of an appreciable increase in liver neoplasms compared to the control group. Kidney enlargement also appeared to be reversible in that mean kidney weights (absolute and relative) in the Group 6 males and females were comparable to control values. However, mineralization of the renal papilla in males, and renal tubule cell pigment in both sexes, was only partially reversible with the incidence and severity approximating values in Group 4 (6,000 ppm). Unlike the liver, treatment-related neoplasia in the kidney of males, and hematopoietic tissue of both sexes, was not reversible. Although no kidney neoplasms were detected in females, malignant tubule cell carcinoma was found in four of the Group 6 males, of which three were killed following the recovery phase. The incidence of mononuclear cell leukemia was 62% and 48% in males and females of Group 6, respectively, compared to 34% and 26% in the control males and females, respectively. Correlating with the increased incidence of leukemia, statistically significant increases in mean spleen weights (absolute and relative) were evident in Group 6 males and females at study termination.

## INTRODUCTION

This study was designed to evaluate the oncogenic potential of Di(isononyl)phthalate when administered to rats in the diet for at least 104 weeks and to provide information on the ability of Di(isononyl)phthalate to cause hepatocellular proliferation and peroxisomal proliferation. Dosing began on May 19/20, 1992, and interim sacrifices occurred on May 22/23, June 1/2, and August 17, 1992, and November 18/19, 1993, and terminal sacrifices were completed on May 25, 1994.

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 was conducted in accordance 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 was audited by the Quality Assurance Unit in accordance with Covance Laboratories Inc. Standard Operating Procedures.

The protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Covance and is presented in Appendix 10. Deviations from the protocol are presented in Appendix 11.

## TEST AND POSITIVE CONTROL MATERIALS

The test material, Di(isononyl)phthalate (referred to as DINP), lot No. QCL 9004-273, was received from the Sponsor on May 22, 1990 (used pretest and Weeks 1-3), on May 13, 1992 (used Weeks 4-37), and on January 15, 1993 (used Weeks 37-106). The test material was described as a clear, colorless liquid with a reported purity of >99% (assumed 100% for dosage calculations), and was stored at room temperature. Methods of

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

The positive control material, WY 14,643; [4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (lot No. CSL-91-315-72-7), was received from ChemSyn Science Laboratories, Lenexa, Kansas, on May 5, 1992, stored at room temperature, and handled as a suspected carcinogen. It was described as white powder with a reported purity of  $\geq 98\%$  and assumed 100% pure for the purpose of dosage calculations.

Information on the methods of synthesis and stability, as well as data on composition or other characteristics which define the positive control article, are on file with the manufacturer, ChemSyn Science Laboratories, Lenexa, Kansas.

Reserve samples (1 g each) of the test material (each shipment), control diet, and positive control were taken prior to initiation of use and stored at room temperature. The samples were subsequently forwarded to the Sponsor after the completion of the in-life phase of the study. In addition, a 5-g sample of the neat test material was taken during Week 13 and sent to a Sponsor-designee at the Eastman Kodak Company.

#### TEST ANIMALS AND HUSBANDRY

A total of 1065 (540 male/525 female) approximately 4-week-old CDF<sup>®</sup>(F-344)Cr1BR rats was received on May 5, 1992, from Charles River Laboratories, Inc., Raleigh, North Carolina. Upon arrival at the animal facility, the animals were housed apart from all other animals in the testing facility. Animals were assigned temporary numbers, acclimated to laboratory conditions for at least 2 weeks, and released for possible 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 (24.2 x 22.0 x 17.3 cm [d x w x h]). The cages and racks were sanitized once every

2 weeks; excreta trays beneath the cages were changed at least three times/week.

Feed and Water - PMI<sup>®</sup> Feeds, Inc. Certified Rodent Diet<sup>®</sup> #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 (an example contaminant analysis is presented in Attachment No. 1 of Appendix 12). Water samples are periodically analyzed for contaminants and specified microorganisms. The water meets the criteria specified for human drinking water in Fairfax County, Virginia. Results of the feed and water analyses are periodically reviewed for compliance to specified limits and are on file at Covance.

No contaminants were reasonably expected to be present in the animal feed and water at levels sufficient to interfere with this study.

Environmental Conditions - Every attempt was made to maintain temperatures at  $72 \pm 6^{\circ}\text{F}$  with a relative humidity of  $50 \pm 20\%$ . A 12-hour light/12-hour dark cycle and 10 or greater air changes/hour was maintained in the room housing the animals. The location of animal racks within the animal room was rotated once every 2 weeks.

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

## METHODS

### Group Assignment and Dietary Dose 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 approximately 1 week prior to initiation of dosing. A total of 915 animals (465 males and 450 females) were 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  $\pm 2$  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.

During the randomization process, each study animal was assigned a unique number and individually housed in a cage affixed with the animal's identification number. A tail tattoo of the last three digits was used to permanently identify each animal.

At initiation of dosing, the animals were approximately 6 weeks of age with body weights ranging from 96 to 134 g for the males and 82 to 109 g for the females. Animals were assigned to groups as indicated in the following table.

GROUP ASSIGNMENT AND DIETARY DOSE LEVELS

Group No.	Group	Dietary Levels ppm	Week of Sacrifice					Total Number of Rats M/F
			1	2 M/F <sup>a</sup>	13 M/F <sup>b</sup>	79 M/F <sup>bc</sup>	104-106 M/F <sup>cd</sup>	
1	Control	0	5/5	5/5	5/5	15/15	55/55	85/85
2	Low	500	5/5	5/5	5/5	0	55/55	70/70
3	Mid-Low	1,500	5/5	5/5	5/5	0	55/55	70/70
4	Mid-High	6,000	5/5	5/5	5/5	15/15	55/55	85/85
5	High	12,000	5/5	5/5	5/5	15/15	55/55	85/85
6	Recovery-High <sup>d</sup>	12,000 (Weeks 1-78)	0	0	0	0	55/55	55/55
7	Positive Control	1,000	5/0	5/0	5/0	0	0	15/0

<sup>a</sup> Hepatocellular proliferation rates and biochemical analyses (protein concentration, cyanide-insensitive palmitoyl-CoA oxidation, and DNA concentration) were measured from five animals/sex from Groups 1-5 and 7 during Weeks 1, 2, and 13, and on five animals/sex from Groups 1, 4, and 5 during Week 104.

<sup>b</sup> Five animals/sex/group were implanted with osmotic minipumps, sacrificed, and the tissues were collected and appropriately processed (hepatocellular prepared to the block stage) for possible hepatocellular proliferation and biochemical analyses. The remaining animals within each group were sacrificed for histomorphological evaluation.

<sup>c</sup> Ten animals/sex/group were designated for histomorphological evaluation; the remaining animals were processed for possible hepatocellular proliferation and biochemical analyses.

<sup>d</sup> Animals assigned to Group 6 were removed from the test diet at Week 78 and were placed on basal diet for the remainder of the study.

### Compound Formulation and Administration

Di(isononyl)phthalate (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 added to a pocket that was made in approximately 200 g of feed in a glass beaker. The test material/feed was mixed in a Waring blender for approximately 2-3 minutes to ensure an apparent homogeneous mixture. (Based on dietary level, multiple premixes were required.) The premixes were added to approximately 5 kg of additional

feed, mixed in a Hobart mixer for 15 minutes (except Group 2 which required 30 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 (Group 2 was mixed for 50 minutes in the twin-shell mixer). Fresh diets were prepared once each week and were 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).

The positive control material, WY 14,643, was assumed to be a 100% active compound for the purpose of dosage calculations. For formulation, the material was weighed on an appropriate balance (mg or kg), added to approximately 200 g of feed, and pre-mixed in a Waring blender for approximately 2 minutes to ensure an apparent homogeneous mixture. The premix was added to the required amount of additional feed, and mixed in a Hobart mixer for a minimum of 5 minutes. Diet formulations of WY 14,643 were stored refrigerated (2-8°C).

DINP was administered in the diet, 7 days per week, for 78 weeks to the Group 6 animals, and for 105 weeks to the animals in Groups 2-5. The Group 6 animals received the basal diet (PMI<sup>®</sup> Feeds, Inc. Certified Rodent Diet<sup>®</sup> #5002) only after Week 78. The positive control males (Group 7) received WY 14,643 in the diet, 7 days per week, for 13 weeks. 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.

Reserve 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<sup>®</sup> lids, and stored frozen. The samples which

were not used for analyses were retained at Covance and were discarded upon issue of the Final Report.

### Analysis of Prepared Formulations

Homogeneity - Homogeneity of the test material in the dietary mixtures was determined prior to the initiation of treatment (Groups 2, 5, and 6), and during Weeks 6 (Group 2), 14 (Groups 2 and 5), and 81 (Groups 4 and 5). 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 - Duplicate samples from the low- and high-dose formulations (500 and 12,000 ppm, respectively) were analyzed to assess 0, 7- and 14-day stability at room temperature.

Routine Concentration - The concentration of the test article at the dietary levels for Groups 1-6 was determined from the samples obtained at Weeks 1, 13, 26, 52, 78, and 104. In addition, the control and 500 ppm dietary level concentration was determined, at the request of the Study Director, on samples obtained at Weeks 2 and 3.

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 Method No. 402 in Appendix 2.

### Observations and Records

Mortality and Clinical Observations - The rats were observed for mortality and moribundity twice daily. A thorough physical examination was conducted at each weighing interval. 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
- appearance
- progression

Body Weight and Food Consumption - Body weights were recorded at randomization, prior to treatment, weekly for Weeks 1-17, and once every 4 weeks thereafter. Food consumption was measured and recorded weekly for Weeks 1-16 and once every 4 weeks thereafter. 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. Blood samples for hematology and serum chemistry evaluations were obtained from the last surviving 10 animals/sex/group during Weeks 26, 52, 78, and 104. Myeloid/erythroid ratios were performed on samples from animals sacrificed at Weeks 79 and 105/106 and when possible, on smears from animals sacrificed in extremis. Samples were obtained via orbital plexus venipuncture from animals that were anesthetized with a gas mixture of 70% carbon dioxide and 30% oxygen. 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                   |

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hematocrit	platelet count
hemoglobin	reticulocyte count
leukocyte differential and cellular morphology <sup>a</sup>	myeloid/erythroid ratios (at necropsy)
leukocyte count	

Serum Chemistry

albumin	inorganic phosphorus
albumin/globulin ratio	potassium
calcium	aspartate aminotransferase
chloride	alanine aminotransferase
creatinine	sodium
gamma glutamyltransferase	total bilirubin
(gamma glutamyl transpeptidase)	total protein
globulin	urea nitrogen
glucose	

<sup>a</sup> The leukocyte differential and cellular morphology analyses were performed for Groups 1 and 5 only.

Urine Chemistry - (presented with the Serum Chemistry)

volume	calcium
osmolality	phosphorus
sodium	creatinine
potassium	creatinine clearance
chloride	

Urinalysis

appearance	occult blood
bilirubin	pH
glucose	protein
ketones	specific gravity
microscopic examination of sediment	urobilinogen

Clinical hematology analyses were performed using a Coulter Counter<sup>®</sup> Model S-Plus IV. Serum and urine chemistry analyses were

performed using a BMD/Hitachi® 704 Chemistry Analyzer. Differential leukocyte counts and cellular morphology quantitations were manually determined from peripheral blood smears. Semiquantitative urinalysis determinations were performed using Ames™ Multistix® or equivalent. Specific gravity was determined with a refractometer.

Terminal Studies

Sacrifice and Gross Pathology - All animals which were 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 food-fasted overnight, weighed the day of scheduled necropsy, given an intraperitoneal injection of sodium pentobarbital, and exsanguinated. Bone marrow smears were obtained from a 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 veterinary pathologist was readily available for consultation. Necropsies included examination of the following:

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

Organ Weights - At one Weeks 79 and 105/106 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
-----------------	--------------------------

spleen	(paired weight)
kidneys (paired weight)	lung
liver	uterus

Organ-to-terminal-body-weight and organ-to-brain-weight ratios were calculated using the fasted terminal body weight recorded at necropsy.

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 <sup>a</sup>	seminal vesicles
eyes with optic nerves <sup>a</sup>	skeletal muscle (thigh) <sup>a</sup>
femur (bone marrow smear) <sup>b</sup>	small intestines (duodenum, jejunum, ileum)
femur with marrow and joint <sup>a</sup>	spinal cord (cervical, lumbar, thoracic)
heart	spleen
kidneys	stomach
large intestines (colon, cecum, rectum)	testes with epididymides
liver	thymus
lung	thyroid with parathyroid
lymph nodes (mesenteric)	trachea
mammary region <sup>a</sup>	urinary bladder
ovaries	uterus with vagina and cervix

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

<sup>b</sup> Smear was fixed in methanol and appropriately stained for analysis of the M:E ratio. The bone used to obtain the smear was not the same femur used for histopathology.

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

#### Postmortem Procedures - Hepatocellular Proliferation/Biochemical Analyses

Animal Selection - The animals selected for the hepatocellular proliferation interim-sacrifice at Weeks 1 and 2 were those animals with the lowest animal identification number assignments within the respective group; all other sacrifices for this analysis were performed utilizing those animals having the highest identification numbers. At the appropriate harvest time, the liver weight was determined, and the following tissues from each satellite animal were preserved in 10% neutral-buffered formalin:

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

The remainder of the liver was immediately frozen at approximately  $-70^{\circ}\text{C}$  and stored frozen for biochemical analysis.

Hepatocellular Proliferation and Biochemical Analyses - The tissue samples from the three liver lobes (right and left lateral, and median), and the duodenum were processed for evaluation of hepatocellular proliferation and analysis of protein concentration, cyanide-insensitive

palmitoyl-CoA oxidation, and DNA concentration from five animals/sex from Groups 1, 5, and 7 sacrificed during Weeks 1, 2, and 13, and from five animals/sex from Groups 1, 4, and 5 sacrificed after 104 weeks of treatment. The procedures are detailed in Attachment No. 3 of Appendix 10.

Histopathology - Hematoxylin-and-eosin-stained sections of liver tissue samples from animals scheduled for evaluation of hepatocellular proliferation in Groups 1, 5, and 7 (sacrificed at Weeks 1, 2, and 13), 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 5.0% one-tailed probability level.

Weekly body weights and food consumption (for Groups 1-6) and the following intervals for mean body weight change (Weeks 1-53 and 1-105) and total food consumption (Weeks 1-52 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.

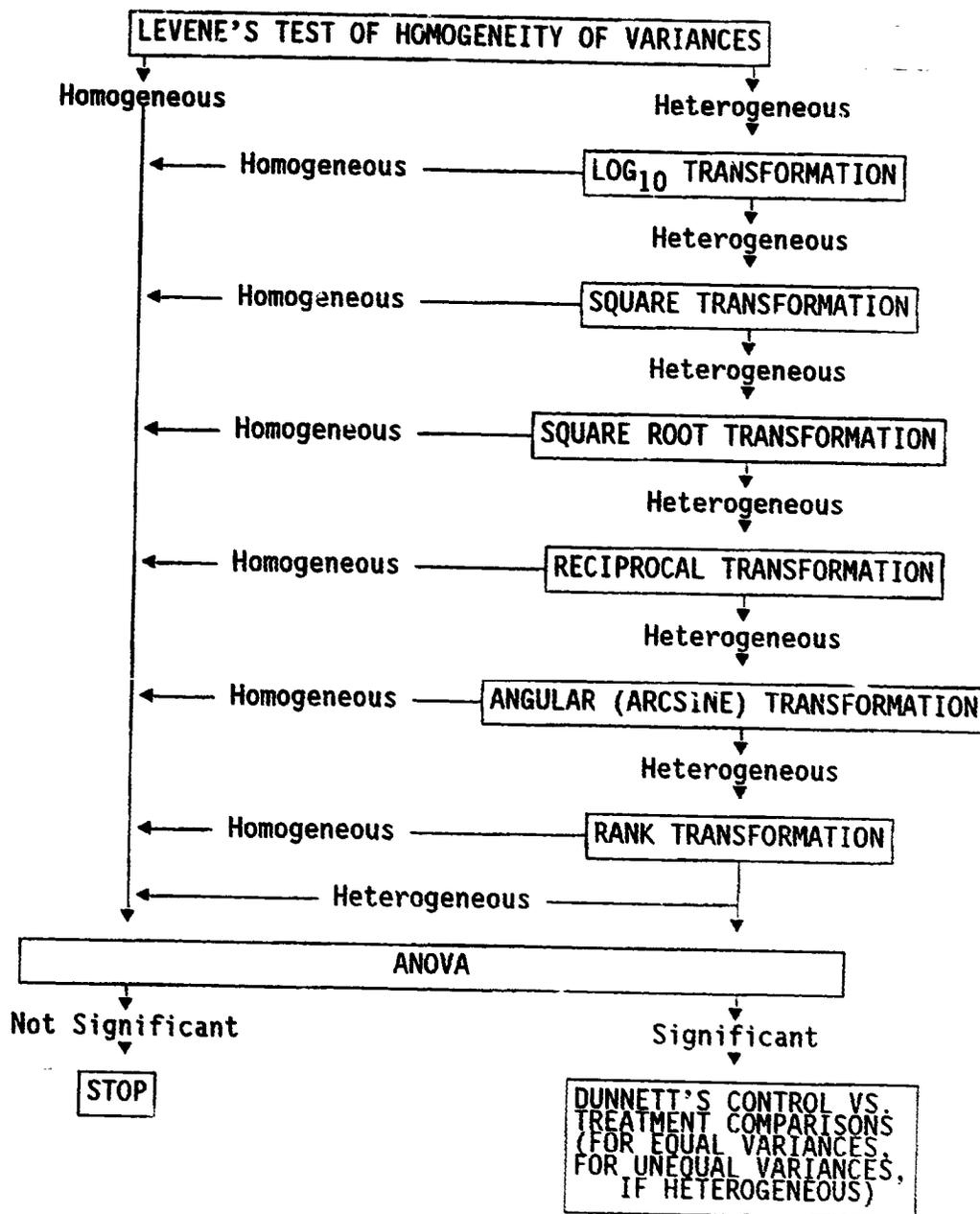
If variances of untransformed data were heterogeneous, a series of transformations was performed in an effort to achieve variance homogeneity. When the series of transformations was not successful in achieving variance homogeneity, analyses were performed on rank-transformed data. Group comparisons were performed at the 5% two-tailed probability level.

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

Specimen, Raw Data, and Final Report Storage

All raw data, documentation, records, protocol, any specimens, 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.

Figure 1  
Flowchart of ANOVA and Related Methods



All parametric comparisons considered variance homogeneity/heterogeneity. All transformations indicated in the flowchart were done on untransformed data.

## 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 - Cells stained with the brown DAB chromogen were observed in the duodenum from all of the animals used in the study. The presence of label in all the animals indicated proper delivery of the BrdUrd label and acceptable immunohistochemical staining. There was no apparent preferential labeling in any of the lobes and the label was random within the lobes, except where necrosis was observed. The necrotic areas were noted but not included in the count.

Microscopic evaluation of H&E-stained liver sections from rats used for evaluation of cell proliferation was performed by a pathologist. In the animals from the terminal sacrifice, several of the rats had leukemia with or without hepatopathy. Animals with leukemia and hepatopathy were not analyzed for cell proliferation since the cell proliferation labeling index could be affected by degenerative/regenerative changes. There were two male rats, one in Group 1 and one in Group 4 that were eliminated. Three female rats were eliminated from evaluation, one in Group 1 and two in Group 5. In addition, two animals that had early leukemia and had elevated labeling indices (one Group 1 and one Group 4 male) were not used in the evaluation since it was not clear if the elevated counts observed were caused by the test article or the leukemia. In the Group 1 male that was eliminated, the labeling index was approximately six times the mean of the labeling index of the remaining three rats. The Group 4 rat that was not used in the evaluation had a labeling index that was approximately five times the mean labeling index of the remaining three rats. Biochemistry (DNA, protein and palmitoyl CoA oxidase) was performed on all livers but the

data from only the animals used for evaluation of cell proliferation is included in the evaluation.

Summary of Labeled Cell Counts for the Liver - The mean labeling index (LI) for each group is presented in the third column of Text Table 1 in the Genetic Toxicology Report.

At the first interim sacrifice, the background labeling indices (Group 1, both sexes) were below 1%. Significant increases were observed in the dosed group (Group 5;  $p \leq 0.05$ ) in both male and female animals. Cell proliferation was not increased by the test chemical at the other three timepoints. The lack of an increase in the LI at Weeks 2, 13 and 104 was observed even though large increases in palmitoyl-CoA oxidase were observed. Large increases in the labeling index ( $p \leq 0.05$ ) were observed in the first sacrifice in the positive control (1000 mg/kg Wy 14,643; Group 7). The positive control response was not considered significantly elevated in the second sacrifice even though the labeling index increased by close to 8-fold. The positive control in the third sacrifice was significantly elevated ( $p \leq 0.01$ ) even though the labeling index in the positive control animals remained less than 1%. The apparent incongruity of the results appears to be related to the variability or consistency of the responses seen among the animals. This is often observed in a weak response. No positive control animals were included for the Week 104 sacrifice.

These results demonstrate that the test chemical induced significant increases in the LI in the liver in male and female rats only during the first week of dosing. At Weeks 2, 13 and 104, the labeling indices for both sexes were similar to the controls. Large increases in the labeling index were observed in the positive control animals at Week 1, but questionable responses were observed at Weeks 2 and 13. The labeling index of Wy 14,643 is known to decrease with time (6,12).

Results from Biochemical Analyses - Palmitoyl-CoA Oxidase Activity - Increases in palmitoyl-CoA oxidase activity were observed in

Group 5 rats (12,000 ppm) at all timepoints. At Week 104, there was also an elevation in palmitoyl-CoA oxidase activity in female rats at 6000 ppm. Male rats treated at 6000 ppm did not have elevated enzyme levels. Animals dosed with 6000 ppm were only evaluated at Week 104. Results are summarized in Text Table 1 (column 4) of the Genetic Toxicology Report and are expressed as nanomoles of NADH generated per minute per mg of protein.

DNA and Protein Analyses - Levels of DNA varied randomly among the groups and at the different timepoints (Text Table 1, column 6). Statistical analysis confirmed the lack of a consistent pattern.

Protein analysis also did not indicate a clear pattern in Groups 4, 5, or 7. Liver weights were significantly elevated in Group 5 animals throughout the study in both male and female rats. The positive control animals included at 1, 2 and 13 weeks were also significantly elevated (Text Table 1, column 7).

Conclusions - Significant increases in the labeling index were observed during Week 1 in the livers of male and female rats treated with the test material. The response was not observed at Week 2, 13, or 104. Increases in palmitoyl CoA oxidase, a monitor of the level of peroxisome proliferation were observed at all four timepoints. Therefore, there is no evidence for sustained cell proliferation associated with the peroxisome proliferation induced by Di(isononyl)phthalate.

#### Analytical Chemistry

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

Homogeneity analyses indicated that the test material was homogeneously mixed; relative standard deviation values were within 3.2%.

Results of stability analyses indicated that the formulations were stable for 14 days at room temperature.

Results of routine concentration analyses indicated that all formulations were within 15% of target. The additional Group 2 samples

that were analyzed during Weeks 2 and 3 averaged 87.8 and 90%, respectively. All other values were within 9% of initial concentration.

### 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 3.

At the beginning of Week 79 (the first day of the recovery period for Group 6), the percent survival in Groups 1-6 was 98, 98, 93, 98, 95, and 98%, respectively, for the males; and 94, 96, 98, 93, 95, and 96%, respectively, for the females. However, at the beginning of Week 104, the percent survival in the males for Groups 1-6 was 74, 71, 78, 66, 54, and 58%, respectively, for the males and 76, 80, 80, 71, 66, and 70%, respectively, for the females.

Statistical evaluation indicated that survival at Week 104 in the Group 5 males was significantly lower than the control group. Analysis of the females revealed no difference in survival between control and treated groups.

Clinical Observations - A summary of daily cageside and weekly physical observations is presented in Table 3. Individual observations are presented in Appendix 4.

The following clinical signs were observed during Weeks 1-105 in males and females of Groups 1-6 and incidences were proportional (in general) to treatment regimen: thin appearance, hunched posture, hypoactivity, prostration, pale body appearance, rough haircoat, urine stains, and few and/or no feces (primarily seen in the males).

Other observed clinical abnormalities occurred sporadically and/or were of the type commonly observed in this species at this laboratory.

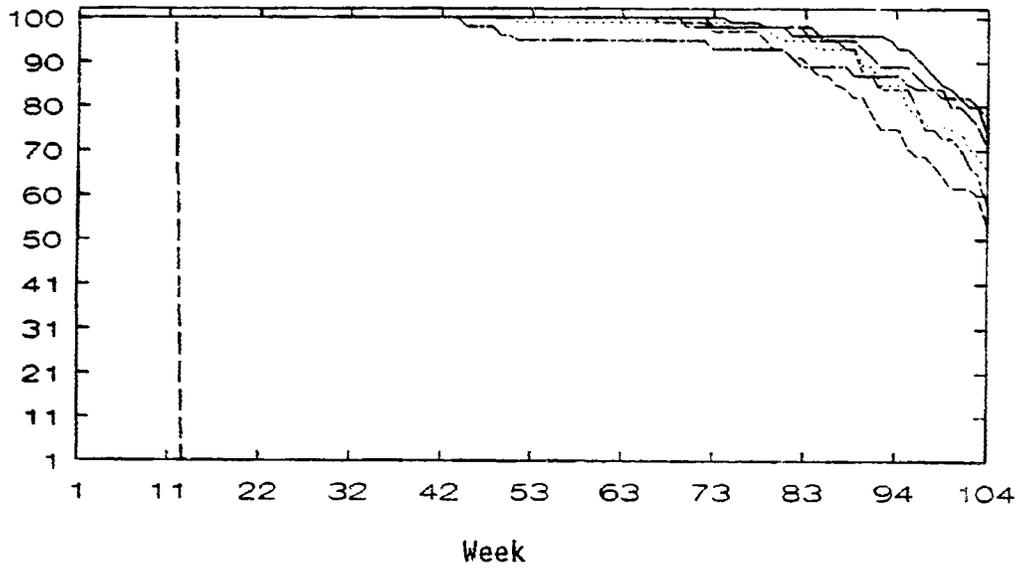
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Figure 2  
Adjusted Survival

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

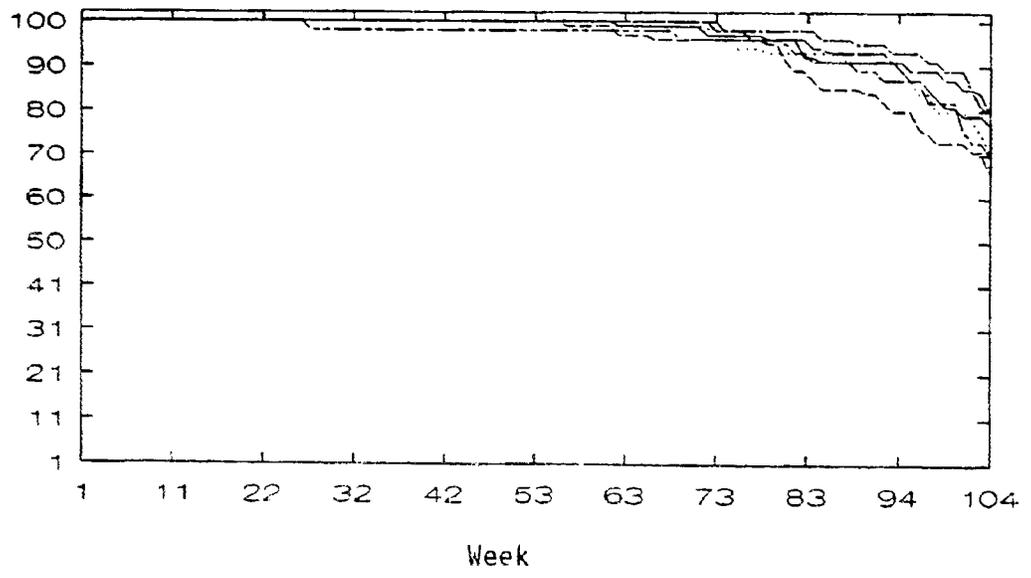
(G)

Males



(G)

Females



Body Weight, and Food and Compound 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 data are presented in Appendix 5. 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 6. Mean compound consumption data are presented in Table 6 and depicted graphically in Figure 5; individual data are presented in Appendix 7.

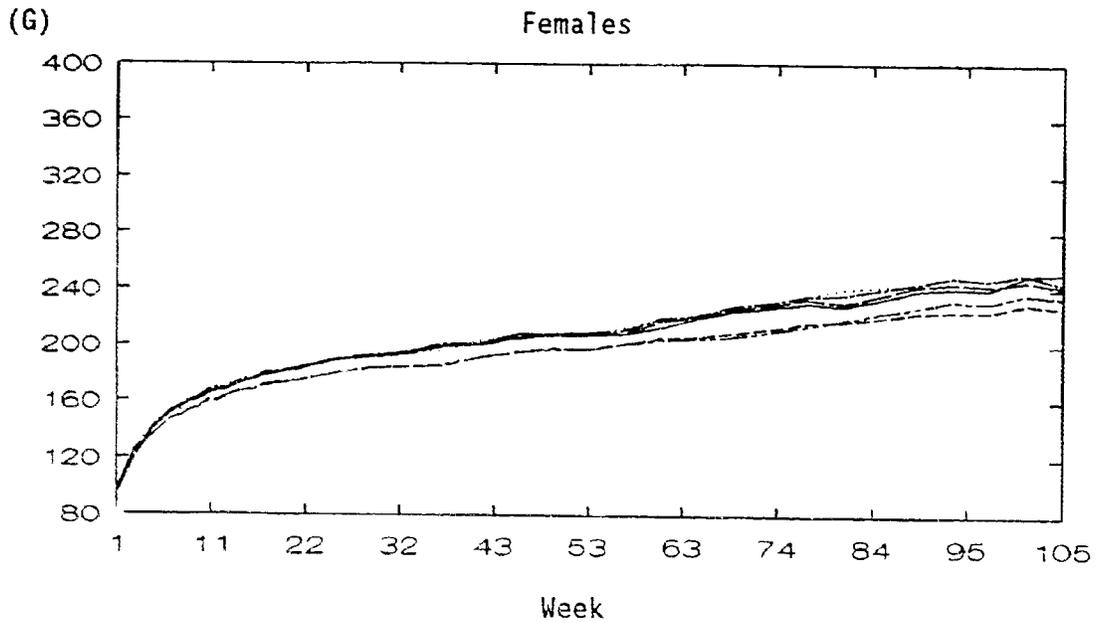
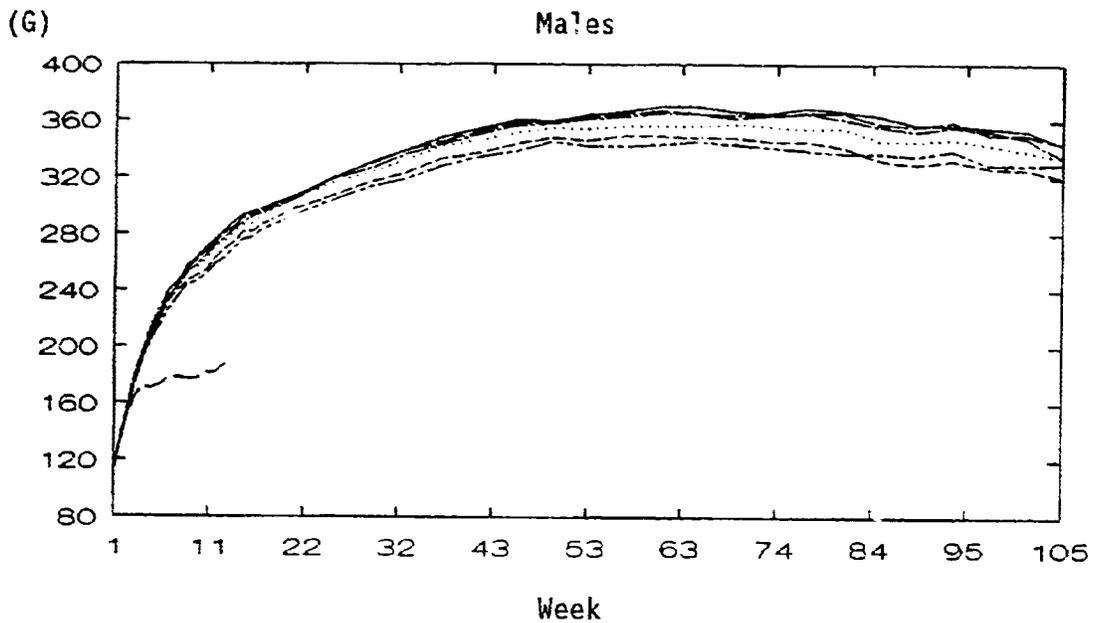
Mean body weights of males and females in Groups 5 and 6 were generally lower than the control animals during the study. When analyzed statistically, the Group 5 and 6 male and female weights were generally significantly lower during Weeks 5-105 and 2-105, respectively.

Throughout the study, mean weekly food consumption values for males and females exhibited sporadic intergroup differences (with occasional statistical significance) lacking any dose- or treatment-related pattern. In females, statistically significant lower mean food consumption values were noted most frequently in Groups 5 and 6. However, even though Group 5 and 6 females were fed the same dietary concentration (12,000 ppm) of Di(isononyl)phthalate, significantly lower mean weekly food consumption values were noted at 18/31 measurement intervals for Group 5, but only 9/31 intervals for Group 6 during Weeks 1-78. Therefore, the statistically significant intergroup differences in mean weekly and total food consumption were spurious findings.

For Weeks 1 through 104, the average daily consumed dose (based on target dietary concentrations of Di(isononyl)phthalate) in Groups 2, 3, 4, and 5, was 29.2, 88.3, 358.7, and 733.2 mg/kg/day, respectively, in males; and 36.4, 108.6, 442.2, and 885.4, respectively, in females. For Weeks 1 through 76 in Group 6 (recovery group), the average daily consumed dose of Di(isononyl)phthalate was 637.3 mg/kg/day in males, and 773.6 mg/kg/day in females.

Figure 3  
Mean Body Weights

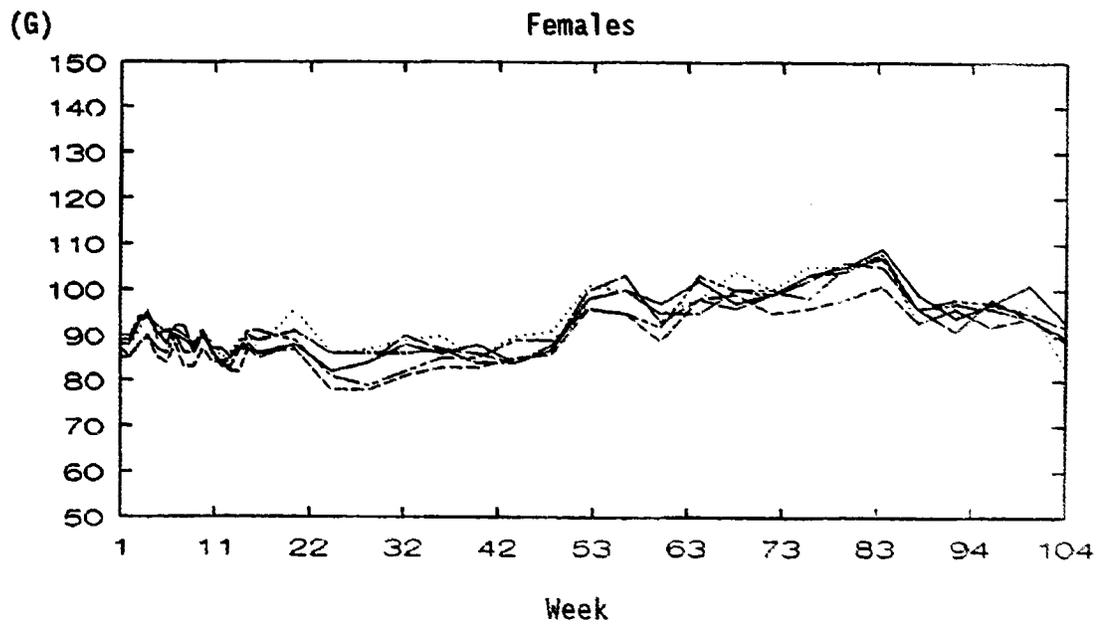
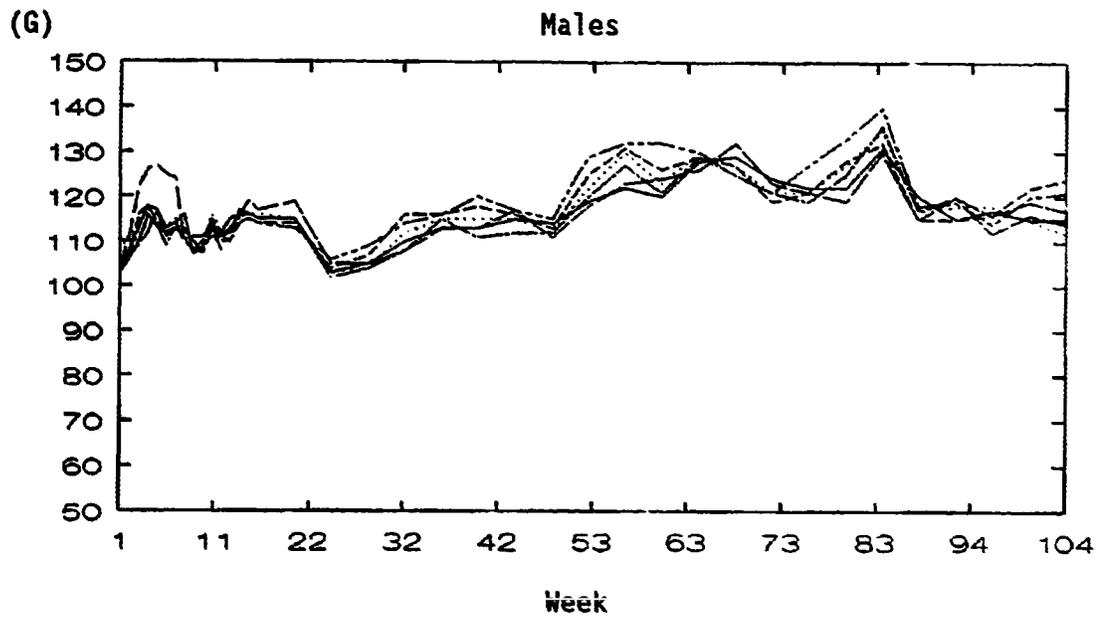
Group 1   Group 2   Group 3   Group 4   Group 5   Group 6



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Figure 4  
Mean Food Consumption

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





### Clinical Pathology

Mean hematology and chemistry values are presented in Tables 7A and 7B, respectively. Individual clinical pathology data are presented in Appendices 8A (hematology), 8B (serum chemistry), and 8C (urinalysis). Findings are further discussed in the Clinical Pathology Report.

Significant changes in the hematology data included mild decreases in the erythrocyte mass (erythrocyte count, hemoglobin, and hematocrit) in Groups 5 and 6 at most intervals; however, the mean values for the Group 6 rats were, in general, not significantly different from controls at Week 104.

Increases (often significant) were observed in the mean values for urea nitrogen, alanine aminotransferase, and aspartate aminotransferase primarily in Groups 5 and 6 throughout the study. The elevations in these analytes corresponded to the histologic findings noted in the kidneys and livers of Group 4 and 6 males and Group 5 animals. Hematopoietic neoplasia (leukemia) was observed in the peripheral blood smears from several Group 5 and 6 animals.

The mean values for urine volume were significantly increased in Group 5 and 6 males at Week 104. The mean creatinine clearance rate was significantly increased in Group 5 females at Week 104; the change was of low magnitude and was not accompanied by a significant change in the mean value for either urine volume, urine creatinine, or serum creatinine. Decreases were observed in the mean values for urine calcium, urine potassium, urine chloride, and urine osmolality in some treated groups (generally Groups 5 and 6) during the study. The significant changes in the aforementioned urinary chemistry tests are of questionable toxicologic importance as they occurred inconsistently over time and did not correlate with concurrent serum chemistry changes.

Urinalyses were generally unremarkable and comparable between control and treated groups at Weeks 26, 52, 78, and 104, with the exception that there was a slight increase in urinary protein in Group 4,

5, and 6 males at Week 26. This transiently elevated urinary protein correlated with the significant increases in serum urea nitrogen in these males at Week 26. The urine specific gravity readings were slightly lower in Group 5 and 6 males relative to controls at Week 104; these decreases correlated with the significantly decreased urine osmolality values observed in these groups at this interval.

#### Terminal Studies

Gross Pathology - Gross pathology findings are summarized in Tables 8A, 8B, and 8C for unscheduled deaths, interim sacrifices, and terminal sacrifice, respectively. Individual gross pathology findings are presented in Appendices 10A (unscheduled deaths), 10B (interim sacrifice), and 10C (terminal sacrifice).

In males and females of Groups 4, 5, and 6, that died or were sacrificed in extremis during the study (unscheduled deaths), the incidence of the following necropsy findings was notably increased: enlarged spleen and granular/pitted/rough appearance of the liver, as well as dark area of the stomach, thoracic cavity fluid, small testis, small seminal vesicle, and uterine mass (however, in animals killed at Week 79, and at study termination, there were no intergroup statistically significant differences in mean absolute weights for testis/epididymis or uterus; and for all deaths combined, there was no histopathological evidence of an effect on spermatogenic activity or the incidence of morphologic abnormalities in the uterus).

At the Week 79 sacrifice, a liver mass was observed in each of two Group 5 males; and various gross-pathologies of the liver (enlarged; irregular shape; granular/pitted/rough appearance; and raised area) were observed in the Group 5 females, when compared to the control group. Small testis and small seminal vesicle were not observed in any males killed from Groups 1, 4 and 5; and the incidence of uterus mass was 0/15, 2/15, and 1/15 in females of Groups 1, 4, and 5, respectively.

At necropsy for study termination, the following abnormalities were observed to have a dose-related increase in Group 4 and 5 males and females: enlarged spleen; enlarged and/or granular/pitted/rough appearance of the liver (seen primarily in the males); dark kidney; dark area of the stomach; various uterine pathologies (cyst, mass, or thickened wall). The incidences for small testis and small seminal vesicle were similar across all groups.

Other observations were incidental to treatment or were commonly seen in this age and strain of laboratory animal.

Organ Weights - Organ weight data are presented in Tables 9A (interim sacrifices - weeks 1, 2, and 13), 9B (interim sacrifice - Week 79 sacrifice), and 9C (terminal sacrifice). Individual data are presented in Appendices 10A (unscheduled deaths), 10B (interim sacrifice - Week 79), and 10C (terminal sacrifice).

Liver organ weights (absolute and relative to body) for all interim sacrifices were significantly increased with dose in animals of Groups 4 and 5, compared to the control group. Spleen and kidney weights (absolute and relative to body) were also statistically increased in Groups 4 and 5 when compared to the control group of animals sacrificed at Week 79. Absolute and relative weights for testis/epididymis or uterus in Groups 4 and 5 were not statistically different compared to Group 1.

In the animals sacrificed after at least 104 weeks of treatment, liver weights (absolute and relative to body or brain) were noted as statistically increased in males and females of Groups 4 and 5. Liver weights in animals of Group 6 (recovery animals) were increased when compared to Group 1 but not significantly. Absolute and relative spleen and kidney weights in females of Group 5 were significantly increased. Statistically significant increases of spleen and kidney weights were observed in females of Group 4. There were no statistically significant differences in absolute and relative weights for testis/epididymis or uterus in any of the Di(isononyl)phthalate groups compared to same sex Group 1 controls.

Histopathology - Expanded microscopic findings are summarized in Tables 10A (unscheduled deaths), 10B (Interim Sacrifices - Weeks 1, 2, 13, and 79), and 10C (Terminal Sacrifices). Individual histopathology findings are presented in Appendices 10A (Unscheduled Deaths), 10B (Interim Sacrifice - Week 79), and 10C (Terminal Sacrifice). The findings are further discussed in the Pathology Report.

One hundred eighty-nine rats (103 males, 86 females) died or were killed in a moribund condition during the study. The most common cause of death was mononuclear cell leukemia in 122 rats (70 males, 52 females), and was most frequently observed in Groups 4, 5, and 6.

Liver changes noted in Group 5 (12,000 ppm) rats sacrificed at Weeks 1, 2, and 13 for cell proliferation studies consisted of increased mitotic activity (Week 1), diffuse hepatocellular enlargement (Weeks 2 and 13), and increased cytoplasmic eosinophilia (Week 13).

Compound-related histomorphologic alterations in animals sacrificed at Week 79, were noted in liver (increased cytoplasmic eosinophilia and diffuse slight or moderate hepatocellular enlargement) and kidney sections from the Group 5 (12,000 ppm) males and females and in the kidney sections from the Group 4 (6,000 ppm) males (mineralization of the renal papilla and increased pigment in renal tubules occurred in the Group 4 and 5 male rats, with the greatest severity in Group 5).

Administration of Di(isononyl)phthalate for at least 104 weeks at levels of 6,000 and 12,000 ppm (Groups 4 and 5, respectively) resulted in compound-related histomorphologic alterations in the liver and kidneys. Liver changes consisting of increased cytoplasmic eosinophilia and hepatocellular enlargement were observed only in the Group 5 (12,000 ppm) animals. An increased incidence of hepatocellular neoplasia was observed in Group 5 rats of both sexes, but was not present in the high-dose recovery group (Group 6). Kidney changes at 104 weeks consisted of mineralization of the renal papilla and increased pigment in tubule cells at 6,000 and 12,000 ppm. Increased mineralization noted in the renal

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papilla of the Group 4, 5, and 6 males was not present in the females. Mononuclear cell leukemia occurred with increased frequency in rats of Groups 4, 5, and 6 and renal tubule cell carcinomas were noted in two Group 5 and four Group 6 males.

Other spontaneous disease lesions, including neoplasms, were of the expected types and severity for rats of this age and strain and are not considered to be an effect of compound administration.

## DISCUSSION AND CONCLUSION

Di(isononyl)phthalate was administered daily to F-344 rats in the diet for at least 104 weeks at dietary concentrations of 0, 500, 1500, 6,000, and 12,000 ppm (Groups 1, 2, 3, 4, and 5, respectively). Rats in Group 6 were administered at dietary concentration of 12,000 ppm for 78 weeks, followed by a 26-week recovery period during which they were administered the basal diet alone.

For Weeks 1 through 104, the average daily consumed dose (based on target dietary concentrations of Di(isononyl)phthalate) in Groups 2, 3, 4, and 5, was 29.2, 88.3, 358.7, and 733.2 mg/kg/day, respectively, in males; and 36.4, 108.6, 442.2, and 885.4, respectively, in females. For Weeks 1 through 76 in Group 6 (recovery group), the average daily consumed dose of Di(isononyl)phthalate was 637.3 mg/kg/day in males, and 773.6 mg/kg/day in females. Therefore, in each of the Di(isononyl)phthalate dose groups, the actual daily dose consumed by females was 21 to 25% greater than that consumed by males.

A treatment-related decrease in survival occurred in males and females of Group 4 (6,000 ppm), Group 5 (12,000 ppm), and Group 6 (12,000 ppm followed by recovery), with a statistically significant decrease in the Group 5 males. Up to Week 79, survival was at least 93% in all groups with survival in all treated groups comparable to that of the controls. However, at Week 104, survival in Groups 1, 2, 3, 4, 5, and 6 was 74%, 71%, 78%, 66%, 54%, and 58%, respectively, in the males; and 76%, 80%, 80%, 71%, 65%, and 70%, respectively, in the females.

Treatment-related clinical abnormalities observed during daily cageside and weekly physical examinations were urine stains and signs of ante-mortem condition, for which the incidences were most notably increased in the Group 4, 5, and 6 males and females. Signs of ante-mortem condition included hunched posture, entire body pale, thin,

hypoactive, few or no feces. However, there was no treatment- or dose-related increase in either sex for the incidence of palpable masses.

Treatment-related depressions of body weight gain occurred in males and females of Group 4 (6000 ppm), as well as Groups 5 and 6 (12,000 ppm); although a rebound in body weight gain occurred in Group 6 animals during the recovery phase (Weeks 79-104). Compared to the control mean values, mean total body weight gain in Groups 4, 5, and 6 during Weeks 1-52 was 3.2%, 7.3%, and 9.3% lower, respectively, in the males; and 0.0%, 9.0%, and 8.1% lower, respectively, in the females. However, mean total body weight gain in Groups 4, 5, and 6 for Weeks 1-104 was 2.7%, 10.2%, and 5.8% lower, respectively, in the males; and 6.5%, 14.9%, and 9.1% lower, respectively, in the females, compared to control values.

There were no treatment-related effects on food consumption. Throughout the study, mean weekly food consumption values in males and females exhibited sporadic intergroup differences (with occasional statistical significance) lacking any dose- or treatment related pattern. In the Group 5 females, mean total food consumption values were significantly lower for Weeks 1-52, and 1-104; however, values for the same time period were not significantly different from control values in the Group 6 females, which received the same dietary concentration (12,000 ppm) of Di(isononyl)phthalate during the first 78 weeks as did the Group 5 females. Therefore, the significantly lower mean total food consumption values for the Group 5 females were spurious findings.

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 the liver and kidneys of Groups 4, 5, and 6. Evaluation of hematology parameters at Weeks 26, 52, 78, and 104 in 10 rats/sex/group, revealed a decrease in erythrocyte mass (erythrocyte count, hemoglobin, and hematocrit) in Group 4 (6,000 ppm), with a more severe decrease evident in Groups 5 and 6 (12,000 ppm) at all intervals. The decreases in

erythrocyte mass correlated with the severity of body weight gain depression in Groups 4, 5, and 6; and were not accompanied by concurrent significant increases in mean values of percentage or absolute counts for reticulocytes. The decrease of erythrocyte mass seemed to be reversible in that at Week 104, mean values for indices of erythrocyte mass in Group 5 remained significantly lower than control values; whereas mean values for Group 6 (after recovery) were generally lower compared to control values, but none of the differences were statistically significant.

Treatment-related serum chemistry findings in Groups 4, 5, and 6, were increased mean values of urea nitrogen, correlating with histologic evidence of kidney toxicity; and increased mean values for aspartate aminotransferase (AST) and alanine aminotransferase (ALT), correlating with histologic evidence of liver toxicity. In Group 4 (6,000 ppm) and Groups 5 and 6 (12,000 ppm), urea nitrogen mean values generally were increased at all intervals (Weeks 26, 52, 78, and 104), with the increases being within 32% of control values in the Group 4 males and females; and within 50% of control values in the Group 5 and 6 males and females. After the 26-Week recovery period, the mean value for urea nitrogen (Week 104) in the Group 6 males remained elevated (approximately 47% greater than the control male value), and was comparable to the mean value for the non-recovery Group 5 males; whereas the mean value for urea nitrogen (Week 104) in the Group 6 females was comparable to the mean value of the control females. Compared to control mean values, there were no significant increases at Week 26 for AST or ALT in any of the Di(isononyl)phthalate dose groups. However, at Weeks 52, 78, and 104, all mean values for AST and ALT in males and females of Groups 4, 5, and 6 were increased compared to control mean values, with many of the differences being statistically significant. After the 26-Week recovery period, mean values for AST and ALT (Week 104) in the Group 6 males and

females were increased compared to control values, with no indication of a reversible effect compared to the Group 5 rats.

There were no treatment-related findings for urine chemistry or urinalysis parameters. Mean values for urine volume were significantly increased in the Group 5 and 6 males only at Week 104, with corresponding decreases in mean values for urine osmolality, specific gravity, and electrolyte concentrations. The increased urine volume in the Group 5 and 6 males was not treatment-related since it occurred only at one analysis interval and with no concomitant effect evident in females of the same dose groups. For mean creatinine clearance rates, there were no significant differences between control and treated groups at all analysis intervals (Weeks 26, 52, 78, and 104); with the exception of a significant increase in the Group 5 (12,000 ppm) females at Week 104. The increase was not accompanied by a significant change in the mean value for either urine volume, urine creatinine, or serum creatinine, and therefore may have been a spurious finding.

Treatment-related findings in the necropsy and organ weight data also indicated the liver and kidneys to be target organs for Di(isononyl)phthalate toxicity. In unscheduled deaths, as well as rats killed at Week 79 and study termination, livers that appeared enlarged and/or granular/pitted/rough were observed with greatest frequency in the Group 4, 5, and 6 males and females. Liver weight data indicated that liver enlargement in males and females occurred only at the 6,000 and 12,000 ppm dietary concentrations, and was detectable after the first week of the study. In rats killed after Weeks 1, 2, and 13, statistically significant increases in mean liver weights (absolute and relative to body weight) were observed only in the Group 4 (6,000 ppm) and Group 5 (12,000 ppm) males and females, with mean values being greater in the Group 5 rats compared to Group 4. At Week 79, mean liver weights (absolute and relative to body weight, and to brain weight) were again increased, with dose, in the Group 4 (6,000 ppm) and Group 5 (12,000 ppm) males and

females. In rats killed at study termination (Week 105), statistically significant increases in mean liver weights (absolute and relative) were again observed only in the Group 4 (6,000 ppm) and Group 5 (12,000 ppm) males and females. However, the liver enlargement appeared to be reversible, in that mean liver weight values (absolute and relative) at study termination in the recovery group (Group 6) were comparable to control mean values, even though males and females in Group 6 had been fed the 12,000 ppm diet up to Week 79. A treatment-related increase in detectable liver masses was evident only in the Group 5 (12,000 ppm) males, and the onset of liver masses appeared to occur after Week 79. At the Week 79 necropsy, liver masses were detected only in 2/15 of the Group 5 males. At Week 105, only the Group 5 males exhibited an incidence of animals with liver masses (10/32; 31%) that was greater than the control incidence (3/41; 7%); and the incidence (2/34; 6%) in the recovery males (Group 6) was less than the control group incidence. In the Group 1, 4, 5, and 6 females, the incidence of animals with liver masses at Week 105 was 0/42, 1/38, 1/37, and 1/39, respectively, and thus no evidence of a treatment-related increase.

The kidneys appearing dark was the only gross abnormality in kidneys exhibiting a treatment-related increase, most notable in rats from Groups 4, 5, and 6. At Week 79, mean kidney weights (absolute and/or relative to body and brain weight) were significantly increased, with dose, in the Group 4 (6,000 ppm) and Group 5 (12,000 ppm) males and females. In rats killed at study termination (Week 105), statistically significant increases in mean kidney weights (absolute and/or relative) were again observed only in the Group 4 (6,000 ppm) and Group 5 (12,000 ppm) males and females. However, the kidney enlargement appeared to be reversible, in that mean kidney weight values (absolute and relative) in the recovery males and females (Group 6) at study termination were comparable to control mean values.

Histopathology findings confirmed that the liver and kidneys were target organs of Di(isononyl)phthalate toxicity. In correlation with the increased liver weights in the Group 4 and 5 rats after Week 1, increased numbers of mitotic cells were observed in livers of 5/5 males and 5/5 females of Group 5 (12,000 ppm) killed after one week of treatment, compared to the controls (Group 1). A second indicator of cell proliferation, the mean labeling index for hepatocytes, was significantly increased in the Group 5 males and females, compared to the Group 1 controls. Additionally, palmitoyl-CoA oxidase activity, an indicator of peroxisome proliferation, was also significantly increased in the Group 5 males and females compared to the Group 1 controls after Week 1.

After Week 2, diffuse hepatocellular enlargement was evident in 5/5 males (mean severity of 2.0, slight) and 5/5 females (mean severity of 1.8, slight) of Group 5; and palmitoyl-CoA oxidase activity was again significantly increased in the Group 5 males and females compared to the Group 1 controls. However, there was no histologic evidence of increased numbers of mitotic cells, and the mean labeling index for the Group 5 males or females was not significantly different from the mean values for the controls (Group 1), suggesting that peroxisome proliferation, but not cell proliferation, was still occurring in the livers of the Group 5 (12,000 ppm) males and females.

After Week 13, diffuse hepatocellular enlargement was again evident in 5/5 males (mean severity of 2.6, moderate) and 5/5 females (mean severity of 2.0, slight) of Group 5; and palmitoyl-CoA oxidase activity was again significantly increased in the Group 5 males and females compared to the Group 1 controls. Again, there was no detectable histologic or biochemical evidence of cell proliferation. Increased cytoplasmic eosinophilia was the only other histologic abnormality in the liver for which the incidence in the Group 5 males (5/5) and females (2/5) was notably increased compared to the Group 1 males (0/5) and females (0/5).

At Week 79, histopathological examination of livers from Groups 1, 4 (6,000 ppm), and 5 (12,000 ppm) indicated treatment-related findings were only present in the 12,000 ppm males and females. In Group 5, diffuse hepatocellular enlargement was again evident in 10/10 males (mean severity of 2.2, moderate) and 9/10 females (mean severity of 1.9, slight); and palmitoyl-CoA oxidase activity was again significantly increased in males and females. Increased cytoplasmic eosinophilia was evident only in livers from Group 5 for which the incidence was 7/10 in males, and 8/10 in females. Additionally, pigment in Kupffer cell/bile canaliculi was evident in 5/10 of the Group 5 males. The first detection of liver neoplasms occurred at the Week 79 sacrifice, but with no treatment-related incidence. Of the 10 rats/sex examined from Groups 1, 4, and 5, hepatocellular adenoma was detected in only one control male and one Group 5 female; and hepatocellular carcinoma was detected in only one Group 5 male.

At study termination, after 104 weeks of treatment, treatment-related histopathological findings occurred with increased incidence only in livers of the Group 5 (12,000 ppm) males and females. In contrast, males and females in Group 4 (6,000 ppm), as well as the recovery group (Group 6 - Di(isononyl)phthalate 12,000 ppm up to Week 79) were remarkably free of treatment-related histologic findings. Diffuse hepatocellular enlargement was evident only in Group 5 males (14/32, mean severity = 0.9) and females (27/37, mean severity = 1.5). As an indicator of peroxisome proliferation, palmitoyl-CoA oxidase activity was again significantly increased in the Group 5 males and females. Palmitoyl-CoA oxidase activity was also significantly increased in the Group 4 (6,000 ppm) females, but not males, and mean liver weights (absolute and relative) were significantly increased in the Group 4 males and females. However, there was no detectable histologic evidence of hepatocellular enlargement in either sex in Group 4. Palmitoyl-CoA oxidase activity was not evaluated for Group 6, so it is unknown whether enzyme activity was

elevated after the recovery period in comparison to the controls. Increased cytoplasmic eosinophilia was evident only in livers from Group 5 for which the incidence was 26/32 in males, and 30/37 in females. In Groups 1, 4, 5, and 6, the incidence of pigment in Kupffer cell/bile canaliculi was 1/41, 0/36, 5/32, and 2/29, respectively, in the males; and 5/42, 5/38, 14/37, and 9/34, respectively, in the females. Most notable were the intergroup differences in the incidences of liver neoplasms. In Groups 1, 4, 5, and 6, the incidence of hepatocellular adenomas was 2/41, 4/36, 8/32, and 4/29, respectively, in the males; and 0/42, 1/38, 0/37, and 0/34, respectively, in the females. In Groups 1, 4, 5, and 6, the incidence of hepatocellular carcinomas was 1/41, 1/36, 9/32, and 1/29, respectively, in the males; and 1/42, 1/38, 3/37, and 1/34, respectively, in the females. Therefore, compared to the controls, the incidence of liver neoplasms was clearly increased in males and females of Group 5 (12,000 ppm), and was not notably increased in either sex of Group 4 (6,000 ppm) or Group 6.

Liver neoplasms were first detected at the Week 79 interim kill, and subsequently detected in unscheduled deaths, as well as animals killed at study termination. It appears that liver neoplasms developed in the Group 5 (12,000 ppm) animals primarily during the last 26 weeks of the study, and did not develop in the Group 6 animals during the 26-week recovery period (after 78 weeks of treatment at 12,000 ppm). As presented in the following table, the incidence of liver neoplasms in all deaths (unscheduled plus interim and terminal kills) mirrored the findings at study termination, with a notable increase in Group 5 males and females.

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Total Incidence of Hepatoceellular Neoplasia												
Sex	Male						Female					
Group	1	2	3	4	5	6	1	2	3	4	5	6
Number Examined	80	50	50	65	80	50	80	50	50	65	80	50
Hepatoceellular												
Adenoma	4	4	2	6	10	5	0	1	0	1	3	1
Carcinoma(s)	1	0	0	1	11	2	1	0	0	1	5	2
Multiple Neoplasms <sup>a</sup>	0	0	0	1	4	0	0	0	0	0	0	1
Total Rats with hepatoceellular Neoplasms	5	4	2	7	17	7	1	1	0	2	8	2

<sup>a</sup> Both adenoma and carcinoma or multiple carcinomas.

Treatment-related histopathological findings in the kidney included an increase in the Group 4, 5, and 6 males, but not females, of the incidence and severity of mineralization of the renal papilla; an increase in the Group 4, 5, and 6 males and females of the incidence and severity of renal tubule cell pigment; and the presence, only in Group 5 and 6 males, of renal tubule cell carcinomas. At the Week 79 interim kill, the incidence and mean severity grade for mineralization of the renal papilla in the Group 1, 4, and 5 males was 5/10 (mean severity = 0.5), 10/10 (mean severity = 2.0), and 10/10 (mean severity = 3.5), respectively. At study termination, the incidence and mean severity grade for mineralization of the renal papilla in the Group 1, 3, 4, and 5 males was 6/36 (mean severity = 0.2), 9/39 (mean severity = 0.2), 30/31 (mean severity = 1.7), 25/27 (mean severity = 2.6), and 29/29 (mean severity = 2.9), respectively. Therefore, mineralization of the renal papilla in males at Di(isononyl)phthalate dose levels of 6,000 and 12,000 ppm was not reversible, in that there was no evidence of a decrease in incidence or severity in Group 6, after the 26-week recovery period, compared to Group 5. Increased incidence and severity of renal tubule cell pigment was evident in the Group 4 and 5 males, and Group 5 females at the Week 79

interim kill. At study termination, increased incidence and severity of renal tubule cell pigment was evident in Group 4, 5, and 6 males and females. In Group 1, 3, 4, 5, and 6 males, at study termination, the incidence and mean severity grade of renal tubule cell pigment was 34/36 (mean severity = 1.2), 39/39 (mean severity = 1.5), 31/31 (mean severity = 2.3), 27/27 (mean severity = 2.9), and 29/29 (mean severity = 2.1), respectively. In Group 1, 3, 4, 5, and 6 females, at study termination, the incidence and mean severity grade of renal tubule cell pigment was 36/37 (mean severity = 1.4), 40/40 (mean severity = 1.2), 33/33 (mean severity = 2.0), 32/32 (mean severity = 2.4), and 34/34 (mean severity = 2.0), respectively. For increased renal tubule cell pigment, Group 3 (1,500 ppm) was the no-effect-level, and severity increased with dose in the 6,000 ppm and 12,000 ppm (Groups 4 and 5) males and females. In Group 6, the severity was comparable to that of Group 4, suggesting that some reversal of the effect in males and females occurred during the recovery phase. Kidney carcinomas were detected only in males, and only in males of Groups 4, 5, and 6. A malignant transitional cell carcinoma was detected in the kidney of one Group 4 male, and malignant tubule cell carcinomas were detected in two Group 5 males, and four Group 6 males. Of the four Group 6 males with malignant tubule cell carcinomas, three were animals killed at study termination.

There was an association between treatment and the occurrence of mononuclear cell leukemia in Groups 4, 5, and 6. The incidence of mononuclear cell leukemia was increased in the Group 4, 5, and 6 males and females, with the highest incidence occurring in Group 6. In unscheduled deaths, mononuclear cell leukemia was the most common cause of death in all groups, but was observed with greater frequency in unscheduled deaths of either sex in Groups 4, 5, and 6. Correlating with the increased incidence of leukemia, the spleen was noted to be enlarged more frequently in unscheduled as well as scheduled deaths from Groups 4, 5, and 6. At Week 79, statistically significant increases of mean spleen weights

(absolute and relative to body and brain weight) were detected only in the Group 5 (12,000 ppm) males and females. However, at Week 105, mean spleen weights (absolute and relative) were increased, but in the absence of a dose-response, in the Group 4, 5, and 6 males and females. For unscheduled deaths, as well as rats killed at Week 79 and study termination, the combined incidence of mononuclear cell leukemia in Groups 1, 4, 5, and 6 was 22/65 (34%), 32/65 (49%), 30/65 (46%), and 31/50 (62%), respectively, in the males; and 17/65 (26%), 29/65 (45%), 30/65 (46%), and 24/50 (48%), respectively in the females.

Other than liver, kidneys, and hematopoietic tissue, there was no evidence of a treatment-related effect. No treatment-related effect on organ weight was evident for the lungs, uterus, testis with epididymis, or brain with stem. Although the testes and/or seminal vesicles were more frequently noted at necropsy as small in the Group 4, 5, and 6 males, mean organ weight values (absolute and relative) for testes at Week 79 and 105 in the Group 4, 5, and 6 males were comparable to mean values for the control males. In male reproductive organs, including testes and seminal vesicles, there was no histologic evidence of treatment-related toxicity or carcinogenicity. Although a greater incidence of uterine gross lesions (cyst, mass, or thickened wall) were observed in the Di(isononyl)phthalate groups compared to the controls, there were no significant intergroup differences in uterus mean weights (absolute and relative) at either the Week 79 or terminal necropsies, and there was no histologic evidence of treatment-related toxicity or carcinogenicity in the uterus.

In conclusion, for F-344 rats, a dietary concentration of 1,500 ppm Di(isononyl)phthalate (Group 3) was the no-effect-level for systemic toxicity, with no evidence of carcinogenic potential. A dietary concentration of 6,000 ppm (Group 4) induced gross and microscopic evidence of liver and kidney toxicity, and an increased incidence of mononuclear cell leukemia in males and females. A dietary concentration of 12,000 ppm (Group 5) also induced gross and microscopic evidence of

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liver and kidney toxicity, as well as an increased incidence of hepatocellular neoplasms in males and females, renal carcinomas in males, and an increased incidence of mononuclear cell leukemia in both sexes. In Group 6, administration of a dietary concentration of 12,000 ppm for 78 weeks, followed by a 26-week recovery period indicated that toxic effects of Di(isononyl)phthalate in the liver, including the induction of neoplasms, were reversible; and toxic effects in the kidney, with the exception of mineralization of the renal papilla in males, were at least partially reversible. However, the incidence of mononuclear cell leukemia in males and females of Group 6 was not decreased compared to Group 5, but was higher in both sexes than for any other study group.

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*May 13, 1998*

Date

## CLINICAL PATHOLOGY REPORT

Summary and Conclusion

The test material, Di(isononyl)phthalate, was administered to Fischer-344 rats at dietary levels of 0, 500, 1500, 6000, 12000, and 12000 ppm (Groups 1-6, respectively) for at least 104 weeks. Hematology, chemistry, and urinalysis tests were performed at Weeks 26, 52, 78, and 104. Differential leukocyte counts and cellular morphology examinations were performed for Groups 1, 5, and 6 at Weeks 26, 52, 78 (Groups 1 and 5 only), and 104. Myeloid/erythroid ratios were determined from bone marrow samples collected from Group 1, 4, and 5 animals at Week 79 and from all groups at study termination (designated as Week 104). Significant changes in the hematology data included mild decreases in the erythrocyte mass (erythrocyte count, hemoglobin, and hematocrit) in Groups 5 and 6 at most intervals; however, the mean values for the Group 6 rats were, in general, not significantly different from controls at Week 104. Increases (often significant) were observed in the mean values for urea nitrogen, alanine aminotransferase, and aspartate aminotransferase primarily in Groups 5 and 6 throughout the study; the elevations in these analytes corresponded to the histologic findings noted in the kidneys and livers of Group 4 and 6 males and Group 5 animals. Hematopoietic neoplasia (leukemia) was observed in the peripheral blood smears from several Group 5 and 6 animals.

Results and Discussion

This laboratory has not established reference ranges for hematology and chemistry parameters in male and female Fischer-344 rats for each study interval included in this report. The mean values for these parameters were therefore compared to reference ranges established by this laboratory for animals in a similar age group (study interval).

Hematology - Significant decreases were observed in the erythrocyte mass, as illustrated in Text Table 1.

Text Table 1  
Significant Alterations in the Hematology Data

Group/Sex	Week:	ERYTHROCYTE				HEMOGLOBIN				HEMATOCRIT			
		26	52	78	104	26	52	78	104	26	52	78	104
3/F		↓								↓	↓		
4/M		↓	↓			↓	↓			↓	↓		↓
4/F			↓		↓					↓	↓		
5/M		↓	↓	↓		↓	↓	↓	↓	↓	↓	↓	↓
5/F		↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
6/M		↓	↓			↓	↓			↓	↓	↓	
6/F		↓	↓	↓			↓			↓	↓	↓	

↓ = Significantly lower than control value,  $p \leq 0.05$ .

These decreases correlated with the significantly lower body weights observed for the Group 5 and 6 rats and are attributed to the administration of the test material; it should be noted, however, that most of the mean values were within the reference ranges established by this laboratory for age-approximated, sex-matched rats. There were no concurrent significant increases in the mean values for percentage and absolute reticulocytes for the affected groups. Significant changes in the erythrocyte indices included decreases for the mean cell volume in Group 5 and 6 females at Weeks 26 and 52 and Group 4 males and Group 5 and 6 females at Week 78 and increases in mean cell hemoglobin in Group 4 females at Week 52 and mean cell hemoglobin concentration in Group 4, 5, and 6 animals at Week 26, Group 3, 4, 5, and 6 females at Week 52, and Group 4, 5, and 6 females at Week 78.

The significant differences in the remaining hematology parameters are considered incidental to the administration of the test

material due to the low magnitude of the changes, the lack of a dose response, the inconsistency of the differences over time, and/or the fact that the mean and most individual values were within the reference ranges established by this laboratory for age- and sex-matched rats; they are listed in this report for documentation purposes. Significant increases were observed in the mean values for total leukocyte count (but not corrected leukocyte counts) in Group 6 males at Week 26 and Group 4 females at Week 78; corrected leukocyte count (but not total leukocyte count) in the Group 5 females at Week 26; and lymphocytes in Group 5 females and Group 6 males at Week 26. The mean basophil count was significantly, but negligibly, decreased in Group 5 females at Week 26.

The cellular morphology findings were generally unremarkable and comparable between control and Group 5 and 6 animals at Weeks 26, 52, 78 (Groups 1 and 5 only), and 104, with the exception of an increase in the incidence of polychromasia in Group 5 and 6 animals at Week 104. There were no significant differences in the mean myeloid/erythroid ratios between control and Groups 4 and 5 at Week 79 or between control and treated groups at Week 104. Evidence of hematopoietic neoplasia was observed or suspected in Group 5 male B27962 at Week 78 and Group 5 males B27950 and B27957, Group 5 female B28044, Group 6 male B28097, and Group 6 female B28147 at Week 104.

Serum and Urine Biochemistry - The significant differences observed in urea nitrogen, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) are illustrated in Text Table 2.

Text Table 2  
Significant Changes in Chemistry Data

Group/Sex	Week:	UREA NITROGEN				AST				ALT			
		26	52	78	104	26	52	78	104	26	52	78	104
3/F							↑						↑
4/M		↑					↑		↑		↑		↑
4/F		↑	↑						↑		↑		↑
5/M		↑	↑	↑	↑		↑				↑		
5/F		↑	↑	↑			↓				↓		
6/M		↑	↑	↑			↑		↑		↑		
6/F		↑	↑	↑			↓		↑		↓		↑

↑ = Significantly higher than control value, p ≤ 0.05.  
AST = Aspartate Aminotransferase  
ALT = Alanine Aminotransferase

The elevations in urea nitrogen are of low magnitude and are not accompanied by increases in the mean creatinine values; they are attributed to the administration of the test material in Group 4 and 6 males and Group 5 animals, however, as they correlate with the histologic evidence of renal involvement and some of the mean values are above the reference ranges established by this laboratory for age-approximated, sex-matched Fischer-344 rats. Similar increases were not observed in the mean values for creatinine in these groups at these intervals; in fact, the mean creatinine values were significantly, but slightly, decreased in Group 5 and 6 females at Week 78.

The mean values for alanine aminotransferase and aspartate aminotransferase were consistently higher in Group 4, 5, and 6 rats than concurrent controls at Weeks 52, 78, and 104; they were above the

reference ranges established by this laboratory for age-approximated, sex-matched Fischer-344 rats for Group 4, 5, and 6 animals at one or more intervals. These elevations in ALT and AST values are attributed to the administration of the test material and correlate with the histologic liver findings noted in Group 4 males and Group 5 males and females.

There were no changes observed for the mean total protein values of any treated group. Significant increases, however, were noted for mean albumin values in Group 4, 5, and 6 animals at Week 26, Group 5 and 6 animals at Week 52, and Group 4 males and Group 5 and 6 animals at Week 78, while significant decreases were observed for mean globulin values in Group 4 females and Group 5 and 6 animals at Weeks 26 and 52, Group 5 animals and Group 6 males at Week 78, and Group 5 males at Week 104. The aforementioned changes may be related to the administration of the test material, but the mechanism for the changes is not apparent from the remainder of the clinical laboratory data.

Significant, but mild, decreases were observed in the mean values for glucose in Group 4 females at Week 26 and Group 3 females at Weeks 52 and 104, total bilirubin in Group 5 and 6 females at Week 26, and sodium in Group 5 males at Week 26 and Group 5 and 6 males at Week 52. Significant increases were noted in mean values for glucose in Group 6 males at Week 52 and total bilirubin in Group 2, 4, and 6 males at week 26. The mean calcium values were significantly decreased in Group 4 animals and Group 5 females and increased in Group 5 males at Week 104; the changes are considered incidental to the administration of the test material due to the low magnitude of the changes and the lack of a dose response.

The mean values for urine volume were significantly increased in Group 5 and 6 males at Week 104. The mean creatinine clearance rate was significantly increased in Group 5 females at Week 104; the change was of low magnitude and was not accompanied by a significant change in the mean value for either urine volume, urine creatinine, or serum creatinine.

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Significant decreases were observed in the mean values for urine calcium in Group 4, 5, and 6 males at Week 26, Group 5 and 6 males at Week 52, Group 5 males at Week 78, and Group 5 females at Week 104; urine potassium in Group 5 and 6 males at Week 104; urine chloride in Group 5 males at Week 104; and urine osmolality in Group 5 and 6 males at Week 104. The decreases in the urine analytes for the males at Week 104 correlate with the increased urine volumes. The mean urine phosphorus values were significantly increased in Group 4, 5, and 6 females at Week 52. The significant changes in the aforementioned urinary chemistry tests are of questionable toxicologic importance as they occurred inconsistently over time and did not correlate with concurrent serum chemistry changes.

Urinalysis - Urinalyses were generally unremarkable and comparable between control and treated groups at Weeks 26, 52, 78, and 104, with the exception that there was a slight increase in urinary protein in Group 4, 5, and 6 males at Week 26. This transiently elevated urinary protein correlated with the significant increases in serum urea nitrogen in these males at Week 26. Other findings included increased crystals in Group 5 and 6 males at Week 78 and a slight decrease in the incidence of bacteria in treated females at Week 52; they do not correspond to changes in the remainder of the clinical laboratory data and are not definitively attributed to Di(isononyl)phthalate administration. The urine specific gravity readings were slightly lower in Group 5 and 6 males relative to controls at Week 104; these decreases correlated with the significantly decreased urine osmolality values observed in these groups at this interval.

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## PATHOLOGY REPORT

General Protocol

The purpose of this study was to evaluate the oncogenic potential of Di(isononyl)phthalate when administered to rats in the diet for at least 104 weeks and to provide information on the ability of Di(isononyl)-phthalate to cause hepatocellular proliferation and peroxisomal proliferation.

The control group (Group 1), consisting of 85 rats/sex, received the basal diet only. The low- and mid-low-dose groups (Groups 2 and 3), consisting of 70 rats/sex/group, received the test article at dose levels of 500 and 1,500 ppm, respectively. The mid-high- and high-dose groups (Groups 4 and 5), consisting of 85 rats/sex/group, received the test article at dose levels of 6,000 and 12,000 ppm, respectively. A recovery high-dose group of 55 rats/sex (Group 6) received 12,000 ppm Di(isononyl)phthalate; the rats in this group were removed from the test diet after 78 weeks of administration. A positive control group (Group 7), consisting of 15 male rats, received WY 14,643 at a dietary concentration of 1,000 ppm.

Five rats of each sex in Groups 1-5 and five males in Group 7 were sacrificed during Weeks 1, 2, and 13 to determine hepatocellular proliferation. Following 78 weeks of compound administration, 15 rats/sex/group were sacrificed in Groups 1, 4, and 5, with 5/sex/group used for hepatocellular proliferation studies. During Week 104, all remaining rats were sacrificed, including 5/sex/group used for hepatocellular proliferation studies.

Hematoxylin-and-eosin-stained sections of three liver lobes (left lateral, median, and right lateral) were evaluated microscopically from the rats in Groups 1, 5, and 7 sacrificed at Weeks 1, 2, and 13 and from five rats/sex/group in Groups 1, 4, and 5 at Week 104.

At Week 79, complete sets of protocol-specified tissues from 10 rats/sex/group in the control and high-dose (Group 5) groups and the liver, testes with epididymides, uterus, spleen, kidneys, and gross lesions from 10 Group 4 rats/sex were examined microscopically.

Excluding the animals designated for analysis of hepatocellular proliferation, complete sets of protocol-specified tissues were examined microscopically from all Group 1 and Group 5 rats which were sacrificed at termination of the study (after 104 weeks) and from all rats that died or were sacrificed in extremis during the study. In addition, liver, testes with epididymides, uterus, spleen, kidneys, and gross lesions were examined from the rats of Groups 2, 3, 4, and 6 sacrificed at termination.

## Results

### Interim Sacrifices

Week 1 Sacrifice - Liver sections examined from the Group 1, 5, and 7 rats revealed increased numbers of mitotic figures in the Group 5 (high dose) males and females and Group 7 (positive control) males. Also, minimal individual cell necrosis was noted in the livers of 4/5 males in Group 7.

Week 2 Sacrifice - Minimal or slight diffuse hepatocellular enlargement was present in liver sections from the Group 5 male and female rats. In the Group 7 rats, moderate diffuse hepatocellular enlargement and increased cytoplasmic eosinophilia were present, and individual cell/focal necrosis was noted in two rats.

Week 13 Sacrifice - In liver sections from Group 5 rats at Week 13, increased cytoplasmic eosinophilia and slight or moderate diffuse hepatocellular enlargement were noted. In Group 7 rats, in addition to increased cytoplasmic eosinophilia and hepatocellular enlargement, pigment was noted in Kupffer cells/bile canaliculi, and hepatocellular necrosis (individual cell or focal) was present.

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Week 79 Sacrifice - Compound-related histomorphologic alterations were noted in liver and kidney sections from the Group 5 (12,000 ppm) males and females and in the kidney sections from the Group 4 (6,000 ppm) males.

Liver changes consisted of increased cytoplasmic eosinophilia and diffuse slight or moderate hepatocellular enlargement in Group 5 males and females and pigment in Kupffer cells/bile canaliculi in 5/10 male rats. Liver sections from the Group 4 rats revealed no evidence of compound effect.

In the kidneys, mineralization of the renal papilla and increased pigment in renal tubules occurred in the Group 4 and 5 male rats, with the greatest severity in the Group 5 rats. Increased pigment also was observed in the kidneys of Group 5 females, but was of slight severity (comparable to the Group 4 males). Kidney sections from Group 4 females were comparable to the control group.

Sections of testes with epididymides revealed no unequivocal difference in spermatogenic activity between control, 6,000 ppm, and 12,000 ppm rats (Groups 1, 4, and 5, respectively). Interstitial cell neoplasms and/or hyperplasia was present in nearly all rats, with an associated effect on spermatogenic activity. The increase in mean spleen weight noted in Group 5 females was due to the presence of mononuclear cell leukemia, with associated splenic enlargement in two of the Group 5 females. Early mononuclear cell leukemia was also noted in a Group 4 female.

#### Terminal Sacrifice

Text Table 1 presents survival data, including the numbers of rats in the various groups at termination of the study.

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Text Table 1  
Survival Data

Sex	Male						Female					
	1	2	3	4	5	6	1	2	3	4	5	6
Group												
Unscheduled Deaths												
Weeks 1-78	1	1	4	1	2	1	4	3	1	4	3	2
Weeks 79-106	13	14	7	18	21	20	9	9	9	13	15	14
Interim Sacrifice												
Week 78	15	-	-	15	15	-	15	-	-	15	15	-
Terminal Sacrifice	41	40	44	36	32	34	42	43	45	38	37	39
Total <sup>a</sup>	70	55	55	70	70	55	70	55	55	70	70	55

<sup>a</sup>Does not include five rats/sex/group in Groups 1-5 sacrificed at Weeks 1, 2, and 13 for cell proliferation studies.

Compound-related histomorphologic alterations were noted in the livers of Group 5 rats and in the kidneys of male rats of Groups 4, 5, and 6. Liver changes in Group 5 rats consisted of increased cytoplasmic eosinophilia in 56 rats (26/32 males, 30/37 females) and hepatocellular enlargement in 57 rats (28 males, 29 females). Spongiosis hepatis also occurred with increased frequency in 12 Group 4 males and 18 Group 5 males compared to 5 Group 1 males. Hepatocellular neoplasms occurred with increased frequency in Group 5 males, as presented in Text Table 2.

Text Table 2  
Hepatocellular Neoplasia - Terminal Sacrifice

Sex	Male						Female					
	1	2	3	4	5	6	1	2	3	4	5	6
Group												
Number Examined	41	35	39	36	32	29	42	38	40	38	37	34
Hepatocellular												
Adenoma	2	2	1	4	8	4	0	0	0	1	0	0
Carcinoma	1	0	0	1	9	1	1	0	0	1	3	1

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In the kidneys, mineralization of the renal papilla and pigment in tubule cells occurred with increased incidence and severity in male rats of Groups 4, 5, and 6, as presented in Text Table 3.

Text Table 3  
Mineralization of Renal Papilla and Tubule Cell Pigment in Male Rats

Group	1	2	3	4	5	6
Number Examined	36	35	39	31	27	29
Mineralization of Renal Papilla						
Minimal	6	11	9	6	2	0
Slight	0	0	0	24	1	2
Moderate	0	0	0	0	22	27
Total	6	11	9	30	25	29
Tubule Cell Pigment						
Minimal	24	21	18	0	0	0
Slight	10	12	21	23	7	26
Moderate	0	1	0	6	17	3
Moderately Severe	0	1	0	2	3	0
Total	34	35	39	31	27	29

As noted, tubule cell pigment was increased in severity in male rats of Groups 4, 5, and 6. This finding is often noted in association with mononuclear cell leukemia, but was also observed in the absence of leukemia.

A variety of spontaneous disease lesions and incidental findings occurred without relationship to treatment. The most common findings were peribronchial/perivascular lymphoid infiltration in the lungs; focal mononuclear cell infiltration, chronic inflammation and bile duct hyperplasia, and inflammation and fibrosis in the liver; and chronic progressive nephropathy and microconcretions in the tubules of the kidneys. Sections of testes with epididymides revealed no unequivocal difference in spermatogenic activity between control and 500, 1,500, 6,000, and 12,000 ppm rats (Groups 1, 2, 3, 4, and 5, respectively) of the recovery rats (Group 6). Interstitial cell neoplasms and/or hyperplasia was present in nearly all rats, with an associated effect on spermatogenic

activity. Heart sections revealed degenerative cardiomyopathy of minimal or slight severity in many of the control and high-dose rats. Mononuclear cell leukemia occurred in rats sacrificed at termination, as presented in Text Table 4.

Text Table 4  
Mononuclear Cell Leukemia - Terminal Sacrifice

Group	1	2	3	4	5	6
Males	14/41	14/35	14/39	16/36	11/32	15/29
Females	10/42	11/38	6/40	14/38	15/37	12/34
Total	24	25	20	30	26	27

Note: Numerals represent the number of rats with the finding over the number of terminal-sacrifice rats per group.

Renal tubule cell carcinomas were noted in three Group 6 males. Other neoplastic findings occurred primarily in the testes and thyroid gland and are typical for rats of this age and strain.

#### Unscheduled Deaths

One hundred eighty-nine rats (103 males, 86 females) died or were killed in a moribund condition during the course of the study.

The most common cause of death was mononuclear cell leukemia in 122 rats (70 males, 52 females), and as noted in Text Table 5, mononuclear leukemia was observed more frequently as the cause of death in rats of both sexes in Groups 4, 5, and 6.

Text Table 5  
Mononuclear Leukemia as Cause of Death

Group	1	2	3	4	5	6
Males	7	8	7	16	18	14
Females	7	5	3	12	13	12
Total	14	13	10	28	31	26

Pituitary neoplasia as the cause of death was noted in 18 rats (6 males, 12 females), and a variety of other neoplastic or inflammatory processes resulted in death of occasional rats.

Discussion

The total incidence of hepatocellular neoplasia is presented in Text Table 6.

Text Table 6  
Hepatocellular Neoplasia

Sex	Male						Female					
	1	2	3	4	5	6	1	2	3	4	5	6
Group	1	2	3	4	5	6	1	2	3	4	5	6
Number Examined	80	50	50	65	80	50	80	50	50	65	80	50
Hepatocellular												
Adenoma	4	4	2	6	10	5	0	1	0	1	3	1
Carcinoma(s)	1	0	0	1	11	2	1	0	0	1	5	2
Multiple Neoplasms <sup>a</sup>	0	0	0	1	4	0	0	0	0	0	0	1
Total Rats with Hepatocellular Neoplasms	5	4	2	7	17	7	1	1	0	2	8	2

<sup>a</sup>Both adenoma and carcinoma or multiple carcinomas.

As noted, the incidence of hepatocellular neoplasia was increased in rats of both sexes in Group 5 when compared to the control and lower-dose groups. The occurrence of hepatocellular neoplasia was not increased in rats of Group 6, which received the test article at the high-dose dietary level of 12,000 ppm for 78 weeks and then received the basal diet for the remainder of the study. The hepatocellular neoplasms occurred late in the study, with the earliest neoplasms noted at the 78-week interim sacrifice in a Group 1 male and in single male and female rats of Group 5. In the 25 Group 5 rats with hepatocellular neoplasms, only 7 rats (3 males, 4 females) died prior to scheduled sacrifice. Hepatocellular neoplasia was not considered the cause of death in any of the rats on study. There was no evidence of an increased incidence and/or

severity of foci of cellular alteration (basophilic or eosinophilic) in rats of Group 5.

As noted previously, mononuclear cell leukemia as the cause of death occurred with greatest frequency in rats of Groups 4, 5, and 6. The total incidence of mononuclear cell leukemia is presented in Text Table 7.

Text Table 7  
Mononuclear Cell Leukemia

Group	1	2	3	4	5	6
Males	22	23	21	32	30	31
Females	17	16	9	29	30	24
Total	39	39	30	61	60	55

Although hepatocellular enlargement and increased cytoplasmic eosinophilia occurred as treatment-related changes in Group 5 rats, the presence of hepatopathy associated with leukemia often precluded the determination of these findings.

In addition to mineralization of the renal papilla and increased tubule cell pigment in male rats of Groups 4, 5, and 6, renal tubule cell carcinomas were present in two Group 5 males (unscheduled deaths) and in four Group 6 males (one death, three sacrificed at study termination).

### Conclusion

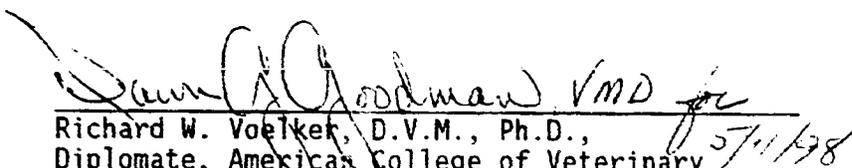
In conclusion, dietary administration of Di(isononyl)phthalate to Fischer-344 rats for at least 104 weeks at levels of 6,000 and 12,000 ppm (Groups 4 and 5, respectively) resulted in compound-related histomorphologic alterations in the liver and kidneys. Liver changes consisting of increased cytoplasmic eosinophilia and hepatocellular enlargement were observed only in the Group 5 (12,000 ppm) animals. An increased incidence of hepatocellular neoplasia was observed in Group 5 rats of both sexes, but was not present in the high-dose recovery group (Group 6). Liver changes noted in Group 5 (12,000 ppm) rats sacrificed at

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Weeks 1, 2, and 13 for cell proliferation studies consisted of increased mitotic activity (Week 1), diffuse hepatocellular enlargement (Weeks 2 and 13), and increased cytoplasmic eosinophilia (Week 13). Kidney changes at 104 weeks consisted of mineralization of the renal papilla and increased pigment in tubule cells at 6,000 and 12,000 ppm. Increased mineralization noted in the renal papilla of the Group 4, 5, and 6 males was not present in the females. Mononuclear cell leukemia occurred with increased frequency in rats of Groups 4, 5, and 6 and renal tubule cell carcinomas were noted in two Group 5 and four Group 6 males.

Other spontaneous disease lesions, including neoplasms, were of the expected types and severity for rats of this age and strain and are not considered to be an effect of compound administration. For all deaths combined, there was no histopathological evidence of a treatment-related effect on spermatogenic activity or the incidence of morphologic abnormalities in the uterus.

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GENETIC TOXICOLOGY REPORT  
PRELIMINARY CELL PROLIFERATION SUMMARY

MEASUREMENT OF CHEMICALLY-INDUCED CELL PROLIFERATION AND PEROXISOME  
PROLIFERATION IN LIVERS OF MALE AND FEMALE  
RATS IN AN ONCOGENICITY STUDY WITH DI(ISONONYL)PHTHALATE

I. INTRODUCTION

The cell proliferation assay described in this study is designed to measure the fraction of cells undergoing cell replication in rat liver cells (1,2). Animals were fed di(isononyl)phthalate (DINP) daily and the livers isolated following administration of bromodeoxyuridine (BrdUrd) in vivo with an ALZET® osmotic pump implanted subcutaneously for 72 hours. The pumps were implanted at several timepoints to determine the pattern of the proliferative response. Quantitation of the fraction of proliferating cells in the tissue sections can be correlated with other endpoints such as toxicity and carcinogenicity.

It is not apparent how cell proliferation acts on the carcinogenic process, but there are numerous processes that can be affected during replication (3-6). Chemically induced cell proliferation may increase the probability of spontaneous mutations as well as increase the probability of converting DNA adducts into mutations prior to a repair process. Unscheduled cell proliferation may also play a role in the expansion of preneoplastic cells leading to the emergence of a fully transformed clone of cells. Some of these examples act by nongenotoxic mechanisms.

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One nongenotoxic mechanism thought to be involved in cell proliferation is hepatocellular peroxisome proliferation. The test chemical, di(isononyl)phthalate, comes from a family of chemicals being investigated for their ability to induce both peroxisome proliferation and cell proliferation (6). Animals exposed to some agents which are known to induce peroxisome proliferation have an increased risk for tumor formation in the liver. For this reason, the activity of palmitoyl-CoA oxidase was also determined from liver homogenates prepared from treated and control livers. The stimulation of this enzyme is related to peroxisome proliferation. Hepatocellular DNA and protein content were also determined.

## II. METHODS

### A. Cell Proliferation

Details of the study design are presented elsewhere in the report. Briefly, Fischer 344 rats were placed in Groups 1 to 7 and administered di(isononyl)phthalate or a positive control compound (Wy 14,643) in the diet. The test compound was administered at 0, 500, 1500, 6000, and 12,000 ppm; Wy 14,643 was administered at 1000 ppm to male rats only for 13 weeks. A high dose recovery group was also included in the dosing but animals were not included for cell proliferation and biochemical analysis. At the end of Weeks 1, 2, 13 and 104, five animals per condition from all available groups were labeled with BrdUrd (20 mg/ml; 10  $\mu$ l/hr) for 72 hours using ALZET<sup>®</sup> osmotic pumps, Model No. 2ML1 (ALZA Corporation, Palo Alto, CA). A single lot of osmotic pumps was used for each sacrifice. ALZET<sup>®</sup> Model No. 2ML1 has a 2000  $\mu$ l capacity with a pump rate of 10  $\mu$ l/hr. At the time of sacrifice, samples of the liver and duodenum were

fixed in 10% neutral Formalin and later embedded in paraffin. Unfixed samples of the livers were frozen for biochemical analysis. At Weeks 1, 2 and 13, Groups 1 (0 ppm), 5 (12,000 ppm) and 7 (Wy 14,643) were analyzed and Groups 2 through 4 saved for possible future analysis. At the end of 104 weeks, animals from Groups 1 (0 ppm), 4 (6000 ppm) and 5 (12,00 ppm) were analyzed and Groups 2 and 3 saved for possible future analysis.

For the cell proliferation portion of the study, five micron paraffin embedded sections from the left lateral, median and right lateral lobes of the livers as well as samples from the duodenum were taken and processed for immunohistochemistry. Each slide was prepared with sections from both liver and duodenum so that the duodenum (a rapidly proliferating organ) could be used as an internal control for delivery of label and immunohistochemical staining. The slides were deparaffinized and rehydrated prior to staining. The slides were stained for determination of cell proliferation as measured by incorporation of BrdUrd into DNA using Biogenix antibodies with peroxidase-conjugated streptavidin and a 3,3-diaminobenzidine tetrahydrochloride (DAB) chromogen and hematoxylin counterstain. Samples from all groups were also processed for analysis by a pathologist.

The section of the duodenum was microscopically examined to ensure that the label was properly administered to the animal. Once label delivery was confirmed, slides from the different lobes were examined for lobular differences. Labeling was similar among the lobes therefore cell counting was performed with sections from the left lateral lobe. The percentage of

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nuclei incorporating label in the liver was determined microscopically at approximately 400 to 600X magnification. The areas to be counted were randomly generated by computer. A 1.0 mm square indexed ocular grid divided into 10 x 10 squares was used to define the counting area. At least 2000 nuclei were examined per animal with a minimum of 3 sections and 6 fields per section.

Any nuclei that were blue were considered unlabeled and any nuclei containing any brown chromogenic hue were considered labeled unless a clear artifact was present. Only hepatocyte nuclei were enumerated. Fields that contained areas of necrosis were not included in the evaluation. The slides were coded for (blind) evaluation as to treatment group.

S-phase nuclei labeling indices for each animal were calculated as follows:

$$\text{Labeled S-phase nuclei (LI)} = \frac{\text{no. of labeled hepatocyte nuclei}}{\text{total no. of hepatocytes counted}}$$

## B. Biochemical Analyses

### 1. Sacrifice

Three days following implantation of the osmotic pumps at each timepoint, four or five animals/sex were sacrificed and livers were frozen for biochemical analyses. DNA, protein and palmitoyl-CoA oxidase analyses were performed. Livers not scheduled for analysis were saved for possible future processing.

## 2. Liver Homogenization

Samples of livers were rinsed in cold homogenization buffer {50 mM tris[hydroxymethyl]aminomethane hydrochloride (tris·HCl), pH 8.3, 154 mM KCl}, minced and homogenized using a Potter-Elvehjem-type homogenizer. Following centrifugation at 3300 x g, the supernatant was filtered through gauze pad/spun glass and stored at -70°C for determination of protein and palmitoyl-CoA oxidase activity.

Preparation of livers for determination of DNA content proceeded as described for protein and enzyme analysis up to the homogenization step. After homogenization, the samples were precipitated in the presence of cold trichloroacetic acid (TCA) and centrifuged. The precipitate was washed twice with 10% cold TCA and then washed with 95% ethanol. The washed precipitate was suspended in 5% TCA and heated to  $90 \pm 3^\circ\text{C}$  for 15 minutes. The samples were then centrifuged, washed once with cold 5% TCA and the supernatants collected. The supernatants were stored at -70°C for later use. At the first three timepoints, 0.75% TCA was used to precipitate the DNA instead of ~7.5% and 1% (instead of 10%) or 0.5% (instead of 5%) TCA was used for the remainder of the extraction due to an error in the SOP. Experiments performed independent of this study showed that, while the amount of DNA recovered was reduced when the lower TCA concentrations were used, the proportion recovered stayed the same. Any increases or decreases in total DNA per mg of protein should be detectable.

### 3. Determination of Palmitoyl-CoA Oxidase Activity

The oxidation of palmitoyl-CoA was determined by measurement of the formation of  $\alpha$ -nicotinamide adenine dinucleotide, reduced from (NADH) at  $A_{340}$  following incubation of substrate, liver homogenate, and cofactors. The reaction was performed in the presence of KCN to inhibit the reoxidation of NADH (7,8).

Liver homogenates containing 100  $\mu$ g of protein in 5  $\mu$ l were mixed to obtain a final concentration of 45 mM Tris·HCl, 1 mM  $\alpha$ -nicotinamide adenine dinucleotide (NAD), 0.1 mM coenzyme A (CoA), 1 mM dithiothreitol (DTT), 75  $\mu$ g/ml bovine serum albumin (BSA), 0.01 mM flavin adenine dinucleotide (FAD), 0.01% Triton X-100 and 1 mM KCN. The samples were incubated at 37°C in a spectrophotometer with temperature controlled cuvette carriage until readings at  $A_{340}$  stabilized. The reaction was then started by adding 40 nM of the substrate (palmitoyl-CoA). Readings were taken at  $A_{340}$  at 1 minute intervals for at least 10 minutes. Nanomoles of NAD reduced per minute was determined by dividing the change in absorbance of samples minus controls per minute by the extinction coefficient (6.22).

### 4. Determination of Protein of Liver.

Protein concentrations of liver homogenates were determined using the Lowry Reagent, Modified (Sigma Diagnostics, St. Louis, MO). This involves a colorimetric reaction between proteins and Folin and Ciocalteu's Reagent.

#### 5. Determination of DNA Content of Liver.

The procedure for the determination of DNA was based on the finding that DNA can be separated from other tissue compounds by its preferential solubility in hot trichloroacetic acid (TCA). Lipids were removed with an alcohol wash. The isolated DNA was then quantitated by means of diphenylamine which produces a colorimetric reaction involving the pentose component of DNA.

#### C. Statistical Analysis

Statistical analysis of labeling index, palmitoyl-CoA oxidase activity, DNA and protein were performed using one-way analysis of variance techniques (9) for each sacrifice interval. Control versus treatment group comparisons were done with Dunnett's t-test or the Student's t-test (10,11). In the case of variance heterogeneity, rank transformation of the data was performed prior to analysis of variance and Dunnett's t-test. The outcome of the statistical analyses are indicated in Text Table 1.

### III. RESULTS AND DISCUSSION

#### A. Cell Proliferation Results

##### 1. General Observations

Cells stained with the brown DAB chromogen were observed in the duodenum from all of the animals used in the study. The presence of label in all the animals indicated proper delivery of the BrdUrd label and acceptable

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immunohistochemical staining. There was no apparent preferential labeling in any of the lobes and the label was random within the lobes, except where necrosis was observed. The necrotic areas were noted but not included in the count.

Microscopic evaluation of H&E stained liver sections from rats used for evaluation of cell proliferation was performed by a pathologist.

In the animals from the terminal sacrifice, several of the rats had leukemia with or without hepatopathy. A summary of the pathology observations for the terminal sacrifice is shown in Text Table 2. Animals with leukemia and hepatopathy were not analyzed for cell proliferation since the cell proliferation labeling index could be affected by degenerative/regenerative changes. There were two male rats, one in Group 1 and one in Group 4 that were eliminated. Three female rats were eliminated from evaluation, one in Group 1 and two in Group 5. In addition, two animals that had early leukemia and had elevated labeling indices (one Group 1 and one Group 4 males) were not used in the evaluation since it was not clear if the elevated counts observed were caused by the test article or the leukemia. In the Group 1 male that was eliminated, the labeling index was approximately six times the mean of the labeling index of the remaining three rats. The Group 4 rat that was not used in the evaluation had a labeling index that was approximately five times the mean labeling index of the remaining three rats. Biochemistry (DNA, protein and palmitoyl CoA oxidase) was performed on all livers but the data from only the animals used for evaluation of cell

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proliferation is included in the evaluation (see Text Table 1). Individual values are shown in Appendix A.

2. Summary of Labeled Cell Counts for the Liver

A summary of the labeled cell counts for each group is shown in Text Table 1. The mean labeling index (LI) for each group is presented in the third column.

At the first interim sacrifice, the background labeling indices (Group 1, both sexes) were below 1%. Significant increases were observed in the dosed group (Group 5;  $p \leq 0.05$ ) in both male and female animals. Cell proliferation was not increased by the chemical at the other three timepoints. The lack of an increase in the LI at Weeks 2, 13 and 104 was observed even though large increases in palmitoyl-CoA oxidase were observed (see Section III.B.). Large increases in the labeling index ( $p \leq 0.05$ ) were observed in the first sacrifice in the positive control (1000 mg/kg Wy 14,643; Group 7). The positive control response was not considered significantly elevated in the second sacrifice even though the labeling index increased by close to 8-fold. The positive control in the third sacrifice was significantly elevated ( $p \leq 0.01$ ) even though the labeling index in the positive control animals remained less than 1%. The apparent incongruity of the results appears to be related to the variability or consistency of the responses seen among the animals. This is often observed in a weak response. No positive control animals were included for the week 104 sacrifice.

These results demonstrate that the chemical induced significant increases in the LI in the liver in male and female rats only during the first week of dosing. At Weeks 2, 13 and 104, the labeling indices for both sexes were similar to the controls. Large increases in the labeling index were observed in the positive control animals at Week 1, but questionable responses were observed at Weeks 2 and 13. The labeling index of Wy 14,643 is known to decrease with time (6,12).

B. Results from Biochemical Analyses.

1. Palmitoyl-CoA Oxidase Activity

Increases in palmitoyl-CoA oxidase activity were observed in Group 5 rats (12,000 ppm) at all timepoints. At Week 104, there was also an elevation in palmitoyl-CoA oxidase activity in female rats at Group 4 (6000 ppm). Male rats treated at 6000 ppm did not have elevated enzyme levels. Animals dosed with 6000 ppm were only evaluated at week 104. Results are summarized in Text Table 1 (column 4) and are expressed as nanomoles of NADH generated per minute per mg of protein.

2. DNA and Protein Analyses

Levels of DNA varied randomly among the groups and at the different timepoints (Text Table 1, column 6). Statistical analysis confirmed the lack of a consistent pattern.

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Protein analysis also did not indicate a clear pattern in Groups 4, 5, or 7.

#### C. Liver Weights

Liver weights were significantly elevated in Group 5 animals throughout the study in both male and female rats. The positive control animals included at 1, 2 and 13 weeks were also significantly elevated (Text Table 1, column 7).

#### IV. Conclusions

Significant increases in the labeling index were observed during Week 1 in the livers of male and female rats treated with the test material. The response was not observed at Week 2, 13 or Week 104. Increases in palmitoyl CoA oxidase, a monitor of the level of peroxisome proliferation were observed at all four timepoints. Therefore, there is no evidence for sustained cell proliferation associated with the peroxisome proliferation induced by di(isononyl)phthalate.

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