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A 90-DAY ORAL TOXICITY STUDY AND A 5-DAY METABOLISM STUDY OF DIISOPROPYL METHYLPHOSPHONATE (DIMP) IN MINK

Final Report

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June 1992

Supported by

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-91-C-1088

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Contracting Officer's Representative: Dr. Jack C. Dacre

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U.S. Army Biomedical Research and Development Laboratory
Fort Detrick, Frederick, Maryland 21702-5010

Unclassified
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Pathology Associates, Inc. (6b. OFFICE SYMBOL (If applicable))	7a. NAME OF MONITORING ORGANIZATION: US Army Biomedical Research and Development Laboratory (USABRDL)
6c. ADDRESS (City, State, and ZIP Code): Post Office Box 26, Jefferson, AR 72079	7b. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21702-5010
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: US Army Medical Research & Development Command (8b. OFFICE SYMBOL (If applicable))	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-91-C-1088
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21702-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62787A; PROJECT NO. 3M162787A878; TASK NO. CA; WORK UNIT ACCESSION NO. 283

11. TITLE (Include Security Classification): A 90-Day Oral Toxicity Study and a 5-day Metabolism Study of Diisopropyl Methylphosphonate (DIMP) in Mink.

12. PERSONAL AUTHOR(S): Thomas J. Bucci, Robert M. Parker, William Wustenberg

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 4/1/91-6/30/92	1992, June	132

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP	DIMP	metabolism	Subchronic
06	11		Lab animals	MINK	rats
24	07				

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

To reinvestigate the potential toxicity of diisopropyl methylphosphonate (DIMP) in mink (Mustela vison), a 90-day dosed-feed study and a pharmacokinetic/metabolic study in mink and rats were conducted.

90-Day Study

Eighty 12-13 month-old brown "Ranch Wild" mink of each sex were randomized by body weight into eight dose groups of ten animals per sex. The animals were caged individually; food consumption and body weight were determined weekly. Blood samples were obtained before treatment began and at weeks 3, 7 and 13. All animals were examined at necropsy and microscopically.

The target dose groups were 0, 50, 450, 2700, 5400 and 8000 ppm. The actual concentrations fed were between 97.6 and 133.5% of the target doses. Males and females in the 8000 ppm group consumed 20% and 24% less food than respective controls and overall mean body weight for both sexes was 18% less than the untreated control group.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT: ☒ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION: Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Virginia M. Miller	22b. TELEPHONE (Include Area Code): 301/619-7325 — 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

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EXECUTIVE SUMMARY

INTRODUCTION

Two studies were conducted to reinvestigate the potential toxicity of diisopropyl methylphosphonate (DIMP) in mink (Mustela vison). One was a 90-day subchronic toxicity dosed-feed study in 160 animals. The other was a pharmacokinetic/metabolic study in 60 mink and 48 rats. Rats were studied to provide a basis for comparison to mink and to a report of an earlier DIMP study in rats.

The Department of Veterinary Pathobiology, College of Veterinary Medicine, University of MN (UMN), St. Paul, MN, conducted the metabolic study and provided formulation of the test rations, animal husbandry, clinical chemistry and hematology for the 90-day study. Necropsy (at UMN) and microscopic examination of the mink were performed by Pathology Associates, Inc, (PAI), Frederick, MD.

The studies were conducted in accordance with "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on the Care and Use of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 85-23, Revised 1985]. The U. S. Environmental Protection Agency's Good Laboratory Practice Standard (Toxic Substances Control Act) was followed.

90-DAY STUDY

One hundred sixty 12-13 month-old brown "Ranch Wild" mink (80 males and 80 females) were randomized by body weight into eight dose groups of ten animals per sex.

The animals were caged individually, and their food consumption and body weight were determined weekly. Blood samples for analyses were obtained from anesthetized animals on four occasions: before treatment began and during weeks 3, 7 and 13. At the end of the study, all animals received gross and microscopic examination.

The test article DIMP was characterized with infrared and ultraviolet/visible spectroscopy, nuclear magnetic resonance analysis and gas and thin-layer chromatography. These analyses revealed the compound to be DIMP with a single impurity (diisopropyl phosphonate) comprising 5.3-5.4% of the sample. Water content was 0.2%. An accelerated stability study showed DIMP and the

impurity to be essentially unchanged after 24 days at -20, 5, and 60°C or at room temperature.

The basal ration for the mink was a commercial product obtained from the same source as the animals. DIMP was incorporated into the basal ration to approximate the target dose and dispensed into individual-portion steel cans of 450-gram capacity (approximately 400 grams per can, calculated to be an excess to permit ad libitum feeding for one animal per day). The cans were capped, color coded for dose, frozen and stored at -20°C. They were thawed 1-2 hours at room temperature before being placed in the cages.

The target dose groups were 0, 50, 450, 2700, 5400 and 8000 ppm. There were also three control groups: one with untreated feed offered ad libitum, and one each given untreated feed in amounts to match the food consumption of the two highest dose groups. There were ten males and ten females in all groups. Test article concentration, homogeneity of mix, and stability of the test article in feed stored at -20°C for 0, 7, 14, 28, 60, 90 and 120 days, were determined. The actual concentrations were found to be between 97.6 and 133.5% of the target doses. The actual doses achieved were judged to be as acceptable as the original target doses, since they effectively spanned the dose range of interest. DIMP was stable in the feed stored up to 120 days at -20°C.

Untreated control rations were analyzed for an extensive list of nutrients and contaminants. All were within acceptable limits.

Males and females in the 8000 ppm group consumed 20% and 24% less food than the 0 ppm controls did, and the overall mean body weight for both sexes of this group was 18% less than the untreated control group. On the day of necropsy, the males weighed 21% less than the control males and the females 22% less than their controls. The difference from the controls was statistically significant at $p > .01$. No other treated groups differed significantly in body weight from the controls. The weight loss was attributed to refusal of food rendered less palatable by the DIMP, rather than to DIMP toxicity, since the untreated animals pair-fed to match the reduced food consumption of the 8000 ppm males lost equal weight during the period that food consumption by both groups was equal.

The data on food consumption, DIMP ingestion, and body weight are summarized below:

Mean Food Consumption, Body Weight, DIMP Ingested
10 Animals/Group; 90 Days

DIMP Dose Group (ppm)	DIMP Actual (ppm in diet)	Feed Consumed (gm/mink/day)		DIMP Consumed (mg/mink/day)		Mean Body Wt. (gm)		DIMP Consumed (mg/kg/day)	
	(µg/mg)	Male	Female	Male	Female	Male	Female	Male	Female
0	0	238.2	169.1	0	0	2084.1	1113.0	0	0
50	57	252.2	167.6	14.4	9.6	2127.3	1062.5	6.7	8.9
450	528	246.1	173.5	129.9	91.6	2047.5	1112.8	63.4	82.3
2700	2930	235.6	170.3	690.3	499.0	2002.8	1096.3	344.8	455.2
5400	6174	240.2	149.1	1483.0	920.9	1985.1	1014.1	747.1	907.7
8000	8990	190.4	128.0	1711.7	1150.7	1697.1	910.7	1008.6	1263.5
0PF1	0	228.2	174.7	0	0	2052.0	1108.8	0	0
0PF2	0	203.0	165.0	0	0	1841.9	1017.9	0	0

Except for weight loss in the 8000 ppm group, there were no clinical signs relating to treatment at any dose level. All animals survived and appeared healthy.

Blood was examined for hematocrit, hemoglobin, erythrocyte (RBC) count, mean cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, RBC morphology, Heinz bodies, total leukocyte count, differential leukocyte count, and platelet count.

There were mild treatment-related changes in a number of RBC characteristics in the 2700, 5400, and 8000 ppm groups. There was an increase in mean corpuscular volume and in numbers of erythrocytes, reticulocytes, platelets and Heinz body-containing RBCs. There was a decrease in hematocrit and mean corpuscular hemoglobin. These changes were mild to moderate, appeared at various times in various groups, and were more consistent at later times and in higher doses. The changes reached statistical significance in some instances. The changes reflected oxidative stress on the RBCs as a result of exposure to the higher doses of DIMP, which caused alteration in hemoglobin (Heinz bodies) and premature destruction of the affected cells in the animal. This stimulated production of new RBCs; immature RBCs are larger, and have reticular remnants, and less hemoglobin per cell. All of these changes resolved completely or nearly completely during the 4-week recovery period. There were no dose-related changes in the 50 or 450 ppm groups.

Clinical chemistry analysis determined values for plasma and RBC cholinesterase, blood urea nitrogen, creatinine, glucose, total protein, albumin, alanine aminotransferase, aspartate aminotransferase, sodium chloride, potassium, total osmolarity, total CO_2, anion gap, total calcium, and inorganic phosphorus.

Although there were random sporadic differences among some analyte values between and within treatment groups over the time of the tests, plasma cholinesterase (PChE) was the only analyte clearly altered as an effect of exposure to DIMP. PChE was decreased in all animals of the 2700, 5400 and 8000 ppm groups. In contrast, the activity of red blood cell cholinesterase was not decreased in the mink exposed to DIMP, and there were no clinical signs of acetylcholinesterase inhibition at any time. At the end of the 13-week treatment period, two mink of each sex from the 2700 and 8000 ppm groups were held for a 4-week recovery period without DIMP in their feed. Their PChE values returned to normal during the first week of recovery.

There were no gross changes detected at necropsy that were attributable to DIMP exposure except for the reduction in body weight of the 8000 ppm group.

Equivocal increase in hematopoietic cell proliferation in the spleens of the 8000 ppm group was the only potential treatment effect noted microscopically. The pathologist concluded that this was a "a probable effect of DIMP exposure."

This microscopic observation supports the hematologic findings, that production of replacement red blood cells was occurring in these animals in response to DIMP exposure. There was no change in spleen weight, and increased hematopoiesis could not be recognized in bone marrow. Spleens of the 8000 ppm group animals held for recovery were normal microscopically.

In conclusion, DIMP appeared to be only slightly toxic to mink at the higher doses employed in this study. The changes noted - decrease in plasma cholinesterase and injury to erythrocytes - were mild and rapidly reversible upon cessation of exposure. The No Observed Adverse Effect Level for DIMP was 73/mg/kg BW/day (average for both sexes of the 450 ppm group), with 400/mg/kg BW/day the Lowest Observed Adverse Effect Level in this study.

METABOLIC STUDY

The principal objectives of the metabolic study were (1) to determine the rate of excretion of DIMP, (2) to determine the identity and relative abundance of any metabolites produced in mink, (3) and to compare these with data obtained in the same manner from rats.

The animals were housed individually in metabolic cages. They were anesthetized and administered either a high dose or a low dose of radioactive DIMP by gavage or were

administered the low dose intravenously. Blood, urine, and feces were collected at intervals over the 120 hours following dosing. The mink (eight of each sex per treatment group) were of the same stock and from the same supplier as for the 90-day study. They were 14-16 months of age at the start of the metabolic study. Groups of eight 10-wk-old Sprague Dawley rats were handled similarly. All animals were fasted overnight before treatment and permitted food and water ad libitum thereafter.

^{14}C-DIMP-5mCi/mmol and 97% pure (labelled on the methyl carbon) - was obtained from Dupont NEN Products, Boston, MA. The mink and rats were each given 40 microcuries/kg body weight, except that the mink dosed intravenously were given 20 microcuries/kg. The radioactive DIMP was diluted with "cold" DIMP from the same stock as that used in the 90-day study to achieve the following total doses (mg DIMP/kg BW):

	High Oral Dose	Low Oral Dose	IV Dose
Rats	660	66	66
Mink	270	27	27

The high dose was approximately an LD_{10} dose for the respective species, based on values in the literature; the low dose was 10% of that amount.

Blood samples were obtained at 1, 2.5, 5, 10 and 30 minutes and at 1, 2, 3, 4, 6, 7 and 24 hours after administration of the DIMP. Urine and feces were collected at 4, 8, 12 and 24 hours and at 2, 3, 4 and 5 days.

In both species, DIMP was absorbed rapidly after oral administration and was metabolized promptly by a saturable pathway to a single primary metabolite, isopropyl methylphosphonic acid (IMPA). This component accounted for virtually all of the recovered radioactivity, which was excreted principally in the urine. Urinary excretion accounted for more than 90% of the recovered radioactivity. By 120 hours, recovery of the administered dose from urine and feces together was 82-87% in male mink, 70-91% in female mink, 86-97% in male rats and 67-72% in female rats. About 75% of the 120-hour totals was excreted within the first 48 hours. There was no evidence of urinary conjugates of DIMP or IMPA.

The high oral dose in each species was not converted proportionately to IMPA and was slower to be eliminated, indicating saturation of the primary conversion pathway. Their were only minor differences between male and female mink in all aspects of metabolism and kinetics. In contrast, female rats converted and eliminated DIMP less efficiently than male rats.

The metabolism of DIMP demonstrated in the male rat and both sexes of mink in this study conformed very well to that reported for rats, mice, dogs and bovine (Hart, 1980; Ivies, 1980). The female rat (not studied previously) was somewhat less efficient

in eliminating DIMP but was not particularly susceptible to DIMP toxicity as reported previously (Biskup, 1978; Hardisty, 1976; Hart, 1976; Hart, 1980).

Taken together, the results of the 90-day subchronic oral toxicity in both sexes of mink and the metabolism study in both rat and mink indicate that the mink has no unique species susceptibility to toxicity from DIMP.

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Fort Detrick, Frederick, MD 21702-5010

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A. INTRODUCTION

1.0 BACKGROUND

Diisopropyl methylphosphonate (DIMP), a by-product of the 1953-1957 manufacture of the military nerve agent Sarin (isopropyl methylphosphonofluoridate), has been recognized for some years as a groundwater contaminant at Rocky Mountain Arsenal, Adams County, CO.

A number of studies have been conducted in several animal species to determine the toxicity of DIMP. The U.S. Environmental Protection Agency (EPA) reviewed those studies comprehensively and issued a Health Advisory (U.S. EPA, 1989) which stipulated that 600 µg/L (600 ppb) of DIMP in drinking water was a concentration at which adverse health effects would not be anticipated to occur in human adults during a lifetime exposure.

The EPA also recommended a Longer-term (7-year) Health Advisory for adult humans to be 30 mg/L(30,000 ppb) of DIMP. To arrive at this value, several animal studies were considered. These included 90-day feeding studies in rats, mice (Hart, 1976) and dogs (Hart, 1980), a three-generation feeding study in rats (Hart, 1980), a one-generation screening study in rats (Hardisty, et al, 1976) and a 26-week drinking water study in rats (Biskup, et al, 1978). There were shortcomings in each of the latter two studies, and all of the first four demonstrated no consistent exposure-related effects at the highest doses tested. The authors of the Advisory based their calculations on the 90-day dog study because it had the most conservative No Observable Adverse Effect Level (NOAEL), 75 mg/kg/day.

The studies having appropriate duration to be considered most specifically, to derive the Lifetime Health Advisory, were the 90-day feeding studies and a 12-month study in mink (Aulerich et al, 1979). The authors of the Advisory concluded that data from the mink study could not be recommended for use in this Advisory because of "high natural mortality in mink and uncertainties concerning the relevance of mink to human health assessment." They therefore used the data from the same 90-day dog study that was used for the Longer-term Health Advisory, because of its conservative NOAEL.

The Shell Chemical Company, a former tenant at Rocky Mountain Arsenal, had earlier reported an acceptable daily intake (ADI) and drinking water guideline for DIMP in humans (Shell, 1988). The Shell authors based their recommendations on the results of the Aulerich et al., 12-month study in mink, and proposed 0.95 mg/kg/day for the ADI and 33 mg/L (33,000 ppb) for the lifetime drinking water guideline.

A consultant reviewed the EPA and Shell Chemical Company recommendations at the request of the State of Colorado and proposed 0.36 µg/L (0.36 ppb) as the lifetime drinking water guideline (Calabrese, 1990). Calabrese used the data from the 12-month mink study as did Shell Chemical Company, but included additional uncertainty factors in his calculation.

To reconcile the disparate recommendations and especially to try to establish the utility of the Aulerich et al., mink study for human risk assessment, the Army Surgeon General requested yet another review, to be conducted by the Committee on Toxicology of the National Academy of Sciences/National Research Council (NAS). The NAS found the Aulerich study inconclusive "because of apparent reporting problems in the Aulerich report, the inadequacy of the pathologic examinations of the animals, uncertainty about the experimental conditions, and lack of original laboratory data" (Doull, 1990). It recommended that the 1989 EPA Health Advisory be followed "until further experimentation is completed." NAS also suggested several studies that it believed would permit more confidence in setting a safe level of DIMP. In brief, NAS recommended replication of the Aulerich study with a larger number of animals and with appropriate histopathologic examination, reproductive and teratogenic toxicity studies in other species, and additional in-vitro mutagenicity studies. NAS recommended that long-term chronic studies in rodents be considered if the new mink studies revealed toxicity.

PAI applied to conduct the 90-day study and a pharmacokinetic/metabolic study as prime contractor under the Army's Broad Area Announcement (BAA) program, and the proposal was accepted. These studies did not replicate the Aulerich work, which had included pregnant and lactating mink exposed to DIMP in feed for 12 months.

The NAS Committee on Toxicology approved the protocol for the 90-day study on 2 October 90, and the pharmacokinetic/metabolic study on 6 November 90. The animals in the 90-day study were placed on dosed feed during the week of 13 May 1991, and were sacrificed during the week of 12 August 1991, except eight animals were held four weeks longer as a recovery group. The pharmacokinetic/metabolic study began during the week of 19 August 1991 and the final replicate was sacrificed during the week of 21 September 1991.

2.0 KEY PERSONNEL AND FACILITIES

2.1 Pathology Associates Inc., Frederick, MD.

- Study Director - Thomas J. Bucci, VMD, PhD, Diplomate, American College of Veterinary Pathologists
- Co-Investigator - Robert M. Parker, PhD, Perinatal Biologist and Toxicologist

- Study Pathologist - Robert Kovatch, DVM, Diplomate, American College of Veterinary Pathologists

- Study Manager - William Wustenberg, DVM, Director of Special Animal Projects

2.2 College of Veterinary Medicine, University of Minnesota, St. Paul, MN

- Administrator - Victor Perman, DVM, PhD, Professor of Pathobiology, Diplomate of the American College of Veterinary Pathologists

- Head of Hospital Laboratories - Douglas J. Weiss, DVM, PhD, Diplomate, American College of Veterinary Pathologists

- UMN provided housing and animal care for the studies, to include formulation of the dosed feed. UMN provided ophthalmoscopic examination of the mink in the 90-day study as well as clinical chemistry and hematologic examination of all blood samples. UMN also conducted the metabolic study in mink and rats, to include determination of total ^{14}C radioactivity of blood, urine and fecal samples from the metabolic study. Methods for and results of the UMN analyses are described in the relevant study sections of the report. UMN also provided the statistical evaluation of the results of its analyses (Appendix 4 A and B).

2.3 Midwest Research Institute, Kansas City, MO (MRI)

- MRI provided chemical analytical services to characterize the test article (DIMP), to document the concentrations of DIMP in the dosed feed and the homogeneity of mix, and to determine the stability of the dosed feed formulation during storage. MRI's detailed report is included in Appendix 1.

2.4 Southwest Research Institute, San Antonio, TX (SwRI)

- SwRI provided identification and quantification of ^{14}C-containing compounds in specimens of plasma, urine and feces from mink and rats administered ^{14}C-DIMP in the metabolic/pharmacokinetic study. SwRI's detailed report is included in Appendix 2.

2.5 Lancaster Laboratories, Inc., Lancaster, PA

- Lancaster Laboratories analyzed the mink ration for macro and micronutrients and for contaminants. Their detailed report is included in Appendix 3.

3.0 GOOD LABORATORY PRACTICE STANDARD

The studies were conducted in compliance with Good Laboratory Practice (GLP) Standards, Environmental Protection Agency, Toxic Substances Control Act (U.S. EPA 1989). All aspects were conducted in accordance with written Standard Operating Procedures (SOPs) of the performing unit, and all raw data and performance documents were maintained in accordance with GLP standards. An administratively separate quality assurance unit (QAU) from PAI monitored the studies to assure adherence to GLPs and approved SOPs. Deviations from the protocol or GLP are noted in the raw data and reflected in Sections B 4.1 and C 4.1 of this final report.

The sponsor of the studies and the Study Director were aware that SOPs did not exist for some of the tasks planned. For example, there was no report in mink of metabolic studies of the type described in 40 CFR 796 (U.S. EPA, 1985). PAI's approach was to use experienced personnel for all critical tasks and to adapt methods established for other species to the mink where none was described for mink. There were instances where peculiarities of the species had to be accommodated. For example, the mink had to be caught and restrained repeatedly (or subjected to prolonged restraint) early in the metabolic study to administer the dose and to obtain five blood samples at specified intervals over the first 30 minutes post-dosing. These multiple stressful manipulations would have confounded normal metabolism if indeed they could even have been accomplished on schedule. We elected instead to anesthetize the animals by simple intramuscular injection before dosing, and were thus able to dose accurately and obtain the initial critically timed blood samples. The obvious trade-off was an unknown effect of anesthesia on metabolism of DIMP.

These reports provide ample description of all procedures to demonstrate that the data are sufficient and reliable.

4.0 CARE AND USE OF LABORATORY ANIMALS

The studies were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 85-23, Revised 1985). All caging, housing, and husbandry met or exceeded the recommendations of the Fur Farm Animal Welfare Coalition's "Standard Guidelines for the Operation of Mink Farms in the United States," March 1989. The UMN Animal Care and Use Committee reviewed and approved the animal use for

these studies.

B. 90-DAY SUBCHRONIC STUDY

1.0 STUDY DESIGN

1.1 Overview

To determine the general toxicity of DIMP to mink during a standard 13-week subchronic study, 160 animals (80 of each sex) were allocated randomly by weight to eight dose groups of ten animals per sex. Five groups received dosed feed ad libitum, one group was the untreated control group, fed ad libitum, and two groups were "pair-fed" to match the group mean food consumption of the two highest dose groups. All received water ad libitum. The mink were placed on dosed feed during the week of 13 May 1991.

1.2 Rationale for Dose Selection

The primary objective of this 90-day study was to reinvestigate the toxicity of DIMP.

Aulerich, et al., (1979) found the acute LD_{50} (single oral dose) for adult female mink to be 503 mg/kg BW, with 95% confidence interval of 379-668 mg/kg. In their 21-day, repeat-dose study, five 8-month old animals of each sex were given 0, 10, 100, 1000, or 10,000 ppm of DIMP in dosed feed. The only treatment-related effect was significantly lower mean body weight in the 10,000 ppm group (1047±49 gm versus 1560±165 in the control). This group also consumed significantly less dosed feed (201 gm/day versus 292 gm in the control). Mean consumption of DIMP in the 10,000 ppm group was 2010 mg/day. Since this exceeds the acute LD_{50} by approximately four-fold, DIMP was not a cumulative toxin in the 21-day study. There were no adverse effects of treatment in any of the other dose groups.

In the Aulerich, one-year chronic study, groups of 30 animals (6 male, 24 females) were given 0, 50, 150 or 450 ppm of DIMP in their diet. The animals were 3 months old at the start. The females were mated, became pregnant and delivered during the study. In the 450 ppm group, there were no deaths before 3 months, there were two deaths by 6 months and three more by 12 months, all in females. The overall mortality was 12/119.

Relative to PAI's 90-day study, at three months into Aulerich's one-year study, the high dose animals (450 ppm) had the same mean body weight as did their controls. We concluded tentatively that 450 ppm would not be very toxic during a 90-day exposure.

In the Aulerich mink there was an unexplained apparent disparity between the single dose oral acute LD_{50} (503 mg/kg) and the 21-day repeat dose (2 grams/kg BW per day for 21 days, with no deaths). Both of these groups were older animals (8 mo) and not pregnant, in comparison with the chronic study, in which the mean consumption of DIMP was approximately 100 mg/kg/day in the 450 ppm group. One speculative explanation for the disparity in response between the single oral LD_{50} and the much larger 21-day repeat dose is that the latter was incorporated in feed, while the former was administered as a bolus to fasting animals.

In an attempt to bridge the dose-response uncertainties, we designated the following doses for this 90-day study: 0, 50, 450, 2700, 5400, and 8000 ppm. The rationale for these doses was to include 50 ppm as virtually certain to be below a 90-day observable effect level, 450 ppm as possibly below the 90-day observable effect level, while repeating the Aulerich 1-year dose, and ranging to 8000 ppm, a dose slightly below the 21-day dose used by Aulerich et. al. The intermediate doses of 2700 and 5400 ppm are approximately equally spread between 50 ppm and 8000 ppm. Our higher doses exceeded Aulerich's chronic high dose, in the expectation that some of the alleged toxic effects seen later in the Aulerich chronic study could be brought about sooner at the higher doses.

1.3 Assessment of Toxicity

The animals were evaluated during the study by periodic measurement of food consumption, body weight, hematological and chemical characteristics, and by clinical examination. At the end of the 90-day period, two animals of each sex from the 8000 ppm and 2700 ppm dose groups were maintained on untreated feed for a 4-week recovery period. All animals were examined by gross necropsy and microscopic examination at the end of the study.

2.0 MATERIALS AND METHODS

2.1 Chemical Composition of Diisopropyl Methylphosphonate

(Dacre and Rosenblatt, 1987) and MRI, 1991 (Appendix 1, this report)

- CAS number: 1445-75-6
- RTECS number SZ9090000 (1983-1984 Supplement)
- Structure: $(CH_3)_2(CH0)_2(CH_3)P=0$
- Molecular Formula: $C_7H_{17}0_3P$
- Molecular Weight: 180.2
- Physical Appearance: clear, slightly viscous liquid
- Density at 23.3°C and 708.7 mmhg: 0.9754±0.0008 g/ml
- Boiling Point: 192.6±0.6 C (747.4 mmhg)
- Solubility in water at 25°C: listed as 1 - 2 g/L (Dacre and Rosenblatt, 1987), however, PAI's tests showed a solubility at room temperature of at least 80 g/L. This amount remained in

solution when temperature was lowered to freezing (approximately 0°C).

- Hydrolysis rate at 10° C: 3.2×10^{-6}M sec^{-1}
- Hydrolysis Half-life: 687 years
- Decomposition Products: (in microwave plasma discharge): methylphosphonic acid, isopropyl methyl phosphonate, phosphoric acid, isopropyl alcohol, and propylene. DIMP forms a number of metal complexes in the absence of moisture.

2.2 Characterization of the Test Article

The DIMP used in this study was stated by the supplier, Lancaster Synthesis, LTD, Windham, NH, to be approximately 97% pure. An aliquot of this batch was analyzed by MRI, Kansas City, MO. The characterization consisted of determination of physical properties, spectroscopy, water analysis, chromatography and an accelerated stability study. The 7 March 91 report from MRI, Appendix 1A, details the methods and instrumentation, the spectra obtained by infrared, proton nuclear magnetic resonance (NMR) and mass analysis, the gas chromatographic profile obtained, and a comparison gas chromatographic major peak profile of diisopropyl methylphosphonate.

In brief, infrared and ultraviolet/visible spectroscopy and nuclear magnetic resonance were all consistent with the structure of DIMP. A slight increase in absorbance was observed between 320 and 200 nanometers with UV and a trace impurity was detected with NMR. Gas chromatography with mass spectroscopy confirmed the major component to be DIMP, with a minor component (approximately 5%) of **diisopropyl phosphonate**. Water content was determined to be 0.2%. Thin-layer chromatography using both methanol and methanol:toluene solvent systems, and gas chromatography, using two different systems, yielded chromatograms with one major peak and one minor peak (> 0.1% of the major peak). The area of the minor peak was 5.3-5.4% of that of the major peak. No other impurities were observed up to 20 minutes after the major peak was eluted.

For the accelerated stability study, DIMP was sealed in teflon-lined vials and held for 24 days at -20°C, 5°C, room temperature, and 60°C, then analyzed by gas chromatographic major peak analysis. The percent of DIMP remaining was 100±0.2, 100±0.1, 99±0.3, and 99.6±0.2, respectively. The 5% impurity (diisopropyl phosphonate) remained between 4.87±0.2% and 4.81±0.1% in the stability test. The toxicological potential of this impurity was reviewed by the sponsor. The impurity posed no known toxicity at the levels used in this study, and the sponsor accepted the test article as characterized above.

2.3 Animal Feed

The basal ration was a commercial product formulated to meet or exceed the minimum nutrient requirements for mink as outlined in the National Research Council Handbook (1982). The diet consisted of commercial cereal, 17% (Special Breeder Mink Cereal, Hager Company, South St. Paul, MN); turkey liver, 12%; beef kidney, 8%; whole cooked eggs, 15%; whole chicken, 12%; whole turkey, 8%; beef lung, 10%; tripe, 6%; Pacific Ocean fish scraps, 12%; alpha-tocopherol acetate (20,000 IU/lb), 0.1%; biotin (100 mg/lb), 0.1%; brewer's yeast, 0.25%; wheat germ oil, 0.25%; vitamin supplement (Hager Company South, St. Paul, MN, Mink Vita Aid™), 0.175%. The consistency adjusted by addition of water, approximately 35% by weight. The test compound was incorporated into this basal ration.

Administration of DIMP in drinking water would have been preferable in the 90-day study, since the issue concerned groundwater and drinking water, but the mink's diet contains approximately 70% moisture, and their daily requirement of 190-225 gm of water is met principally through feed. (Full-grown animals consume 200-285 gm of feed/day). They need only 25-50 ml of drinking water daily (Joergensen, 1985). Dosed water would therefore contain high and possibly unpalatable concentrations of DIMP. Mink also play with their water source and spill unquantified amounts, so determining the actual doses consumed by this route would require considerable approximation. Thus we used the more cumbersome, but less uncertain route of dosed feed.

For each dose level of DIMP, two separate lots of feed were prepared as described in Section 2.4, below. One lot of each dose was prepared during 28 Mar - 4 Apr 91; the second lot was prepared during 21 May - 6 June 91. The individual-portion cans were sealed with "pop-tops," color coded by dose group, frozen and stored at -20°C until feeding. The first lot lasted until Day 50 of the study for each dose group and the second lot was fed from Day 50 to the end of the study. Cans were removed from the freezer and thawed 1-2 hours at room temperature before being placed in the cages.

One can per 100 was selected randomly and shipped to MRI to analyze for DIMP content, lot homogeneity, and stability. The stability tests documented the DIMP content in samples stored at -20°C for 0, 7, 14, 28, 60, 90 and 120-day intervals, and in others held 24 hours at room temperature. Specific instrumentation and analytical methods used for these analyses are detailed in the MRI report (Appendix 1B).

One can per 100 from each batch of control feed was selected at random for nutrient and contaminant testing and stored at -20°C, thawed 24 hours at 4°C and mixed together in a 91 kg-capacity ribbon blender. Two samples of approximately 1 kg were taken after mixing for one hour, and a second pair was taken after mixing for 1.25 hours. These samples were placed in plastic freezer bags and refrozen at -20°C. One sample taken at one hour and one taken at 1.25 hours were retained at UMN. The other set was shipped frozen to Lancaster Labs, Lancaster, PA, for the following analyses: moisture,

crude protein, crude fat, crude fiber, aflatoxin (B1, B2, G1, G2), free fatty acids, peroxide value, aerobic plate count, arsenic, cadmium, lead, mercury, vitamin E, organochloride and organophosphate pesticides including alpha BHC, beta BHC, gamma BHC, lindane, delta BHC, heptachlor, aldrin, heptachlor epoxide, DDE, DDD, DDT, HCB, mirex, methoxychlor, dieldrin, endrin, telodrin, chlordane, toxaphene, PCB's, ronnel, ethion, trithion, diazinon, methyl parathion, ethyl parathion, malathion, endosulfan I, endosulfan II, and endosulfan sulfate. Analytical methods and results are listed in Appendix 3. All values were within acceptable ranges; in particular, organophosphate insecticides were below detectable limits.

2.4 Formulation and Verification of Dosed Feed

2.4.1 Formulation

The basal mink ration was obtained from North Branch Fur Farm, North Branch, MN, the supplier of the mink. It was delivered to UMN by truck in steel drums containing about 200 kg each. Two 362 kg batches of dosed feed were prepared for each dose group. Because the stability of DIMP in feed was unknown at the time the doses were prepared, a 10% excess was added. The following quantities of DIMP were incorporated into a DIMP/water premix for each dose group:

Target Dose (ppm; mg/kg)	Grams of DIMP per 362 kg dosed feed
0	0
50	21.0
450	189.0
2700	1134.3
5400	2268.5
8000	3360.8

The DIMP for each dose was weighed into a Nalgene brand plastic carboy, diluted to 17 L with tap water and mixed for 5 minutes by agitation.

The DIMP premix was added to 185 kg of basal ration in a single-screw, 454-kg capacity ribbon mixer, and mixed for 20 minutes; another 160 kg of basal ration was added and mixed for 30 minutes.

The dosed feed was transferred to steel drums of 225-kg capacity and immediately dispensed into individual-portion cans. Canning was accomplished with a hydrostatic sausage stuffer equipped with a metering head. (EZ-PAK Stuffer, model PO529, Mandeville Co., Inc., Minneapolis, MN). Approximately 400 gm of dosed feed were dispensed into epoxy-lined 450-gm capacity, 307/306 steel cans (American National Can Company, St. Paul, MN). An aluminum "pop-top" lid was sealed onto each can with an electric can sealer (Freund Can Co., Chicago, IL. Catalog No. EL 12253-1).

Each can was color-coded by dose. The sealed cans, 100 per steel freezer basket, were frozen and stored at -20°C until used.

Batches of dosed feed were mixed in ascending order of dose, starting with untreated control feed. The food-handling equipment was thoroughly emptied between batches, washed daily with hot water and sanitized 15 minute with 5% sodium hypochlorite solution.

2.4.2 Verification of Target Doses and Homogeneity of Mix

To determine actual DIMP concentrations in dosed feed samples, MRI weighed approximately 40 gm of feed into 200 ml centrifuge bottles that contained ten 5/16" stainless steel ball bearings. A 100 ml aliquot of methanol was measured into the bottle, and the bottle was sealed. The DIMP was extracted into the methanol with 15 minute of shaking on a wrist-action shaker. The methanol extracts were clarified, diluted, filtered and injected onto a gas chromatographic system for quantification of DIMP content. The analyses were performed in triplicate. A complete description of the analytic procedure, instrumentation and results are contained in the 21 January 92 MRI report in Appendix 1B.

MRI found the Lot 1 samples to contain 98.6 to 133.5% of the target concentrations of DIMP. The formulations were considered homogeneous, based on standard deviations of less than 6.1% for the found/theoretical DIMP concentrations. Lot 2 had 97.6 - 119.5% of the target concentrations and was equally homogenous, with standard deviations of less than 5.1% for the found/theoretical DIMP concentrations. The control samples contained no detectable DIMP.

The target versus actual concentrations of DIMP in each lot are tabulated in Table B 2.4.2.

Table B 2.4.2 DIMP in Dosed Feed, µg/gm (ppm)
Target Concentration Versus Actual

Target Concentration	Lot 1 (50 days)	Lot 2 (40 days)	*Actual Concentration
50	56.7	56.9	56.7
450	581	464	528
2700	2776	3129	2930
5400	6207	6146	6174
8000	9032	8957	8990

* Weighted average, Lot 1 + Lot 2, 90 days.

The rationale for selecting the target doses is given in Section B 1.2 of this report. The actual doses achieved were judged to be as acceptable as the original target doses, since they effectively spanned the dose range of interest. **In this report, the treatment groups are referred to by their original Target Dose designations, but their DIMP consumption was calculated on the basis of Actual Concentrations.**

2.4.3 Stability Study

DIMP in mink feed at the 50 ppm dose level (from Lot 2) was analyzed by MRI to determine its stability during storage at room temperature for 24 hours (to simulate feed in the animal cage) and frozen at -20°C for 7, 14, 28, 61, 90 and 120 days (to simulate storage of the dosed feed prior to serving it). The analytic system was the same as that referred to in Section 2.4.2, and is described in detail, together with the results of the stability test, in the same MRI report in Appendix 1B.

The analyses showed "no statistically significant loss of chemical when stored in sealed containers for 120 days at -20°C. In addition, dosed feed stored for 24 hours open to air and light showed no statistically significant loss of chemical" (Appendix 1B).

2.5 Animals and Animal Husbandry

One hundred sixty, 12-month-old brown "Ranch Wild" type mink (80 males and 80 females), obtained from North Branch Fur Farm, North Branch, MN, were randomized by body weight into eight experimental groups as outlined in Table B 2.5. Mink used had been vaccinated at eight weeks of age against Pseudomonas pneumonia, canine distemper, mink virus enteritis, and Type C botulism (Biocom DP, United Vaccine Co, Madison WI) and were free of Aleutian disease virus.

Table B 2.5 Dose Groups for DIMP 90-Day Subchronic Study

Sex	Dose Level	Animal Numbers
Male	0 ppm	10
Male	50 ppm	10
Male	450 ppm	10
Male	2700 ppm	10
Male	5400 ppm	10
Male	8000 ppm	10
Male	Pair-Fed Control[a]	10
Male	Pair-Fed Control[b]	10
Female	0 ppm	10
Female	50 ppm	10
Female	450 ppm	10
Female	2700 ppm	10
Female	5400 ppm	10
Female	8000 ppm	10
Female	Pair-Fed Control[a]	10
Female	Pair-Fed Control[b]	10

[a] Group 0PF1 = untreated ration to match food consumption of 5400 ppm dose group

[b] Group 0PF2 = untreated ration to match food consumption of 8000 ppm dose group

The animals were quarantined for approximately 6 weeks and randomly assigned by animal number to seven rooms in the Veterinary Science Building, College of Veterinary Medicine, UMN (room numbers 345B, 345K, 345L, 345M, 118, 116, 114). Animals of all dose groups and both sexes were represented randomly in all rooms.

Animals were held individually in galvanized wire mesh cages, 30 cm wide by

37.5 cm high by 60 cm long. Each cage was also equipped with a wire mesh "nest box," approximately 22 x 22 x 45 cm atop the rear of the cage, to which the mink had unrestricted access. Lighting in the animal rooms was controlled to approximate the outdoor daylight:dark cycle at the time the animals were quarantined. At the beginning of the quarantine period, the time clocks were inadvertently set so that rooms 345 B, K, L, and M received 13.5 hours of light and 10.5 hours dark, and rooms 118, 116 and 114 received the intended setting, 12.5 hours of light and 11.5 hours of dark. The time differential, discovered midway through the study, was maintained for the duration of the study. Animal rooms were held at 23±5°C and 30 - 70% relative humidity.

The mink cages, color-coded for dose, were on metal supports, with plastic-backed absorbent-lined paper on the floor beneath. Feces were removed from the cages, and the papers were removed from the floor and replaced daily. Fresh cages were provided every fourth week; used cages were moved to a separate room, scrubbed manually with a quaternary ammonium disinfectant and rinsed with hot water under high pressure.

Although there were no federal or local regulatory requirements to do so, feces-soiled absorbent paper, uneaten food and all other solid waste that potentially contained DIMP were collected into special containers for disposal by a Minnesota-licensed hazardous waste disposal firm under contract to UMN.

The cages were equipped with aluminum drinking cups that were cleaned and refilled twice daily. Residual water was withdrawn with a "wet-dry" vacuum and was replaced with fresh water. Individual mink that habitually played in their water and caused spillage were attended to more frequently.

An ophthalmic examination of all mink was conducted during allocation and again prior to necropsy. Topical 1% tropicamide drops were used to dilate the pupils; examination by indirect ophthalmoscope was performed with the animals restrained manually.

Clinical observations were recorded twice daily. Animal body weights and 24-hour feed consumption were recorded once weekly for all animals. For daily feeding, an individual "pop-top" can containing approximately 400 gm of feed was opened and placed in a customized holder that had been fabricated into the cage, the color code of the can matching that of the cage card. The can was replaced with a fresh one each day. To determine food consumption, the can and contents were weighed when dispensed, and again 24 hours later; the difference was recorded as food consumption. If any spilled food was noted at that time, it was collected and weighed as accurately as possible. Animal care personnel identified the animals by cage number and also color code. They were unaware of dose group identity.

Food weights and individual animal body weights were collected with Mettler brand balances coupled to a lap-top computer, both mounted on a wheeled cart. The data

were collected manually and also entered into the proprietary LABCAT software system (Innovative Programming Associates, Princeton, NJ).

The 400-gm daily food ration was sufficiently excessive to provide ad libitum feeding. All animals were fed their respective ration ad libitum except the pair-fed control animals. Pair-fed undosed control groups were fed to match the food consumption of the two highest dose groups. When the food consumption of either of the two highest dose groups was less than 90% of that of the untreated control animals, the treated group's mean daily food consumption was offered to its respective pair-fed cohort during the following week. When the treated group's feed consumption was greater than 90% of the untreated controls', the pair-fed cohort was fed ad libitum. The purpose of the pair-fed controls was to allow estimation of any weight loss in the high dose groups due to food refusal.

2.6 Hematology and Clinical Chemistry

Chemical and hematological examination of blood specimens from all mink was conducted by the Hospital Laboratories, College of Veterinary Medicine, UMN. Blood samples (approximately 6 ml) were obtained from each animal at allocation, during weeks 3, 7, and at termination of the study (week 13). Samples were collected by jugular venipuncture with a syringe and 22-gauge needle, prior to morning feeding, with mink under anesthesia (ketamine 40 mg/kg BW:xylazine 1 mg/kg BW, administered intramuscularly). The animals recovered from anesthesia in 15-20 minutes. Blood was collected into two tubes, one plain to allow blood to clot (approximately 4 ml) and one containing potassium EDTA anticoagulant (2 ml). The samples were submitted to the laboratory for processing within one hour.

For leukocyte differential cell counts and red cell morphology, two wedge blood films were made and stained with a Hema-Tek Model 4480 (Miles Scientific Naperville, IL) stainer using a Hema-Tek Stain Pak 4481 (Miles Scientific, Naperville, IL), a Wright's stain. For reticulocyte counts, 2 drops of blood were mixed with new Methylene Blue dye, incubated for 15 minutes at room temperature, remixed, and two wedge films were prepared. Reticulocytes were enumerated per 1000 erythrocytes and expressed as a percent. Heinz body slides were prepared by the method of Schwab and Lewis (1969). Two thin wedge films were made, Heinz bodies were quantified per 1000 erythrocytes (500 on each slide) and expressed as a percent. Two hundred leukocytes were enumerated as to cell type, i.e., polysegmented neutrophil, immature neutrophil, eosinophil, basophil, monocyte, lymphocyte, blast cell or other(s). Leukocyte data were reported as a percent cell type and as absolute number. Uncommon cell types were subjectively scored as rare or present.

Erythrocyte morphology was determined at 1000 magnifications on the Wright's stained films. Characteristics evaluated were:

- Anisocytosis - Variation in cell size, increased size indicative of increased rate of production of erythrocytes.

- Polychromasia - Cells having uneven staining, tending to be more basophilic than normal, caused by decreased hemoglobin content, indicative of increased rate of production of erythrocytes.

- Howell-Jolly Bodies - Basophilic nuclear fragments present in circulating erythrocytes, indicative of increased rate of production of erythrocytes.

- Eccentrocytes - "Dimpled" circulating erythrocytes associated with conditions that produce Heinz bodies, presumably due to interaction between the cell membrane and the denatured hemoglobin internally. Commonly seen in early oxidant injury to hemoglobin.

- Keratocytes - Abnormal fragmented erythrocytes that may be associated with removal of Heinz bodies from them by the spleen ("pitting" of Heinz bodies).

- Schizocytes - Erythrocyte fragments resulting from mechanical injury and subsequent fragmentation of erythrocytes.

- Echinocytes - Shrunken, RBC with spiked periphery dehydration or membrane alteration.

Established criteria (Bessis, 1973; Jain, 1986; Viskochil et al., 1978; Perman, 1991) were used to classify the morphologic changes, and they were quantified subjectively as "absent" or "rare" and, when in greater number, graded as 1+ to 4+. These data were treated statistically as categorical data, and the analysis is included in the statistician's report, Appendix 4A.

Quantification of erythrocyte indices, platelets and leukocytes was done on a Coulter Model S+IV (Coulter Electronics, Hialeah, FL). The complete blood cell count included white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean erythrocyte cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and platelets. Results and histograms were printed in duplicate. Quantitative data were entered into the Biovation Hematology Console (Biovation Co. Richmond, VA) for data management.

The blood collected for serum chemistry was allowed to clot at room temperature. Within one hour of collection, the clot was rimmed with an applicator stick and the tube centrifuged for 10 minutes at 3000 rpm in a Beckman GP Centrifuge (Beckman Instruments, Palo Alto, CA). The fibrin-free serum was transferred to clean polystyrene tubes and capped. All serum samples were refrigerated until analyzed. Remaining serum was frozen (-20°C).

The analyses for blood urea nitrogen, sodium, potassium, chloride, total carbon dioxide, glucose, calcium, creatinine, and phosphorus, and calculation of anion gap and osmolarity were done on an Astra-8 Analyzer system (Beckman Instruments, Brea, CA) using the manufacturer's recommended methodology. The analyses for serum cholinesterase, albumin, alanine aminotransferase and aspartate aminotransferase were conducted with a Beckman CX4 analyzer (Beckman Instruments, Brea, CA). The methods in the manufacturer's manual (Beckman Instruction 015-2477712-6) were followed except for albumin, which was measured by the method of Poumas (1972), using Boehringer Mannheim Diagnostic-PN 5107242H reagent.

The instruments were coupled to a computer system which created a database and printed data in chart form. All data were analyzed on spreadsheets and verified prior to statistical analysis. The statistician's report is in Appendix 4A.

At the end of the 90-day treatment period, two males and two females from both the 8000 and 2700 ppm groups were allowed a 4-week recovery period on untreated feed. Food consumption, body weight and blood parameters that differed significantly from control values at the end of the 90 days were monitored weekly. These eight animals were subjected to the same necropsy and histopathology procedures as the 90-day cohort.

2.7 Necropsy and Histopathology

At the termination of the study, all animals were anesthetized for collection of the final blood sample and were then euthanized by carbon dioxide asphyxiation while anesthetized. A detailed gross necropsy was performed on all treated and control animals.

Necropsy, tissue collection and processing, and histopathologic evaluations followed the procedures of the National Toxicology Program (NTP) for 13-week studies (National Toxicology Program, 1987) and the Standard Operating Procedures of Pathology Associates, Inc. The NTP "read down" procedure, in which 44 tissues from all high dose and control animals are examined first microscopically, was used. The organ weights of all groups were compared by analysis of variance and group means were compared with Dunnett's T-test.

2.8 Statistical Analysis

Body weight, food consumption, blood chemistry and continuous hematology data were analyzed using repeated measures, with an incomplete block design (Winer, 1971; Steel and Torie, 1980). Contrasts (Steel and Torie, 1980) were used to compare the untreated control values to all other treatment values as well as to determine if linear, quadratic or cubic effects existed. All times were compared to the first time as well as polynomial effects of time. The repeated option of PROC GLM of SAS (SAS, 1987) was used to perform the analyses.

Morphologic changes in erythrocytes were analyzed as categorical variables, with repeated measures (Koch et al, 1977). The variables anisocytosis, polychromasia and formation of eccentrocytes and keratinocytes each had three categories (rare, +1, +2), while Howell-Jolly bodies and schizocytes formation each had two categories of response (rare, +1). Contrasts were used to compare 1) values from untreated controls to all other treatment values, 2) the 5400 and 8000 ppm dose groups to their respective pair-fed controls, and 3) the allocation time (Week 0) to all other times (Freeman, 1987). The analyses were performed with the "Repeated" option of ROC CATMOD of SAS (SAS, 1987), using weighted least squares.

The males and females were analyzed separately. The detailed statistical report is included as Appendix 4A.

The organ weights of all groups were recorded at necropsy and were compared by analysis of variance; group mean organ weights were compared with Dunnett's T-test.

3.0 RESULTS

3.1 Food Consumption and Body Weight

3.1.1 Food Consumption

Group mean daily food consumption, measured weekly, is tabulated by sex in Tables B 3.1.1 a & b and plotted in Figures B 3.1.1 a-f. Data for individual animals are compiled in Appendix 5.

Table B 3.1.1a Group Mean Food Consumption (grams) Male, N=10
Day of Study

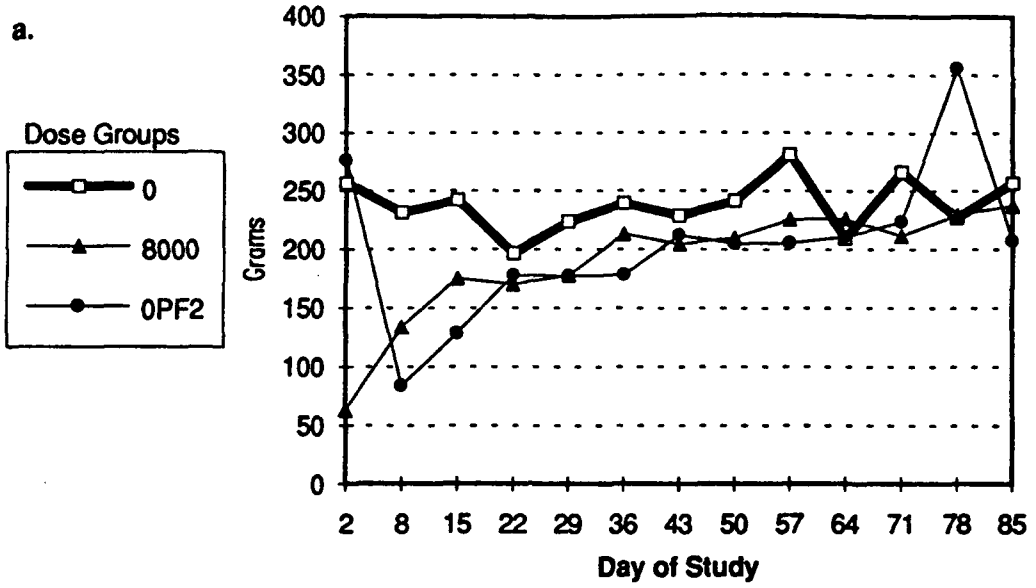
Dose Group		2	8	15	22	29	36	43	50	57	64	71	78	85
0	Mean	256.6	230.8	241.7	196.3	223.2	239.0	228.3	241.5	281.1	209.0	266.4	226.8	256.5
	SD	36.9	17.3	45.1	29.5	59.8	52.9	26.9	27.1	35.2	45.7	64.7	41.5	33.6
50	Mean	286.7	236.1	259.2	226.4	238.8	232.9	225.7	238.0	265.2	249.0	264.4	269.4	287.4
	SD	19.8	52.2	33.0	39.5	52.4	53.6	60.9	47.9	62.8	55.5	59.3	54.2	38.6
450	Mean	284.9	240.2	258.6	221.7	221.4	227.8	195.5	266.5	259.7	227.5	276.6	270.0	249.4
	SD	64.9	41.9	52.1	43.3	79.7	41.3	77.1	65.9	51.8	59.5	51.5	47.8	72.0
2700	Mean	174.5	223.0	238.3	208.8	215.2	221.3	220.2	240.2	264.8	242.5	284.2	250.8	279.2
	SD	82.7	39.6	46.7	34.9	47.9	47.7	57.5	47.2	62.2	47.6	46.3	48.9	53.9
5400	Mean	144.5	226.2	240.7	226.5	219.5	243.3	229.5	253.3	268.7	252.1	267.6	276.8	273.4
	SD	83.1	45.2	41.1	43.9	58.4	41.6	49.1	45.4	41.1	30.4	51.5	54.7	38.3
8000	Mean	63.1	133.4	174.9	169.8	177.7	213.2	204.2	209.9	225.7	226.2	211.3	229.2	237.1
	SD	51.2	56.4	31.6	44.1	34.9	28.7	25.5	35.7	37.4	30.9	49.2	40.6	25.1
0PF1	Mean	260.0	160.6	267.1	247.8	219.7	214.2	198.2	245.6	221.5	196.5	258.1	223.7	253.5
	SD	54.0	42.4	82.4	60.0	49.4	57.3	70.7	73.7	53.4	26.8	47.1	37.9	56.6
0PF2	Mean	275.9	83.4	128.3	177.4	176.9	178.1	212.0	204.6	205.3	210.8	223.5	356.1	207.0
	SD	54.1	80.3	23.1	2.7	5.5	2.0	4.3	3.3	25.7	5.3	9.4	62.4	11.0

Table B 3.1.1b Group Mean Food Consumption (grams) Female, N=10
Day of Study

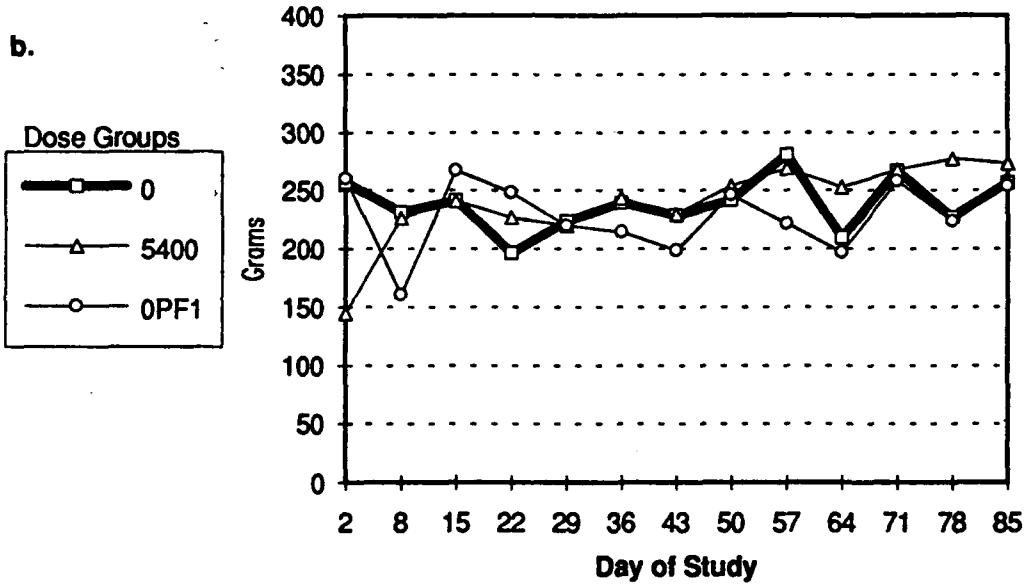
Dose Group		2	8	15	22	29	36	43	50	57	64	71	78	85
0	Mean	196.7	165.2	168.5	133.2	166.7	141.7	158.5	149.7	173.6	161.3	201.9	172.6	209.3
	SD	46.7	50.7	43.6	41.8	54.9	64.0	35.7	62.5	32.3	47.8	38.9	30.8	44.7
50	Mean	173.8	159.5	149.9	129.5	174.1	163.1	155.1	148.8	191.8	169.4	193.6	179.0	190.6
	SD	65.9	44.3	28.0	24.4	28.3	39.1	33.8	63.1	40.5	40.3	37.5	44.9	44.6
450	Mean	186.6	180.8	179.0	156.1	161.7	187.8	147.4	199.4	170.0	143.2	189.0	168.2	186.9
	SD	90.6	31.3	50.4	28.8	54.3	46.7	68.6	69.2	38.2	30.3	45.9	23.9	47.0
2700	Mean	166.9	173.0	172.9	160.6	144.4	142.3	156.3	183.0	183.0	167.2	194.7	179.8	190.4
	SD	79.5	33.0	48.5	37.7	32.2	35.6	24.9	66.7	38.1	26.2	46.2	26.2	47.3
5400	Mean	64.3	152.5	155.1	137.9	117.2	151.0	149.9	157.3	161.9	165.0	178.4	160.3	187.3
	SD	57.0	47.7	32.9	31.1	57.4	21.3	21.8	32.8	28.3	33.5	41.2	28.4	29.3
8000	Mean	37.1	99.5	133.3	122.0	117.0	134.6	136.7	146.2	140.4	144.5	147.5	147.7	157.3
	SD	39.7	26.7	45.8	26.4	32.4	28.2	29.3	25.8	11.2	32.7	40.1	24.7	34.5
0PF1	Mean	187.6	116.0	150.6	217.1	177.2	108.2	240.5	172.7	200.4	160.0	186.2	178.7	175.6
	SD	48.2	72.8	78.3	67.6	19.3	14.2	47.8	34.4	30.3	47.4	27.6	25.7	20.5
0PF2	Mean	201.7	52.5	93.2	135.4	202.6	111.0	253.6	132.5	230.1	176.2	138.2	242.6	175.5
	SD	39.6	23.7	21.1	5.3	59.5	15.7	54.7	6.5	35.5	31.2	7.5	47.1	49.6

Figures B 3.1.1 a-c Weekly Food Consumption - Males

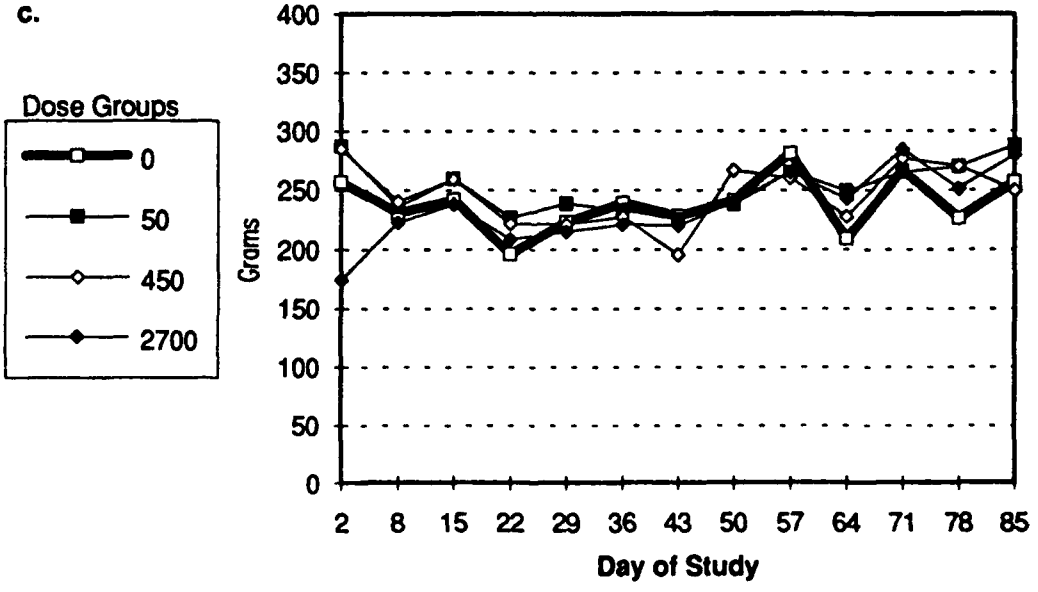
a.



b.

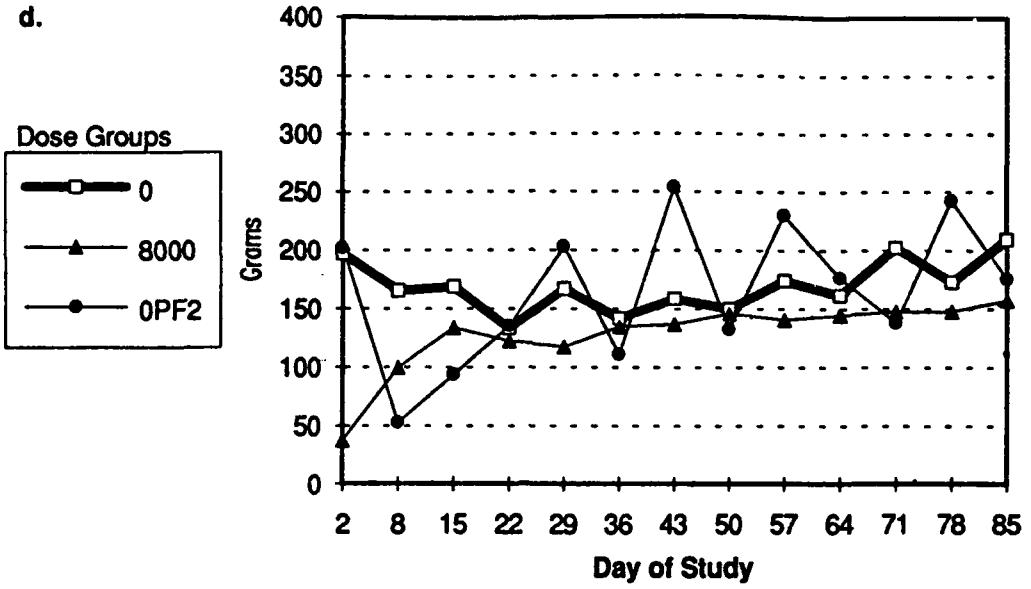


c.

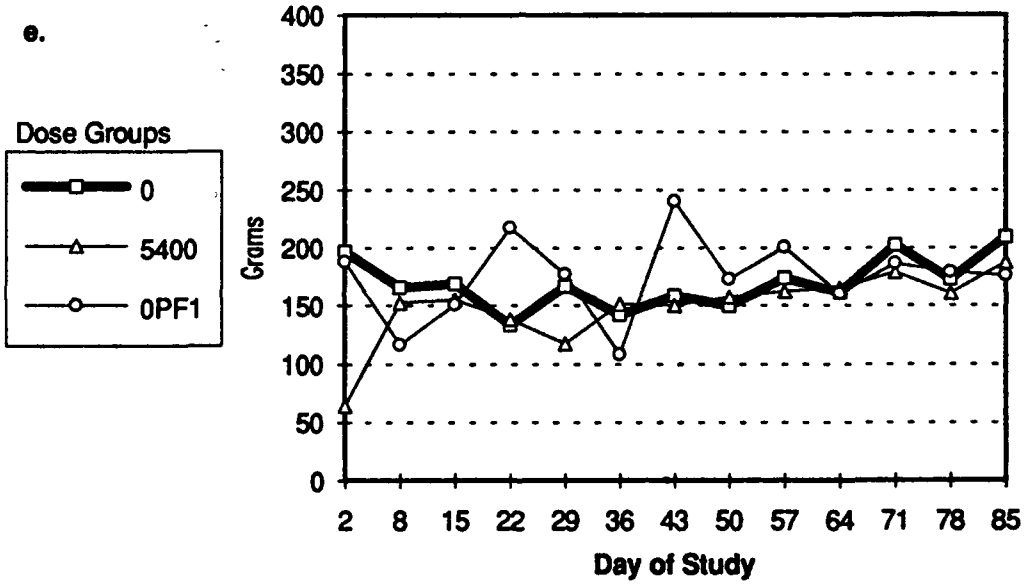


Figures B 3.1.1 d-f Weekly Food Consumption - Females

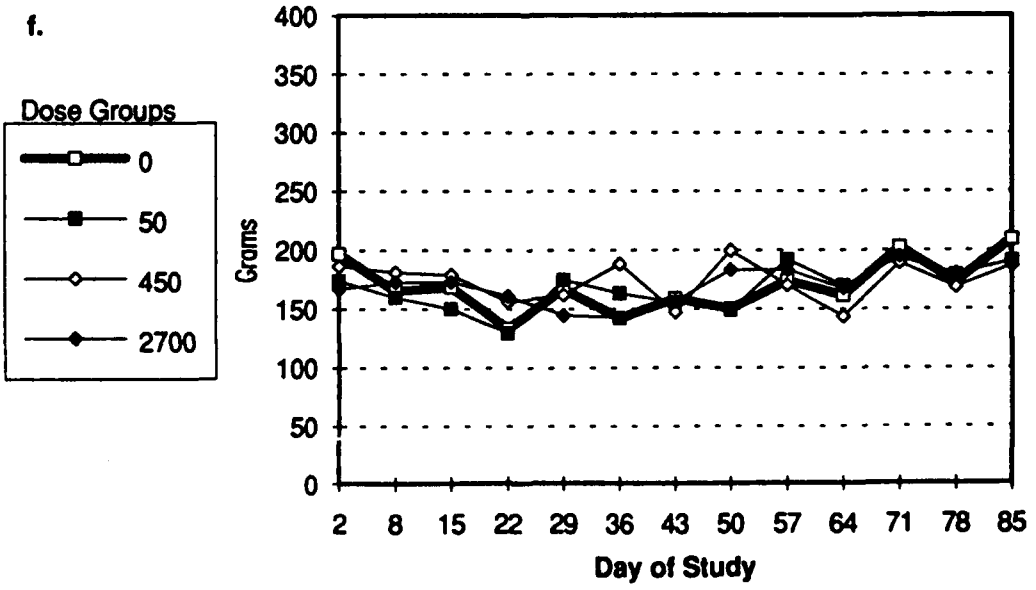
d.



e.



f.



Overall, males and females of the 8000 ppm group consumed 20% and 24% less food than their respective 0 ppm controls, a difference statistically significant at $p < 0.01$. The 5400 ppm females ate 12% less overall than the 0 ppm females, also a significant decrease. All other treatment groups consumed as much food as the 0 ppm group. There was a consistent trend of increasing food consumption over the duration of the study for all groups (note Figures B 3.1.1 a-f).

The higher-dosed animals refused food, i.e., ate less, and they also lost weight (see Section B 3.1). Because of the weight loss, their decrement in food consumption was smaller on the basis of per-kilogram of body weight than it was on the basis of grams consumed per day, when compared to 0 ppm animals that had no significant weight loss. The mean food consumption per kilogram of body weight averaged over all 13 weeks is listed by treatment group in Table B 3.1.1c. On this basis, the female 8000 ppm group consumed only 7.6% less than the untreated controls, while there was no substantial difference among the other groups. The equivalent nutrition thus provided per kilogram of body weight lends increased validity to comparisons of clinical chemistry and hematology among groups.

Table B 3.1.1c Group Mean Food Consumption (grams/kg BW/day)

Dose Group	Gm Food/ kg BW/day	
	Male	Female
0	114.3	151.9
50	118.6	157.7
450	120.2	155.9
2700	117.6	155.3
5400	121.0	147.0
8000	112.2	140.6
0PF1	111.2	157.6
0PF2	110.2	162.1

The attempt to "pair feed" a control group to match the weekly voluntary food consumption of the two higher dose groups was only partially successful because of the mink's ability to overcompensate for previous food deprivation. The pair-fed group was permitted ad-libitum feeding when the treated cohort ate at least 90% of the amount eaten by the 0 ppm group. When a treated group ate less than 90% of that consumed by the 0 ppm group, the ration of the pair-fed cohort was restricted during the following week to the amount eaten by the treated group. If the treated group then ate 90% or more

during the succeeding week, the pair-feds were allowed ad-libitum feeding for the next seven days. When this occurred, they often more than made up for the previous restriction.

Because the food consumption pattern in the female 5400 and 8000 ppm groups allowed their pair-fed cohorts several unrestricted feeding weeks, these females consumed less than their pair-fed cohorts (Table B 3.1.1b, Figure B 3.1.1 d & e). Food consumption by the male 5400 ppm group was within 90% of the male 0 ppm group (and not statistically different) so restricted pair-feeding was not involved for that group (Figure B 3.1.1a).

The pair-fed cohort for the male 8000 ppm group did provide evidence that the decrement in food consumption, and not toxicity, was responsible for the weight loss observed in the treated animals. For the first nine weeks of the study, this group and their pair-fed cohort were well matched for food consumption, and their body weight changes were equivalent. Their weekly food consumption is listed in Table B 3.1.1a and Figure B 3.1.1a and their weekly body weights are in Table B 3.1.2a and Figure B 3.1.2a, for the entire 13 weeks. Their performance for the first nine weeks, while the food consumption was accurately matched, is tabulated in Table B 3.1.1d, and also compared to the 0 ppm group for the same interval. During this period, the treated group's mean body weight decreased 20% from their own starting weight and was 24% less than the concurrent 0 ppm control. Their pair-fed cohorts lost similar weight, 18 and 21%, respectively. (Percentages are rounded.)

Table B 3.1.1d Effect of Pair-Feeding on Body Weight, Males
*(Treatment Days 1-64)

Dose Group	0	8000	0PF2
Mean Daily Food Consumption			
Gm/animal	234.8	179.8	185.3
Gm/kg BW	112.1	102.4	99.9
Percent Loss in Body Weight			
Versus Day 1, within group	2.0	17.9	13.1
Versus 0 ppm, Day 64		22.8	17.4

*Days 8-71 for Group 0PF2, since pair-feeding followed the weekly measurements of food consumption by treated groups.

We concluded that the weight loss demonstrated by the male 8000 ppm group was the result of food refused and inferred that the same was true for the female 8000 and 5400 ppm groups, with lower mean food consumption and mean body weights than the 0 ppm females.

3.1.2 Body Weight

At the beginning of the study there were no differences among the mean body weights of all groups. Dose-related weight loss in both sexes of the 8000 ppm animals began with the first measurement and continued for the duration of the study. The differences were statistically significant beginning with the Day 15 weights. The female 5400 ppm group lost less weight than the 8000 ppm group, although their mean weight also became statistically less than the 0 ppm group's by Day 64 measurement.

Compared with their pair-fed controls, the 8000 ppm females had lower body weights beginning on Day 43; this difference was also present in the males on Day 85. The 5400 ppm females were significantly lighter in weight than their pair-fed controls, beginning on Day 78. However, as noted in Section 3.2, the pair-fed data are reliable only for the male 8000 ppm group (0PF2) for the first 64 days, because the food consumed was not equivalent at other times or in other groups. For this reason, clinical chemistry or hematology values of pair-fed groups was not used for comparison with the treated groups. They were not rigidly pair-fed, yet their food intake was manipulated so they could not qualify as additional true untreated controls. The weekly mean body weights for each group are listed by sex in Tables B 3.1.2a and 2b, and plotted in Figure B 3.1.2 a - f. Data for individual animals are compiled in Appendix 5.

Table B 3.1.2a Group Mean Body Weight (Grams) Male, N=10

Day of Study

Dose Group		0	2	8	15	22	29	36	43	50	57	64	71	78	85	90
0	Mean	2138	2213	2162	2098	2088	2103	2079	2038	2055	2059	2014	2041	2045	2069	2060
	SD	230	235	225	234	240	272	275	275	247	287	270	284	289	294	280
50	Mean	2128	2207	2153	2128	2137	2178	2170	2119	2127	2104	2072	2080	2093	2112	2102
	SD	214	142	156	154	175	197	202	210	211	208	184	178	161	165	149
450	Mean	2134	2175	2117	2060	2062	2087	2062	2003	2030	2024	1983	1985	1996	2002	1993
	SD	206	205	223	223	260	256	270	273	269	284	262	258	257	273	242
2700	Mean	2151	2145	2096	2033	2041	1953	2003	1977	2000	1961	1931	1945	1936	1943	1927
	SD	228	234	241	249	267	339	257	252	252	253	247	227	250	244	226
5400	Mean	2142	2078	2053	1984	2042	2002	1972	1959	1984	1937	1917	1916	1934	1941	1916
	SD	199	166	164	160	178	185	176	195	205	216	222	237	251	249	255
8000	Mean	2128	1992	1885	1762	1795	1707	1661	1607	1648	1577	1554	1540	1535	1542	1524
	SD	209	163	123	135	141	151	144	144	145	141	141	141	148	150	146
0PF1	Mean	2136	2170	2093	2000	2086	2081	2031	2016	2022	2037	2001	2016	2025	2039	2027
	SD	205	223	216	176	209	215	228	229	222	209	201	205	201	192	193
0PF2	Mean	2135	2221	2143	1833	1757	1833	1766	1657	1719	1676	1664	1693	1765	1894	1873
	SD	218	273	258	203	317	188	168	190	176	199	201	213	197	181	212

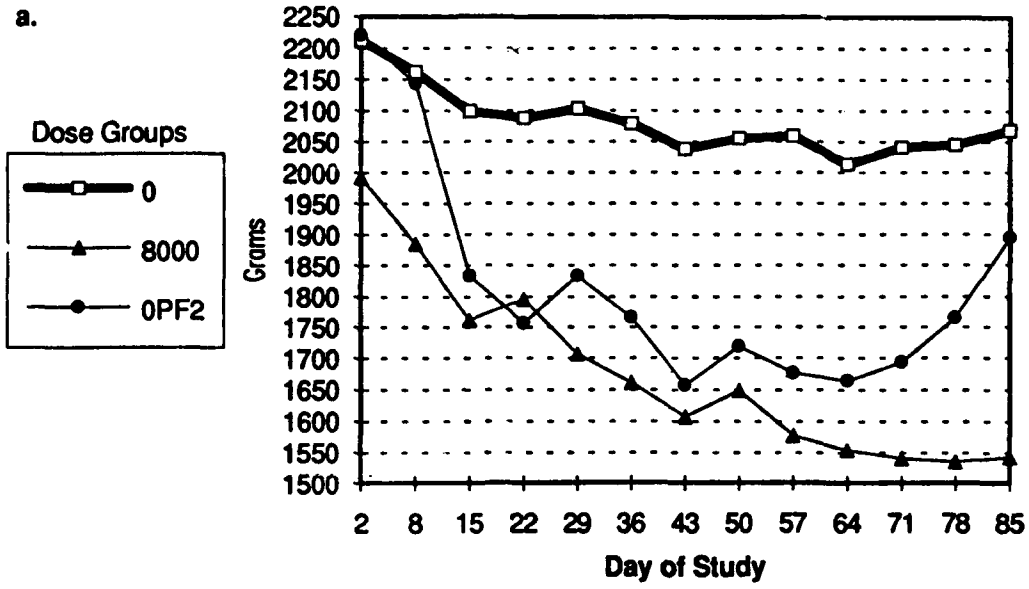
Table B 3.1.2b Group Mean Body Weight (Grams) Female, N=10

Day of Study

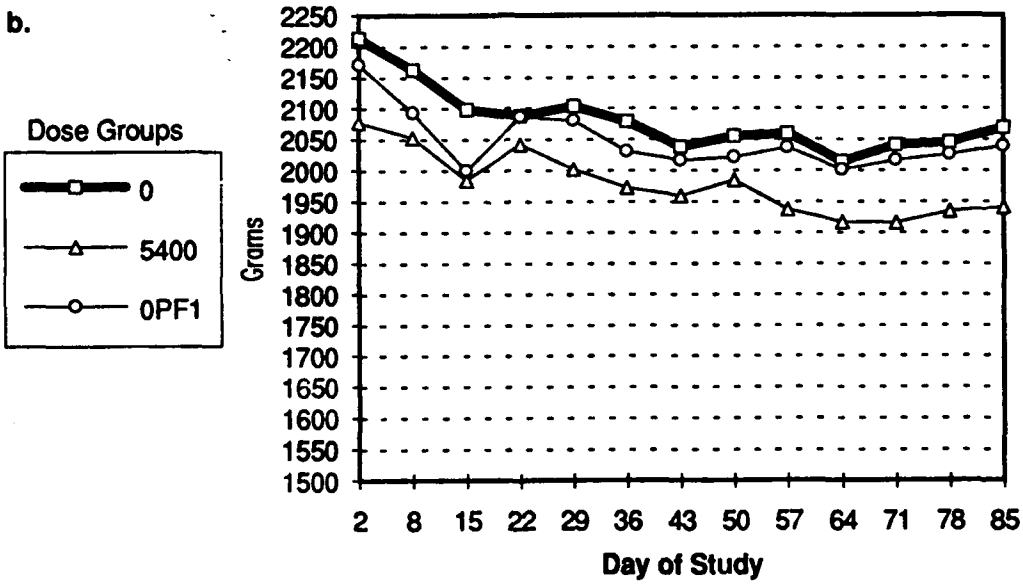
Dose Group		0	2	8	15	22	29	36	43	50	57	64	71	78	85	90
0	Mean	1161	1191	1159	1113	1157	1112	1095	1078	1090	1097	1080	1090	1094	1092	1086
	SD	142	132	136	140	209	170	176	185	181	194	192	190	187	203	182
50	Mean	1166	1191	1146	1097	1088	1089	1057	1024	1033	1020	993	1002	1003	1018	1011
	SD	136	111	116	116	110	101	108	112	115	113	118	110	119	120	135
450	Mean	1173	1175	1162	1130	1128	1126	1125	1083	1116	1107	1079	1078	1064	1081	1065
	SD	131	134	142	168	174	191	205	206	198	202	191	187	174	187	156
2700	Mean	1174	1189	1175	1128	1132	1120	1107	1073	1084	1060	1036	1041	1042	1049	1034
	SD	123	88	79	71	71	74	89	88	94	89	79	94	86	107	99
5400	Mean	1196	1109	1091	1050	1060	1020	999	979	1007	976	947	952	945	947	934
	SD	175	125	125	116	112	100	102	116	114	109	107	112	111	91	99
8000	Mean	1169	1060	1004	949	939	896	879	849	888	856	840	833	836	838	824
	SD	144	91	77	79	84	88	86	79	82	78	71	67	56	53	54
0PF1	Mean	1175	1206	1150	1015	1146	1132	1115	1062	1108	1106	1076	1077	1083	1100	1081
	SD	126	130	125	129	133	142	143	145	146	165	162	161	166	151	163
0PF2	Mean	1178	1177	1094	915	934	971	980	978	1009	1005	1004	991	1005	1024	1003
	SD	157	140	149	159	144	115	104	103	85	120	101	92	112	101	118

Figures B 3.1.2 a-c Weekly Body Weights - Males

a.



b.



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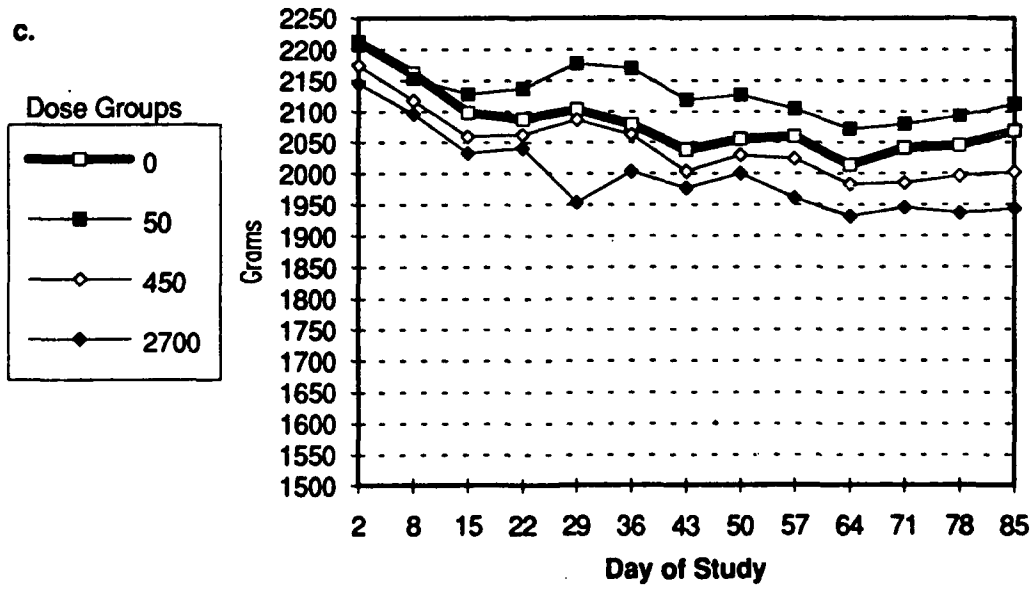
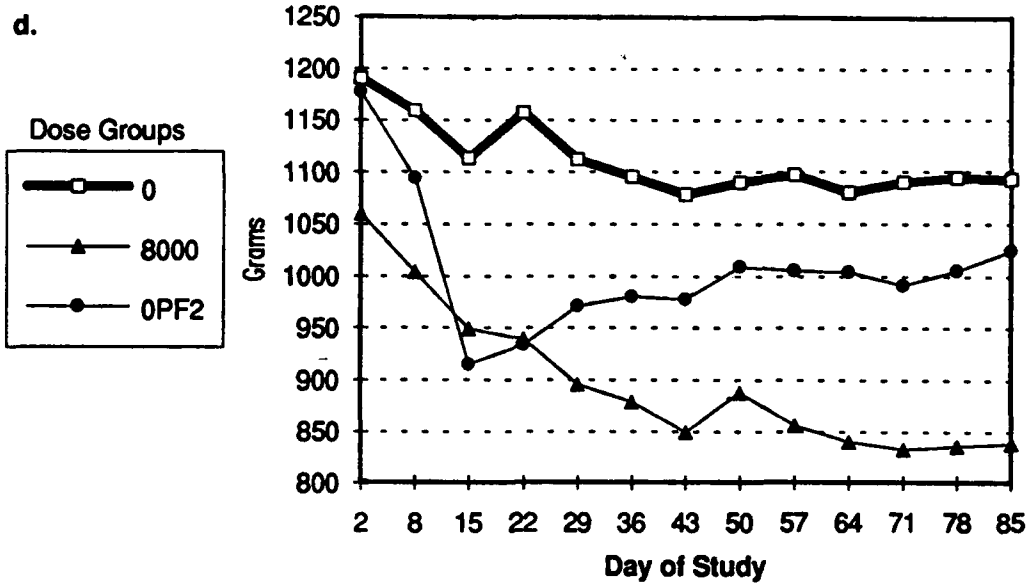
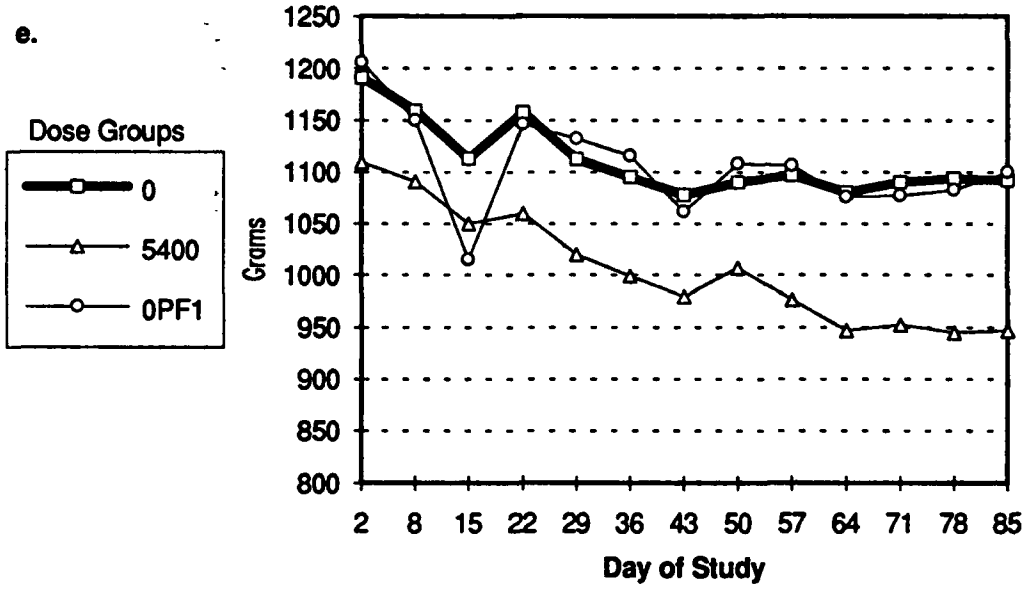


Figures B 3.1.2 d-f Weekly Body Weights - Females

d.



e.



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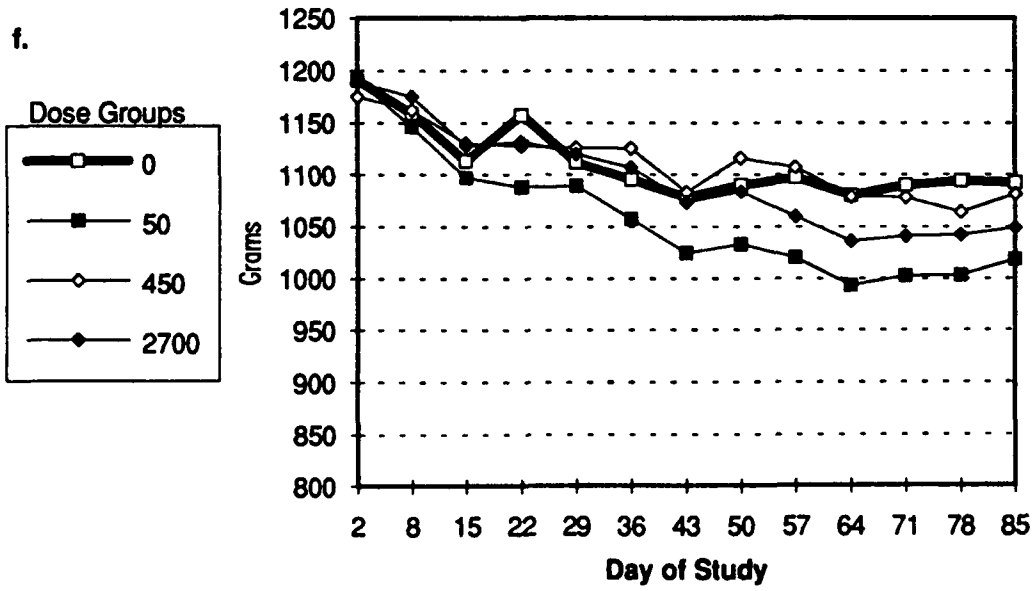


3.1.3 DIMP Consumption

Table B 3.1.3 summarizes the overall mean values for food consumption, body weight and DIMP ingestion for each group. DIMP ingestion is listed per animal as well as per kilogram of body weight, both calculated on the basis of actual DIMP content of the feed, as listed in Table B 2.4.2.

Table B 3.1.3 Group Mean Food Consumption, Body Weight, DIMP Ingested - 10 Animals/Group; 90 Days

DIMP Dose Group (ppm)	DIMP Actual (ppm in diet)	Feed Consumed (gm/mink/day)		DIMP Consumed (mg/mink/day)		Mean Body Wt. (gm)		DIMP Consumed (mg/kg/day)	
	(µg/mg)	Male	Female	Male	Female	Male	Female	Male	Female
0	0	238.2	169.1	0	0	2084.1	1113.0	0	0
50	57	252.2	167.6	14.4	9.6	2127.3	1062.5	6.8	9.0
450	528	246.1	173.5	129.9	91.6	2047.5	1112.8	63.4	82.3
2700	2930	235.6	170.3	690.3	499.0	2002.8	1096.3	344.7	455.2
5400	6174	240.2	149.1	1483.0	920.9	1985.1	1014.1	747.1	907.7
8000	8990	190.4	128.0	1711.7	1150.7	1697.1	910.7	1008.6	1263.5
0PF1	0	228.2	174.7	0	0	2052.0	1109.8	0	0
0PF2	0	203.0	165.0	0	0	1841.9	1017.9	0	0

3.2 Clinical Observations and Ophthalmology

There were no clinical signs or ophthalmologic findings related to treatment throughout the study, except for the food refused and weight loss in the 8000 ppm group. The ophthalmologist's report and a summary of the varied and random clinical observations are included in Appendix 5.

3.3 Hematology and Clinical Chemistry

Blood for hematological and clinical chemical data was obtained from all animals during the week preceding dosing (Week 0 or Allocation), again during weeks 3 and 7, and at necropsy at the end of Week 13. The data were transferred either manually or electronically to spreadsheets and analyzed statistically using the PROC GLM program of SAS (1987, Version 6, SAS/STAT Guide for Personal Computers, SAS Institute Inc., Cary, NC). The statistician's report is in Appendix 4A. Individual animal values and group mean summaries for all data are included in Appendix 7 for Hematology and in

Appendix 8 for Clinical Chemistry.

The values of the pair-fed groups have been excluded from the tables and figures in this section (they are available in Appendices 7 & 8). In the figures, the asterisks denote a statistically significant difference from the concurrent 0 ppm control, with $p \leq 0.05$.

3.3.1 Hematology

3.3.1.1 Hematocrit (HCT)

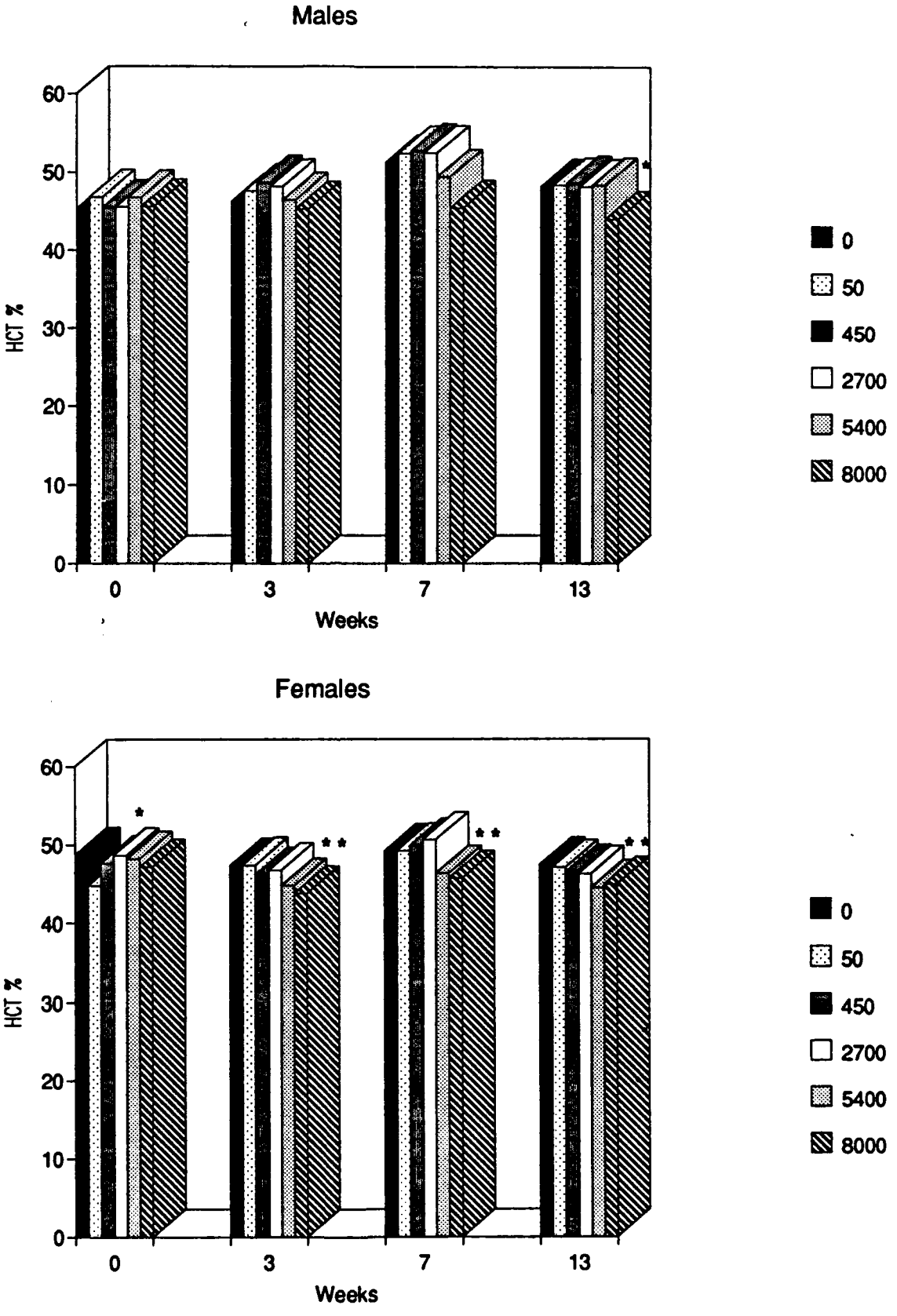
The group means are summarized in Table B 3.3.1.1 and are plotted in Figure B 3.3.1.1.

The 8000 ppm males had significantly lower HCT than their 0 ppm controls at week 13 ($p< 0.01$). The 5400 and 8000 ppm females had significantly lowered HCT compared to the 0 ppm females, beginning at Week 3 ($p< 0.02$), when the effect was linear with dose. There was no significant treatment effect at other dose levels. The 50 ppm females had significantly lower HCT than the control group at Week 0.

Table B 3.3.1.1 Hematocrit (%)

Dose Level	Sex	Week 0	Week 3	Week 7	Week 13
0	Male	45.31±2.86	46.24±1.49	51.25±2.11	48.15±2.11
50	Male	46.76±3.94	47.53±1.79	52.37±2.43	48.29±2.44
450	Male	45.43±2.89	48.63±3.13	52.69±2.07	48.58±2.53
2700	Male	45.48±2.92	48.14±3.58	52.45±4.07	48.14±2.20
5400	Male	46.71±3.60	46.40±2.71	49.41±2.08	48.33±3.51
8000	Male	45.56±2.28	45.23±2.29	45.40±2.00	44.01±2.12
0	Female	48.68±3.11	47.29±2.61	49.15±4.51	47.41±3.75
50	Female	44.74±2.90	47.39±2.59	49.20±2.68	47.12±2.12
450	Female	47.60±1.85	46.13±1.41	49.86±2.58	46.44±2.84
2700	Female	48.64±3.36	46.69±2.99	50.63±1.49	46.30±3.87
5400	Female	48.18±3.55	44.74±2.92	46.33±3.88	44.51±2.26
8000	Female	47.15±2.93	43.79±2.62	45.71±4.92	44.95±2.39

Figure B 3.3.1.1 Hematocrit (%)



The asterisks denote a statistically significant difference from the concurrent 0 ppm control, with $p \leq 0.05$.

3.3.1.2 Hemoglobin (HGB)

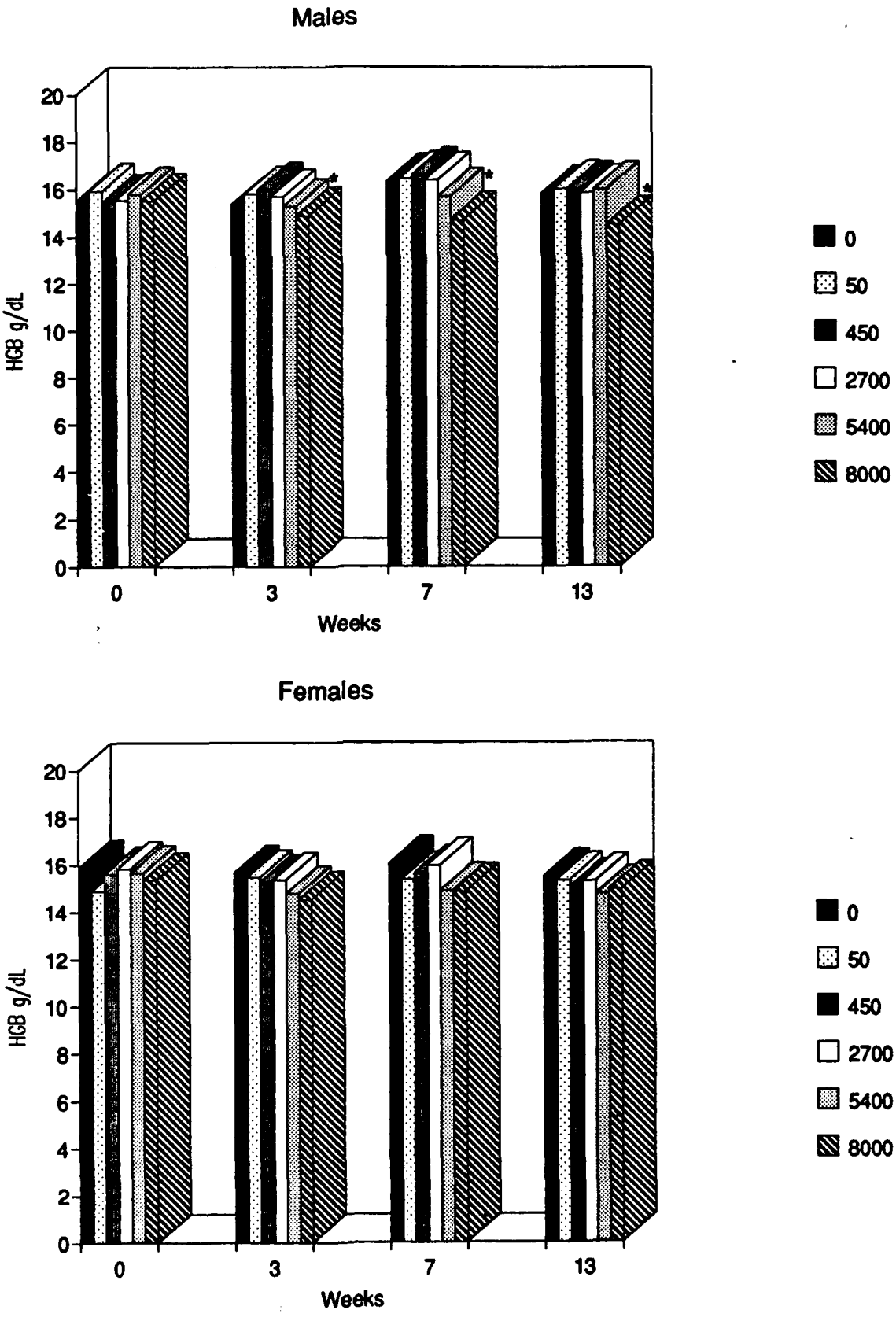
The group means are summarized in Table B 3.3.1.2 and plotted in Figure B 3.3.1.2.

The 8000 ppm males had lower HGB at Weeks 3, 7 and 13 than did their 0 ppm controls ($p < 0.02$); there was a significant ($p < 0.01$) time effect as well, the later value being more depressed. The 5400 and 8000 ppm females also had reduced HGB overall that was linear with dose. The difference from 0 ppm was significant at $p < .03$. There were no treatment-related effects in the lower dose groups.

Table B 3.3.1.2 Hemoglobin (g/dL)

Dose Level	Sex	Week 0	Week 3	Week 7	Week 13
0	Male	15.52±1.00	15.36±0.61	16.31±0.81	15.80±0.65
50	Male	15.91±1.42	15.79±0.58	16.43±0.60	15.99±0.69
450	Male	15.43±1.03	15.96±1.08	16.60±0.69	15.96±0.77
2700	Male	15.52±1.08	15.67±1.13	16.38±1.36	15.82±1.04
5400	Male	15.76±1.14	15.27±0.93	15.69±0.51	15.95±1.28
8000	Male	15.48±0.81	14.89±0.78	14.71±0.58	14.49±0.59
0	Female	15.86±0.99	15.63±0.76	16.01±0.99	15.46±1.03
50	Female	14.88±0.59	15.44±0.91	15.34±0.87	15.29±0.72
450	Female	15.59±0.69	15.25±0.37	15.62±0.76	15.00±0.95
2700	Female	15.81±1.13	15.33±0.88	15.93±0.59	15.25±1.23
5400	Female	15.63±1.09	14.77±0.99	14.87±1.27	14.72±0.74
8000	Female	15.33±0.91	14.52±0.83	14.84±1.45	14.92±0.76

Figure B 3.3.1.2 Hemoglobin (g/dL)



The asterisks denote a statistically significant difference from the concurrent 0 ppm control, with $p \leq 0.05$.

3.3.1.3 Erythrocyte Count (RBC)

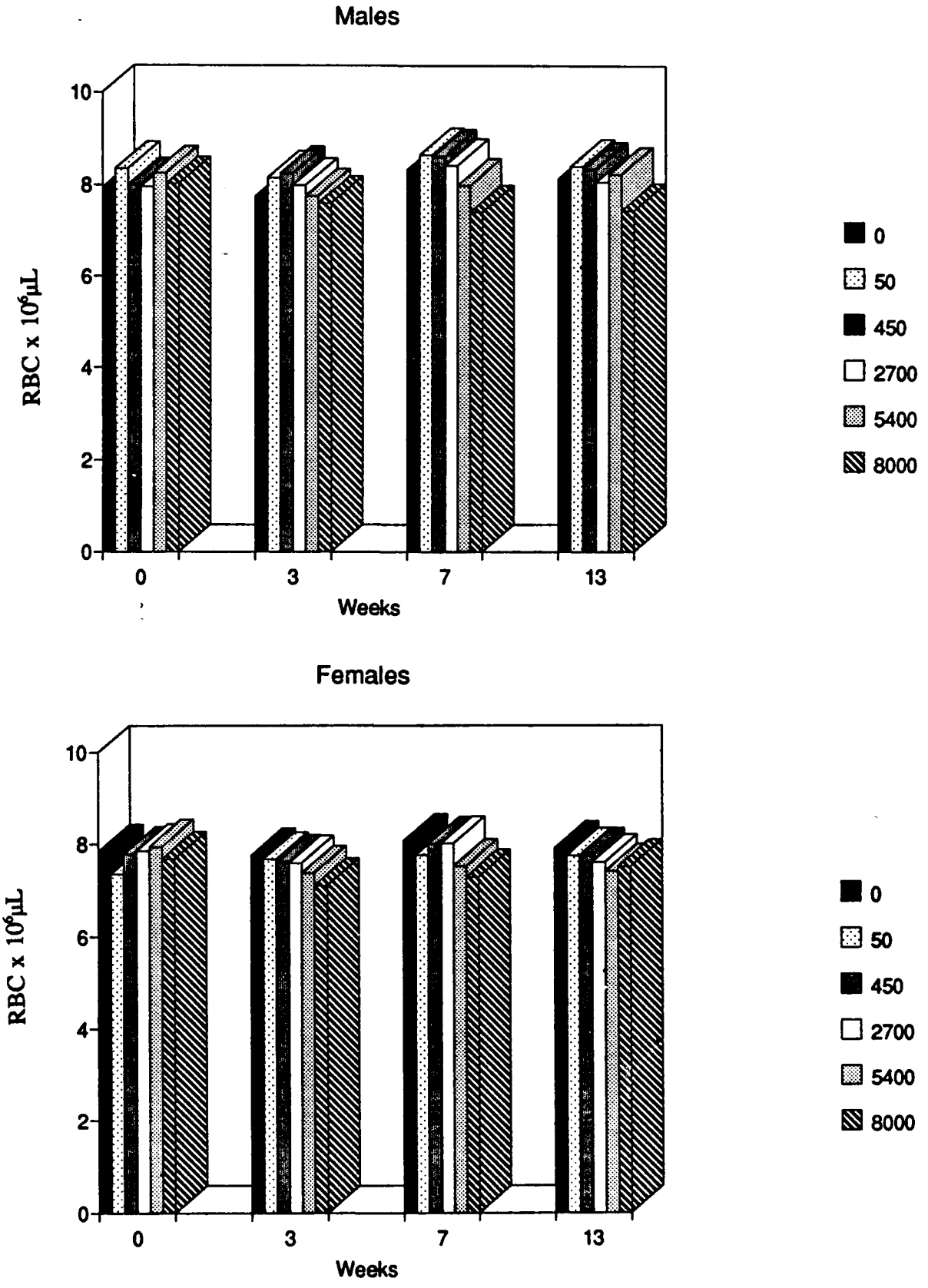
The group means are summarized in Table B 3.3.1.3 and plotted in Figure B 3.3.1.3.

The 5400 and 8000 ppm groups had a slight dose-related decrease in RBC count when values for Weeks 3 through 13 were averaged and compared with either the respective Week 0 value, or the 0 ppm average. The lower doses had no effect on RBC count.

Table B 3.3.1.3 Erythrocyte Count ($10^6/\mu L$)

Dose Level	Sex	Week 0	Week 3	Week 7	Week 13
0	Male	7.95±0.75	7.74±0.50	8.33±0.64	8.13±0.57
50	Male	8.35±0.82	8.15±0.29	8.65±0.47	8.39±0.56
450	Male	8.00±0.76	8.24±0.55	8.61±0.50	8.33±0.48
2700	Male	7.96±0.64	7.99±0.63	8.41±0.88	8.05±0.60
5400	Male	8.25±0.56	7.76±0.41	7.98±0.30	8.22±0.54
8000	Male	8.02±0.50	7.60±0.50	7.40±0.37	7.44±0.44
0	Female	7.84±0.31	7.75±0.46	8.06±0.78	7.90±0.44
50	Female	7.35±0.55	7.67±0.50	7.76±0.47	7.75±0.45
450	Female	7.78±0.29	7.60±0.24	8.00±0.46	7.75±0.50
2700	Female	7.85±0.53	7.59±0.42	8.01±0.22	7.61±0.61
5400	Female	7.94±0.60	7.37±0.61	7.52±0.80	7.41±0.32
8000	Female	7.68±0.52	7.11±0.44	7.29±0.95	7.51±0.61

Figure B 3.3.1.3 Erythrocyte Count ($10^6\mu L$)



3.3.1.4 Erythrocyte Indices: MCV, MCH, MCHC

There were no significant dose-related effects on these RBC indices, although they varied somewhat over time in most groups.

3.3.1.4.1 MCV

The means for all groups of males increased over time, with largest MCV at Week 7. The increase in females was not evident at Week 3; the Week 13 means were decreased for all female groups. For both sexes, the increase was not related to dose.

3.3.1.4.2 MCH

The MCH decreased slightly in most females over the course of the study, while the Week 3 value for males increased compared with other times.

3.3.1.4.3 MCHC

The MCHC tended to decrease slightly in all groups by Week 7, then to recover by Week 13.

3.3.1.5 Reticulocytes

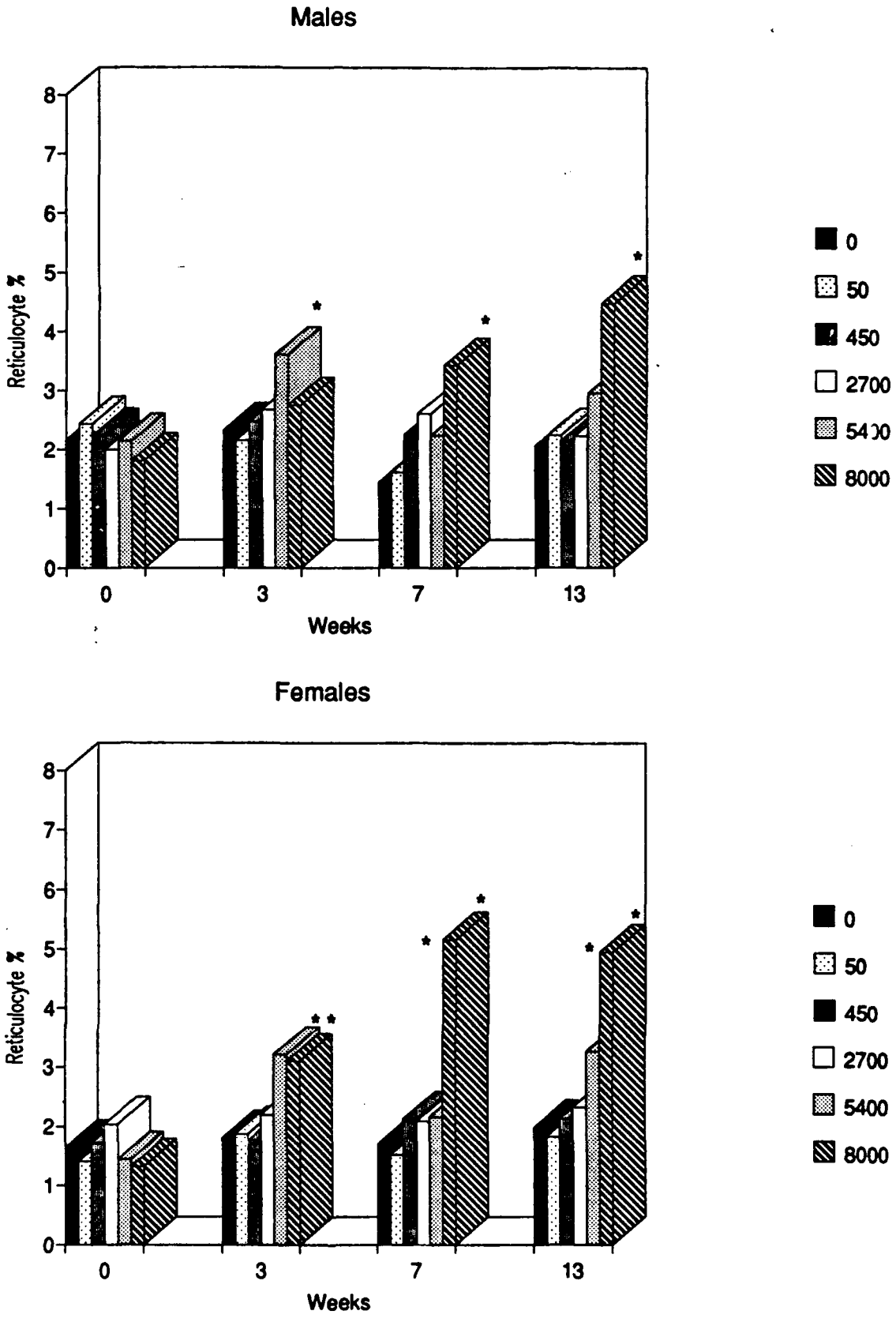
The group means are summarized in Table B 3.3.1.5 and plotted in Figure B 3.3.1.5.

The mean number of reticulocytes in the 2700, 5400, and 8000 ppm groups exceeded the 0 ppm value at each time point after Week 0. The overall mean (Weeks 3-13) was significantly larger for both sexes of the 8000 ppm group, and the male 5400 ppm group, the weekly means increasing with time. At Week 13 the reticulocyte counts for the male and female 8000 ppm groups approached 4.5-5.0%, versus approximately 2.0% in controls at Week 13, significant at $p = 0.0001$. The respective pre-treatment value (Week 0) for the 8000 ppm groups was also approximately 2%.

Table B 3.3.1.5 Reticulocytes (% x RBC)

Dose Level	Sex	Week 0	Week 3	Week 7	Week 13
0	Male	2.16±1.28	2.32±0.75	1.43±0.63	2.05±0.66
50	Male	2.43±0.67	2.15±0.72	1.60±0.68	2.23±1.06
450	Male	2.22±0.96	2.46±0.51	2.24±0.94	2.14±0.54
2700	Male	2.00±0.67	2.67±1.01	2.60±1.47	2.22±0.88
5400	Male	2.14±1.06	3.61±1.09	2.23±1.17	2.96±0.57
8000	Male	1.81±0.80	2.75±1.17	3.42±1.42	4.48±2.45
0	Female	1.64±0.78	1.80±0.71	1.70±0.68	1.98±0.77
50	Female	1.41±0.62	1.87±0.71	1.52±0.99	1.83±0.54
450	Female	1.71±0.58	1.76±0.63	2.12±0.87	2.13±0.92
2700	Female	2.03±0.48	2.19±0.59	2.09±0.76	2.33±0.97
5400	Female	1.44±0.42	3.21±1.32	2.17±0.82	3.27±1.70
8000	Female	1.32±0.69	3.10±1.58	5.16±2.19	4.95±2.62

Figure B 3.3.1.3 Reticulocytes (% x RBC)



The asterisks denote a statistically significant difference from the concurrent 0 ppm control, with $p \leq 0.05$.

3.3.1.6 Heinz Bodies

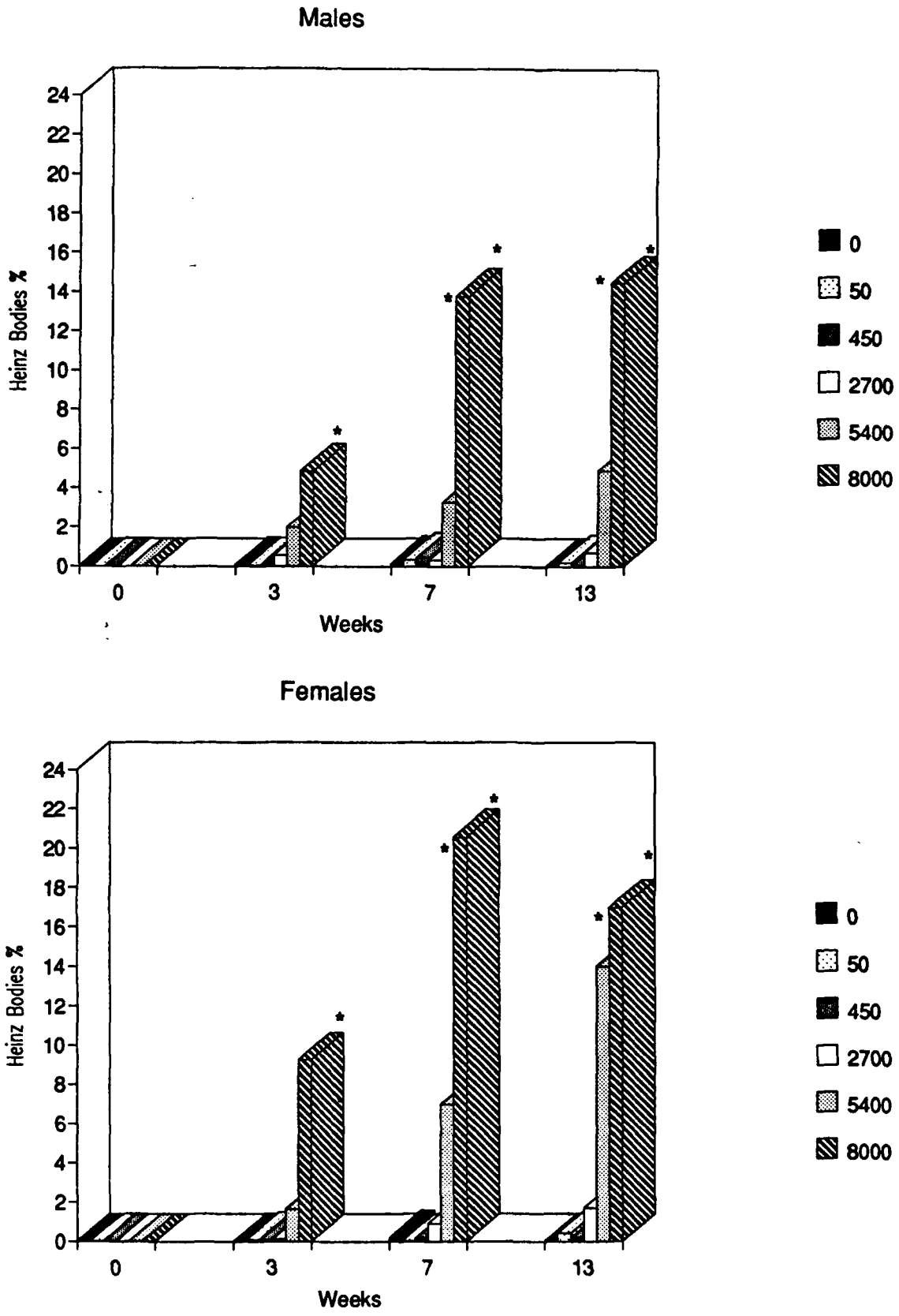
The group means are summarized in Table B 3.3.1.6 and plotted in Figure B 3.3.1.6.

Most treated animals had increased numbers of Heinz-body-containing RBCs at each sampling time. The increase for the 8000 ppm groups (from 0% to 5% in males and to 9% in females) reached statistical significance by Week 3. At Week 13, the female 5400 ppm group also had statistically significant increases.

Table B 3.3.1.6 Heinz Bodies (% x RBC)

Dose Level	Sex	Week 0	Week 3	Week 7	Week 13
0	Male	0.00±0	0.07±0.11	0.13±0.24	0.00±0.00
50	Male	0.00±0	0.04±0.07	0.32±0.53	0.20±0.31
450	Male	0.00±0	0.07±0.08	0.41±0.96	0.03±0.07
2700	Male	0.00±0	0.56±0.86	0.32±0.69	0.70±0.73
5400	Male	0.00±0	1.98±2.42	3.26±2.12	4.88±5.62
8000	Male	0.00±0	4.85±9.99	13.78±7.71	14.45±8.48
0	Female	0.01±0.03	0.01±0.03	0.19±0.16	0.02±0.06
50	Female	0.01±0.03	0.08±0.10	0.02±0.04	0.41±0.89
450	Female	0.00±0	0.05±0.11	0.15±0.25	0.17±0.16
2700	Female	0.01±0.03	0.12±0.14	0.90±0.92	1.70±1.91
5400	Female	0.01±0.03	1.65±1.65	6.98±6.03	14.01±9.22
8000	Female	0.01±0.03	9.24±12.93	20.56±8.22	16.99±6.65

Figure B 3.3.1.6 Heinz Bodies (% x RBC)



The asterisks denote a statistically significant difference from the concurrent 0 ppm control, with $p \leq 0.05$.

3.3.1.7 Platelets

The group means are summarized in Table B 3.3.1.7 and plotted in Figure B 3.3.1.7

Females had dose-related increase in platelet counts, in the 5400 and 8000 ppm groups compared with the 0 ppm group ($p < 0.02$) averaged over all times. The 450 ppm males had a slightly lower platelet count than the 0 ppm group at all times, including Week 0. This effect was significant at $p = 0.01$, although there was no time effect, i.e., the group's mean number did not change in comparison to its own Week 0 value, discounting treatment as the cause of the lower count.

3.3.1.8 Erythrocyte Morphology

Abnormal erythrocyte morphology was recorded as schizocytes, keratocytes, anisocytes, echinocytes, eccentrocytes, and polychromasia. Tabulation of the categorical data on RBC morphology for each animal is included in Appendix 7. The general pattern was one of mild to moderate changes affecting the 2700, 5400 and 8000 ppm groups inconsistently but in an approximate dose-dependent and time-dependent way. The prevalence of animals affected with anisocytosis, polychromasia, Howell-Jolly bodies and eccentrocytes paralleled and was consistent with the more quantitative findings of Heinz bodies, hematocrit, RBC count and reticulocyte count. The latter three responses do not differentiate between RBC injury caused by DIMP and the possible effect of blood loss caused by sampling, however, the control group was sampled similarly.

3.3.1.9 Leukocytes (WBC)

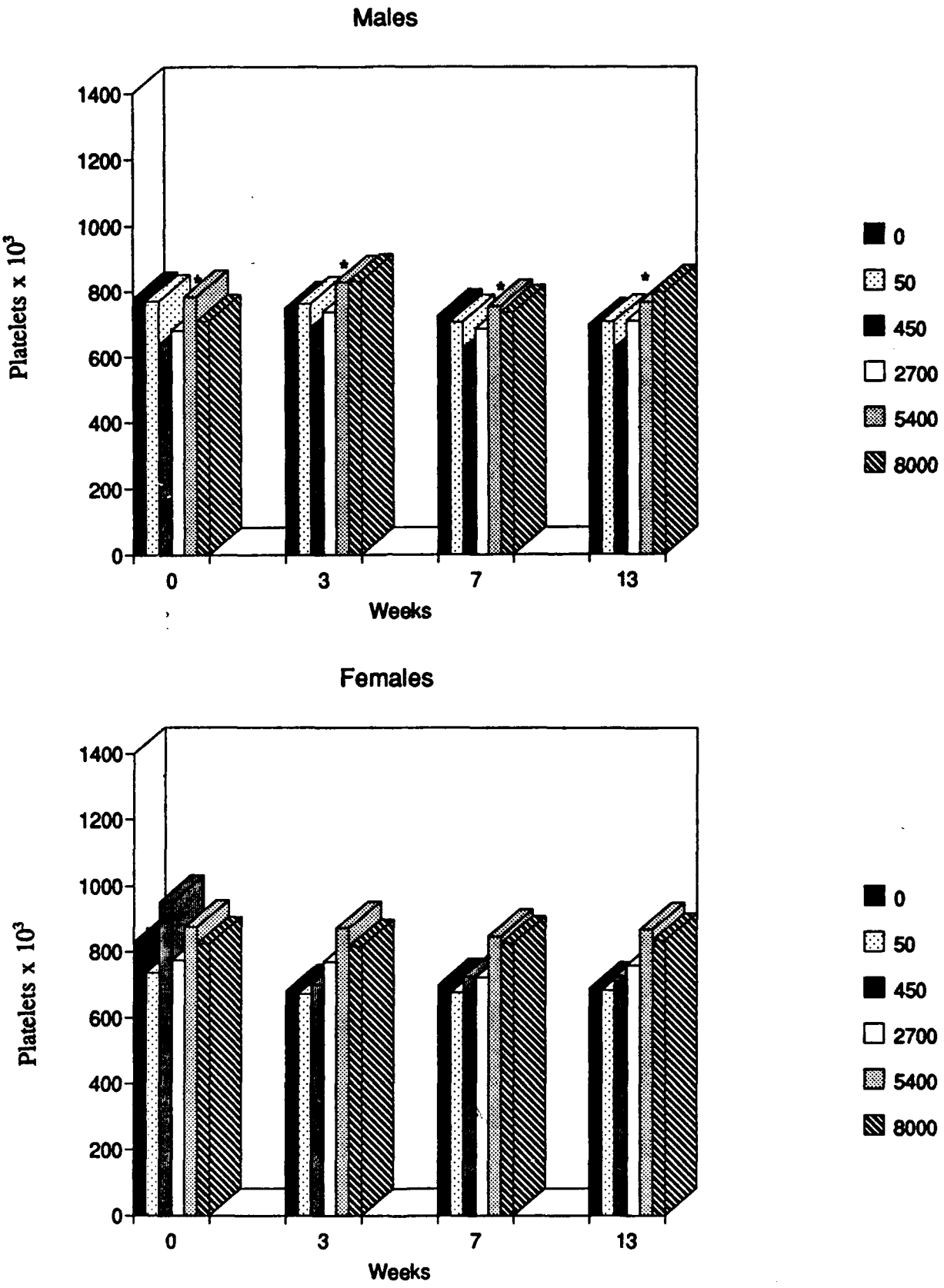
Total and differential leukocyte counts were enumerated as absolute counts per microliter of blood.

There were no overall effects of treatment on total or differential counts of WBC.

Table B 3.3.1.7 Platelets ($10^3/\mu L$)

Dose Level	Sex	Week 0	Week 3	Week 7	Week 13
0	Male	778.40±102.76	749.20±72.59	724.80±86.14	697.40±80.31
50	Male	770.10±114.75	763.40±130.77	707.10±102.55	708.40±110.99
450	Male	637.90±93.30	688.70±111.39	624.70±109.18	626.50±138.80
2700	Male	681.10±183.64	737.90±154.86	685.30±169.31	710.90±104.78
5400	Male	783.80±68.26	827.90±82.58	753.90±113.21	767.20±100.53
8000	Male	708.70±113.91	831.30±129.02	736.60±109.17	798.70±100.33
0	Female	828.30±289.84	681.10±102.70	699.50±67.64	689.20±65.01
50	Female	735.56±199.17	673.80±91.43	677.40±89.07	685.70±107.24
450	Female	951.00±429.64	701.60±70.04	725.50±87.21	726.90±112.97
2700	Female	774.90±87.49	769.50±113.06	723.50±66.00	761.70±58.84
5400	Female	875.90±232.25	872.40±197.51	848.40±136.89	870.40±123.68
8000	Female	825.50±197.82	818.30±186.60	827.90±117.88	836.70±119.66

Figure B 3.3.1.7 Platelets ($10^3/\mu L$)



The asterisks denote a statistically significant difference from the concurrent 0 ppm control, with $p \leq 0.05$.

3.3.2 Clinical Chemistry

3.3.2.1 Plasma Cholinesterase (PChE)

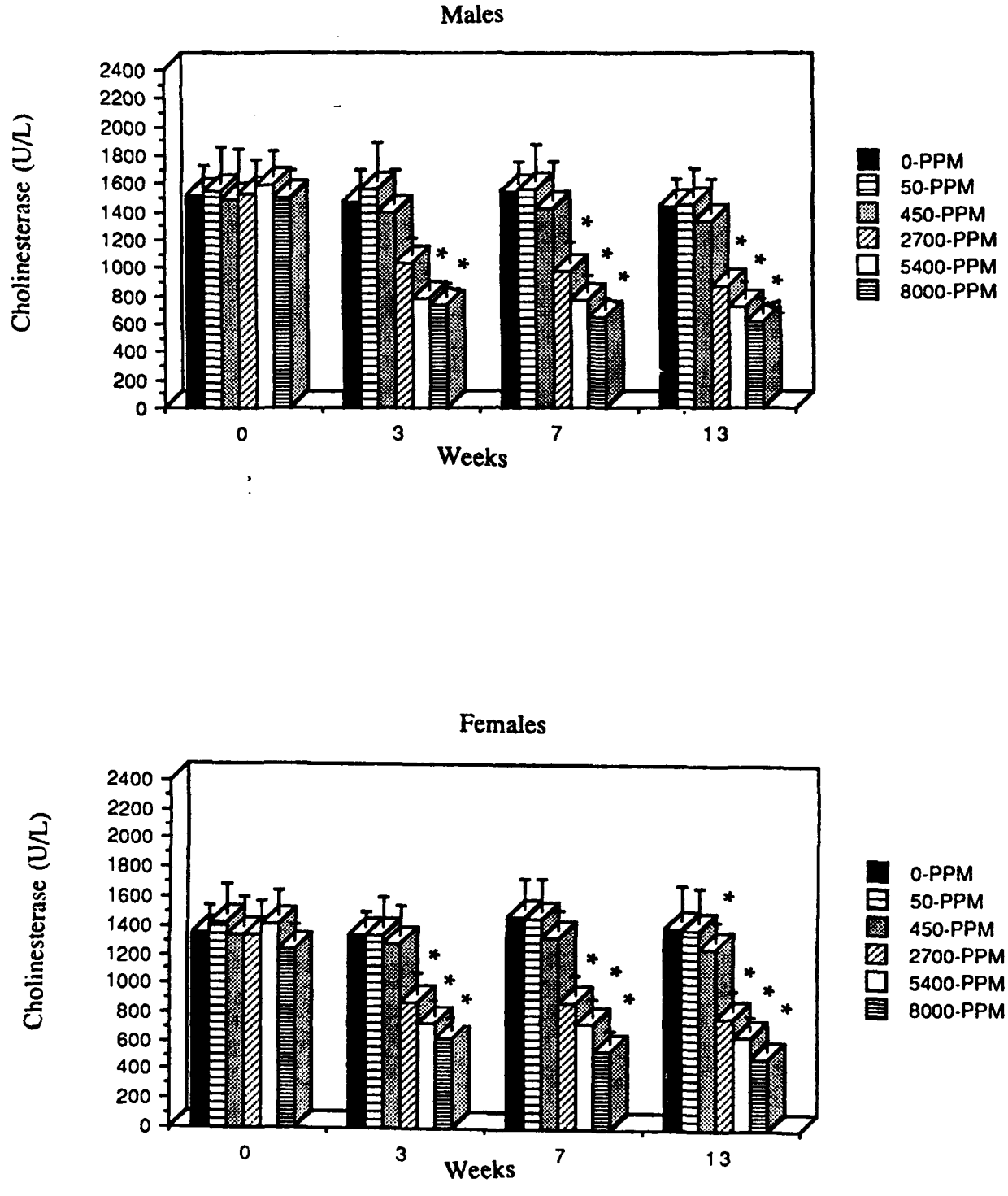
The group mean values are summarized in Table B 3.3.2.1 and plotted in Figure B 3.3.2.1.

Both sexes of the 2700, 5400 and 8000 ppm groups had lowered PChE in comparison to 0 ppm ($p = 0.0001$). At Week 13 the values for the two upper dose groups were 50% or less of those of the controls, as well as 50% or less of each group's own pre-treatment mean value. The general pattern showed a dramatic decrease by Week 3, with smaller continued decrements to Week 13. The decrease in the 450 ppm group was small numerically, i.e., biologically, although it did reach a level of $p = 0.05$ statistically in the females at Week 13, in comparison to the 0 ppm controls at Week 13. However, the Week 13 values for both sexes at 450 ppm were not significantly different from their respective Week 0 value (Appendix 8).

Table B 3.3.2.1 Plasma Cholinesterase (PChE, units/L)

Dose Level	Sex	Week 0	Week 3	Week 7	Week 13
0	Male	1519.67±144.84	1469.10±172.27	1542.10±157.93	1446.70±134.14
50	Male	1545.22±247.56	1563.90±263.22	1563.00±248.98	1460.70±183.65
450	Male	1486.30±297.67	1393.70±240.50	1427.90±273.63	1340.50±227.17
2700	Male	1522.70±187.59	1029.80±123.99	982.00±144.22	865.70±87.24
5400	Male	1595.40±178.24	784.20±120.96	763.30±115.51	722.10±118.39
8000	Male	1503.70±131.40	733.30±85.18	646.00±73.66	609.50±117.00
0	Female	1351.40±130.25	1332.60±104.66	1467.00±205.92	1395.10±236.96
50	Female	1424.20±202.24	1342.80±194.97	1460.10±213.82	1387.30±224.75
450	Female	1331.11±213.68	1276.40±203.54	1324.90±123.12	1252.10±126.21
2700	Female	1341.30±165.79	872.40±152.22	874.20±130.47	771.30±128.77
5400	Female	1410.60±178.55	722.50±101.14	722.10±118.17	646.60±85.69
8000	Female	1236.80±111.33	620.40±90.20	541.40±90.84	479.60±50.01

Figure B 3.3.2.1 Plasma Cholinesterase (PChE)



The asterisks denote a statistically significant difference from the concurrent 0 ppm control, with $p \leq 0.05$.

3.3.2.2 Blood Urea Nitrogen (BUN)

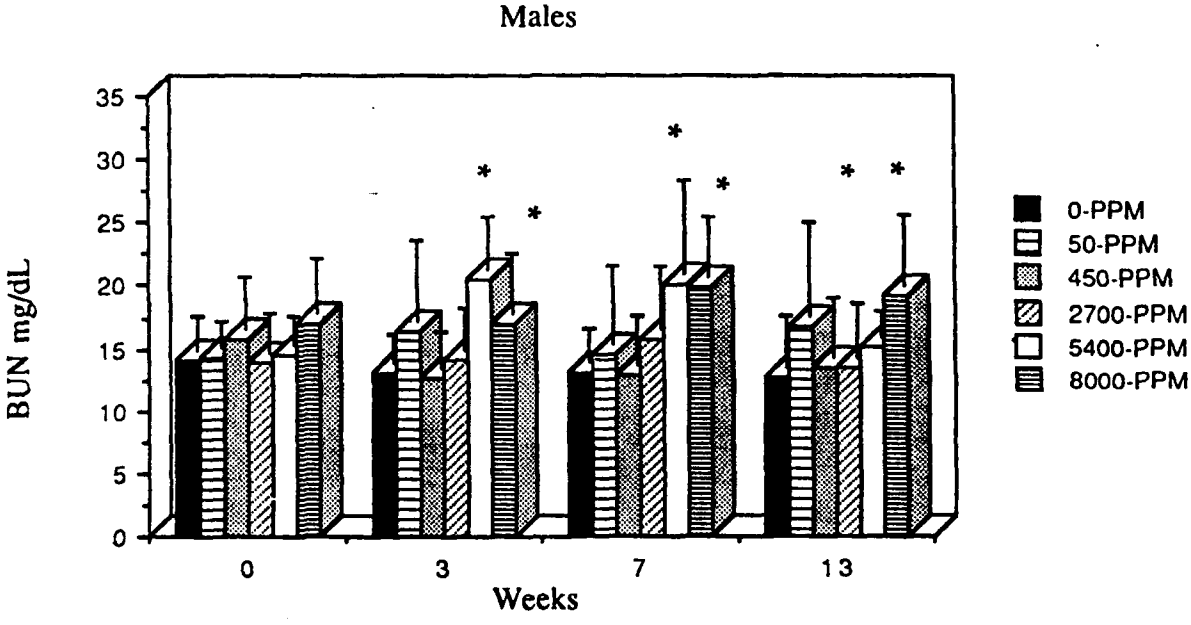
The group mean values are summarized in Table B 3.3.2.2 and plotted in Figure B 3.3.2.2.

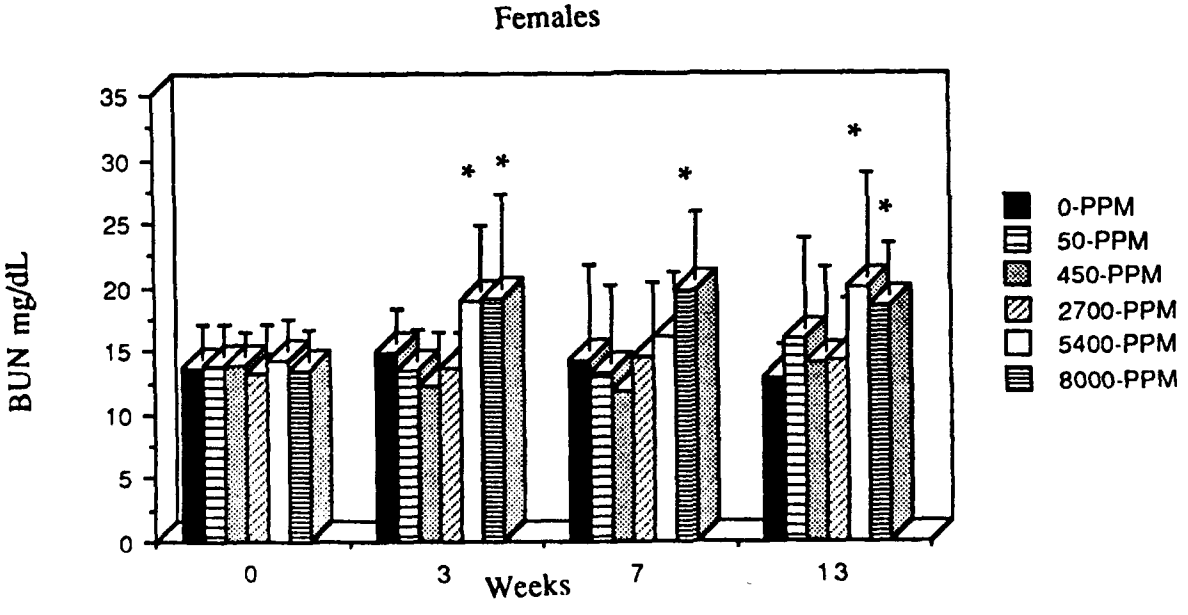
BUN was increased moderately with dose in both sexes at 5400 and 8000 ppm ($p = 0.05$). The absolute increase in mean value was not large (15 - 20 versus 13 mg/dL), but was the result of modest change in many animals of each group. No other groups were affected.

Table B 3.3.2.2 Blood Urea Nitrogen (BUN, mg/dL)

Dose Level	Sex	Week 0	Week 3	Week 7	Week 13
0	Male	14.11±2.57	13.00±2.26	13.00±2.83	12.70±4.00
50	Male	14.22±2.05	16.40±6.38	14.40±6.28	16.70±7.60
450	Male	15.80±4.08	12.60±2.84	12.90±3.84	13.50±4.70
2700	Male	13.80±3.08	14.10±3.28	15.80±4.98	13.50±4.35
5400	Male	14.40±2.46	20.40±4.17	20.10±7.39	15.10±2.08
8000	Male	16.90±4.43	17.00±4.83	19.80±4.80	19.30±5.52
0	Female	13.70±2.58	14.80±2.70	14.30±6.67	12.80±1.75
50	Female	13.80±2.49	13.40±2.46	13.20±6.03	15.90±7.08
450	Female	13.78±1.86	12.30±3.33	11.90±1.85	14.10±6.61
2700	Female	13.30±2.95	13.60±2.17	14.50±5.08	14.20±4.13
5400	Female	14.30±2.31	18.90±5.09	16.00±4.40	20.00±8.10
8000	Female	13.40±2.55	19.20±7.28	19.70±5.42	18.60±3.89

Figure B 3.3.2.2 Blood Urea Nitrogen (BUN, mg/dL)





The asterisks denote a statistically significant difference from the concurrent 0 ppm control, with $p \leq 0.05$.

3.3.2.3 Glucose

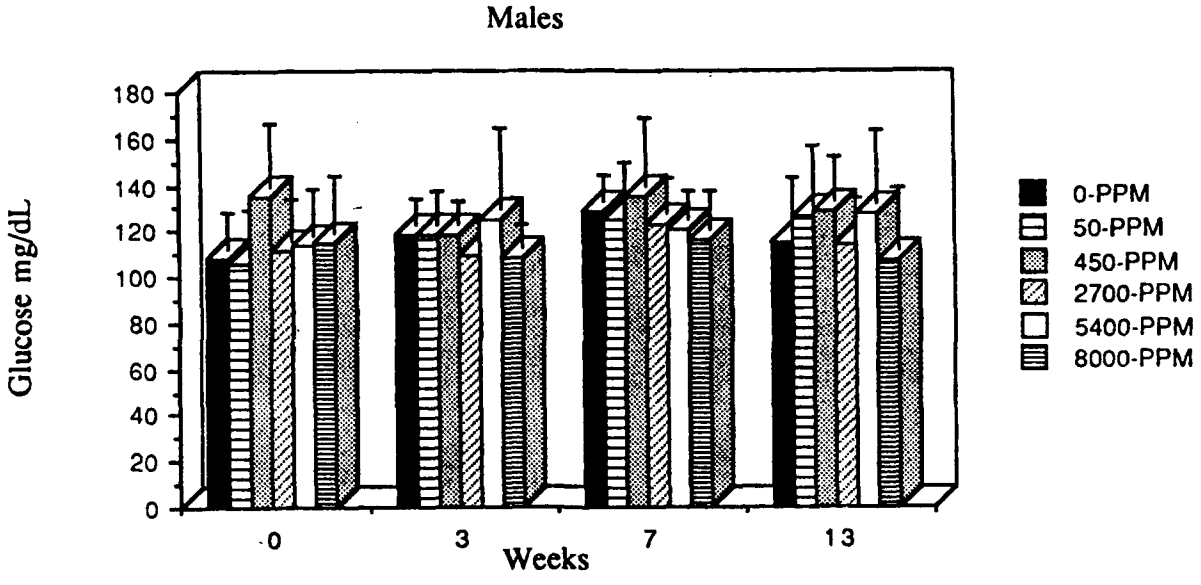
The group mean values are summarized in Table B 3.3.2.3 and plotted in Figure B 3.3.2.3.

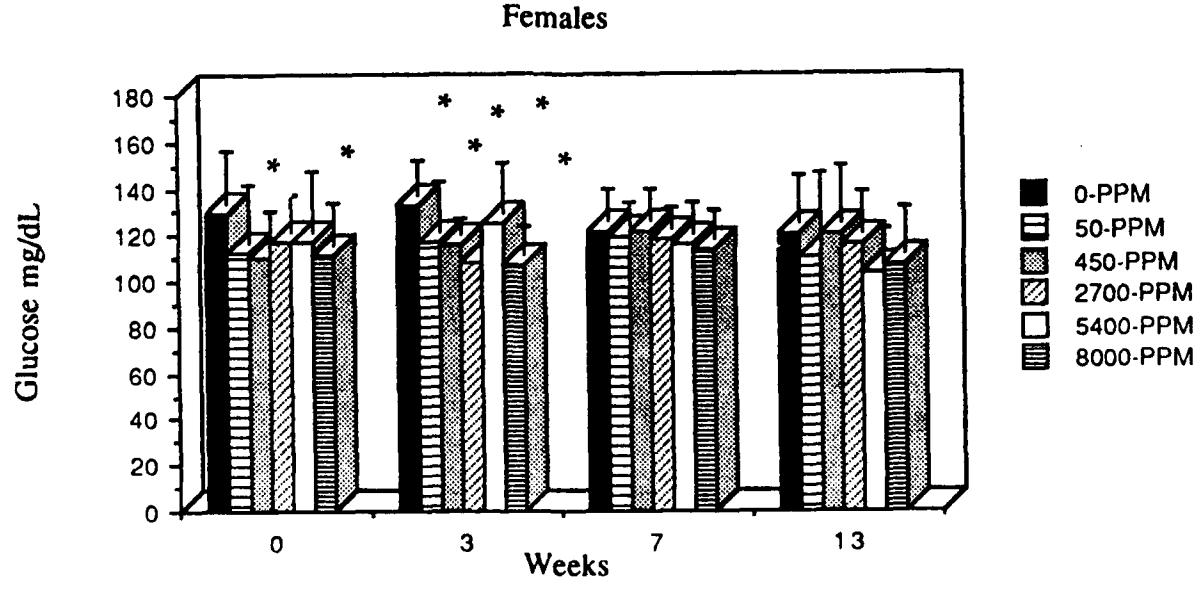
Females in the 50 and 8000 ppm group had slightly lower glucose values than the 0 ppm control when averaged over all sampling times, approximately 110 mg/dL versus 125 ($p = 0.05$). The female controls had high values during Weeks 0 and 3, and the 450 ppm males were consistently slightly high (129 mg/dL overall). There were no clear treatment-related effects on glucose concentration.

Table B 3.3.2.3 Glucose (mg/dL)

Dose Level	Sex	Week 0	Week 3	Week 7	Week 13
0	Male	107.89±15.50	117.70±11.65	127.90±11.44	115.30±22.74
50	Male	106.11±19.02	117.60±15.52	125.10±20.30	125.60±26.05
450	Male	135.00±27.55	118.20±10.43	134.60±29.60	128.10±18.84
2700	Male	111.50±17.91	109.00±13.03	122.80±15.58	114.00±14.95
5400	Male	113.70±20.0	124.40±35.39	120.10±12.71	126.90±31.79
8000	Male	115.10±24.15	108.50±9.96	115.80±16.79	107.00±26.89
0	Female	129.70±22.13	132.90±14.33	121.60±13.08	120.80±19.76
50	Female	112.40±24.68	116.60±22.30	119.60±10.16	110.80±30.47
450	Female	110.33±15.53	116.00±7.12	121.30±13.86	120.20±24.61
2700	Female	117.30±15.11	107.90±10.59	117.70±9.15	116.20±17.84
5400	Female	116.70± 26.52	125.40±20.36	115.80±13.66	103.70± 4.38
8000	Female	110.00± 17.86	107.20±12.23	114.10±11.78	107.30±19.48

Figure B 3.3.2.3 Glucose (mg/dL)





The asterisks denote a statistically significant difference from the concurrent 0 ppm control, with $p \leq 0.05$.

3.3.2.4 Albumin

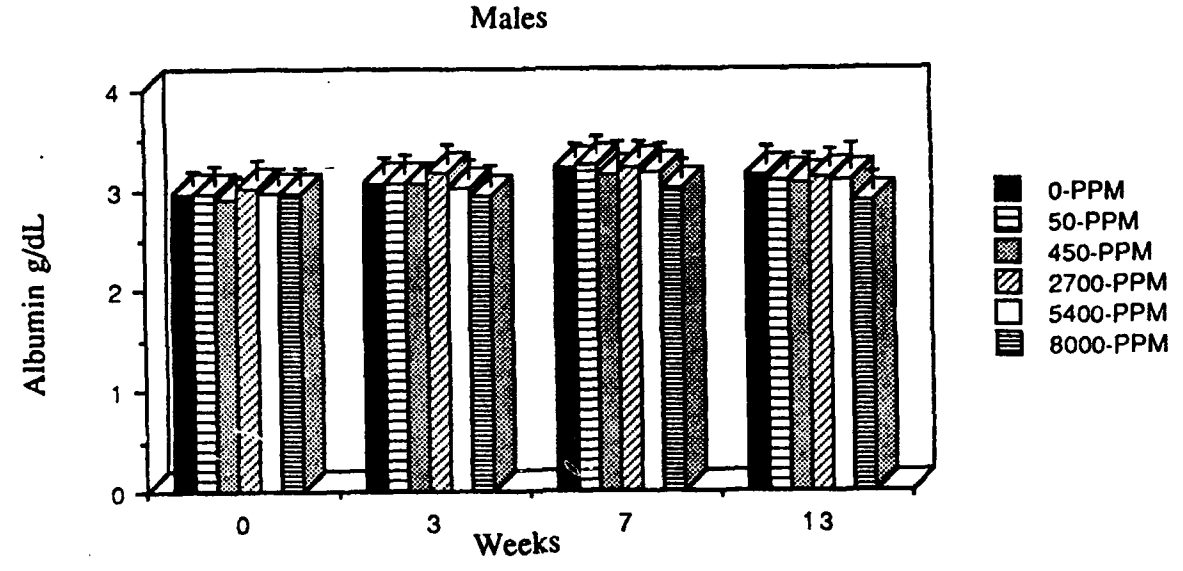
The group mean values are summarized in Table B 3.3.2.4 and plotted in Figure B 3.3.2.4.

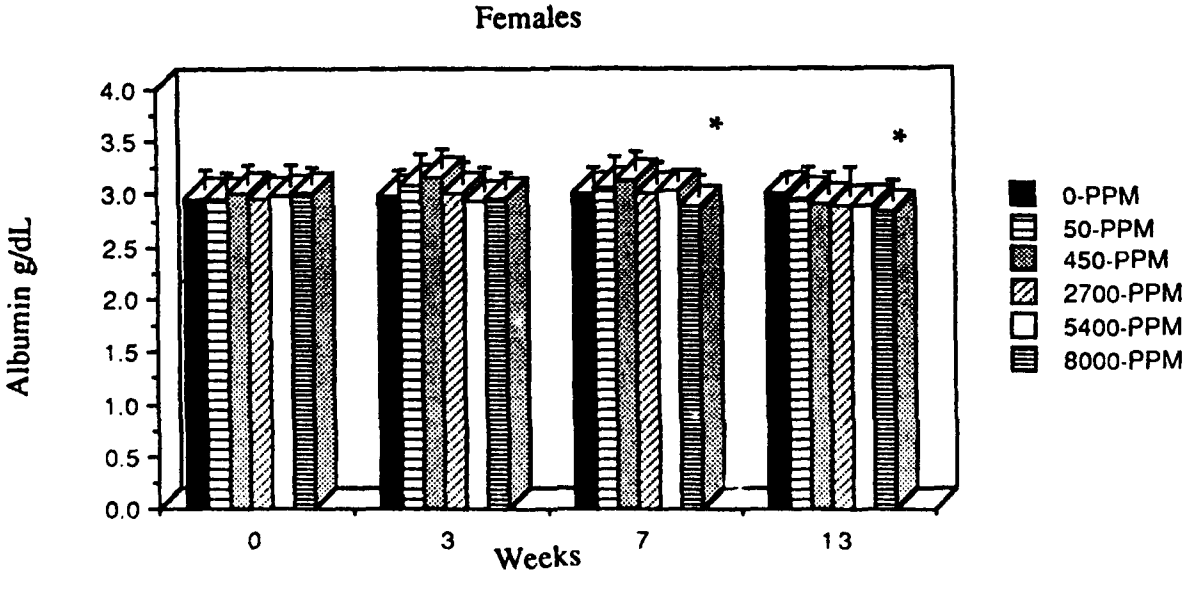
Females in the 8000 ppm group had slightly reduced albumin concentration at Weeks 7 and 13, 2.90 and 2.83 g/dL respectively, compared with 3.00 in the control. Their own pre-treatment mean was also 3.00 g/dL. This change was significant at p = 0.03. No other groups were affected.

Table B 3.3.2.4 Albumin (g/dL)

Dose Level	Sex	Week 0	Week 3	Week 7	Week 13
0	Male	2.97±0.12	3.07±0.16	3.22±0.13	3.15±0.19
50	Male	2.98±0.12	3.08±0.18	3.26±0.16	3.10±0.15
450	Male	2.91±0.14	3.06±0.10	3.16±0.22	3.07±0.19
2700	Male	3.03±0.16	3.17±0.18	3.22±0.17	3.11±0.20
5400	Male	2.98±0.13	3.03±0.18	3.18±0.18	3.10±0.26
8000	Male	2.98±0.14	2.95±0.21	3.02±0.19	2.90±0.16
0	Female	2.96±0.18	2.99±0.15	3.02±0.15	3.00±0.08
50	Female	2.97±0.15	3.08±0.21	3.05±0.20	2.97±0.18
450	Female	3.01±0.17	3.15±0.18	3.13±0.18	2.92±0.20
2700	Female	2.96±0.13	3.00±0.20	3.02±0.18	2.89±0.28
5400	Female	2.99±0.19	2.95±0.20	3.03±0.11	2.88 0.11
8000	Female	3.00±0.15	2.96±0.14	2.90±0.19	2.83 0.21

Figure B 3.3.2.4 Albumin (g/dL)





The asterisks denote a statistically significant difference from the concurrent 0 ppm control, with $p \leq 0.05$.

3.3.2.5 Red Blood Cell Acetylcholinesterase

To confirm the plasma cholinesterase (PChE) values measured at UMN and to determine RBC acetylcholinesterase concentrations (AChE) in 24 representative mink, PAI analyzed samples taken at the 13-week bleeding. Refrigerated whole blood was shipped to PAI in Jefferson, AR, where the cholinesterase determinations were performed on a Roche Cobas Fara II Analyzer (Hoffman La Roche Nutley, NJ). Whole blood cholinesterase was measured on the day of arrival. The plasma was frozen and plasma cholinesterase was measured three days later.

The manufacturer's recommended calorimetric kinetic enzyme method, based on the hydrolysis of propionylthiocholine to thiocholine by cholinesterase, was used (Sigma Chemical Co., St. Louis, MO, Cat # 422).

Three animals of each sex, from each of the 8000, 2700, 0 and 0PF2 groups, were studied. The individual values are listed in Appendix 9. The means with standard deviation for each group of three are tabulated below, excluding the PF animals (which were normal).

Table B 3.3.2.5 Plasma and Red Blood Cell Cholinesterase
13 Weeks; N=3/group; Mean±Std Dev

Dose Group	HCT (decimal)	Whole Blood ChE (U/L)	Plasma ChE (U/L)	RBC AChE (U/L)*
0 Male	.462	2165±175	1365±37	3097±359
0 Female	.450	2216±28	987±58	3718±157
2700 Male	.481	1972±260	563±76	3492±145
2700 Female	.451	2057±294	495±63	3958±474
8000 Male	.441	1729±37	512±140	3272±130
8000 Female	.434	1624±436	322±20	3322±1110

*Red Blood Cell acetylcholinesterase content (RBC AChE) was calculated with the formula: RBC AChE = $\frac{\text{Whole Blood ChE - [Plasma ChE x (1-HCT)]}}{\text{HCT}}$

The plasma cholinesterase values were significantly lower in the 2700 and 8000 ppm groups, confirming the UMN finding (Table B 3.3.2.1). There was no effect of treatment on RBC acetylcholinesterase.

3.3.2.6 All Other Chemical Values

There were no changes in the remaining analytes and calculated values that were regarded as potentially treatment-related. The unaffected characteristics were:

creatinine	chloride
total protein	phosphorus
alanine aminotransferase	total calcium
aspartate aminotransferase	carbon dioxide
sodium	osmolarity (calculated)
potassium	anion gap (calculated)

3.4 Recovery Animals

Two animals of each sex from the 2700 and 8000 ppm dose groups were placed on untreated feed for four weeks, beginning on the 91st day of the study. Food consumption and body weight data were collected weekly as in the first 13 weeks, and blood was obtained and analyzed in the same manner. Since only Heinz bodies and related erythrocyte characteristics, and plasma cholinesterase had been affected by DIMP ingestion during the 90-day study, these data were compiled during the recovery period. Individual animal data are included in the respective appendices.

3.4.1 Food Consumption and Body Weight

The animals offered untreated feed ad libitum for four weeks at the end of the 13-week treatment period responded with an immediate (Week 14) increase in food consumption that decreased slightly over the final three weeks. Their body weights, delayed by about one week, increased approximately in parallel. The changes were more marked in the males. The average food consumption throughout the study for the two animals is each group is listed in Table B 3.4.1a and plotted in Figure B 3.4.1a. The body weights are depicted similarly in Table B 3.4.1b and Figure B 3.4.1b.

Table B 3.4.1a Food Consumption, Recovery, Weeks 14-17
(2700 and 8000 ppm, N=2/sex)

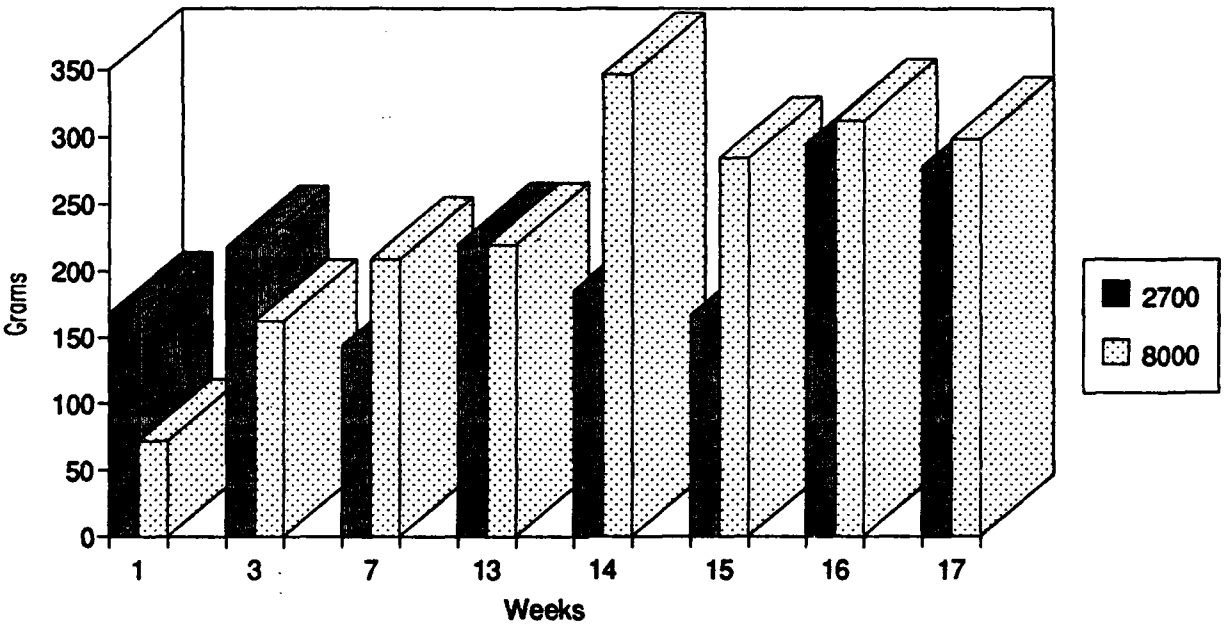
Dose	Sex	Week 1	Week 3	Week 7	Week 13	Week 14	Week 15	Week 16	Week 17
2700	Male	168.0	217.0	143.5	219.5	185.0	166.5	294.5	278.5
2700	Female	123.5	154.0	141.5	142.0	188.0	158.5	118.5	159.0
8000	Male	72.0	162.0	208.5	219.5	346.5	284.0	312.5	299.0
8000	Female	19.5	123.5	136.5	145.0	162.0	145.0	157.5	128.5

Table B 3.4.1b Body Weight, Recovery, Weeks 14 -17
(2700 and 8000 ppm, N=2/sex)

Dose	Sex	Week 0	Week 3	Week 7	Week 13	Week 14	Week 15	Week 16	Week 17
2700	Male	2059	1915	1818	1794	1935	1921	2022	2024
2700	Female	1105	1036	979	913	972	979	985	968
8000	Male	1941	1822	1641	1404	1682	1772	1910	1959
8000	Female	996	918	854	820	959	940	973	953

Figure B 3.4.1a Food Consumption, Recovery, Weeks 14-17
(2700 and 8000ppm, N=2/sex)

Males



Females

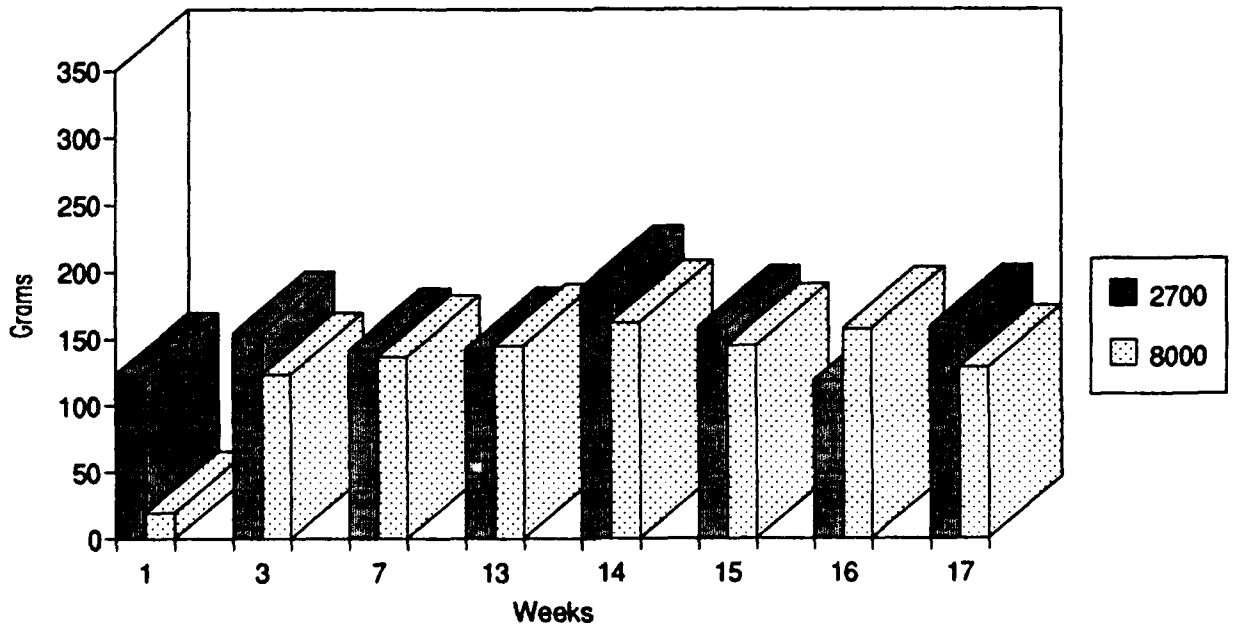
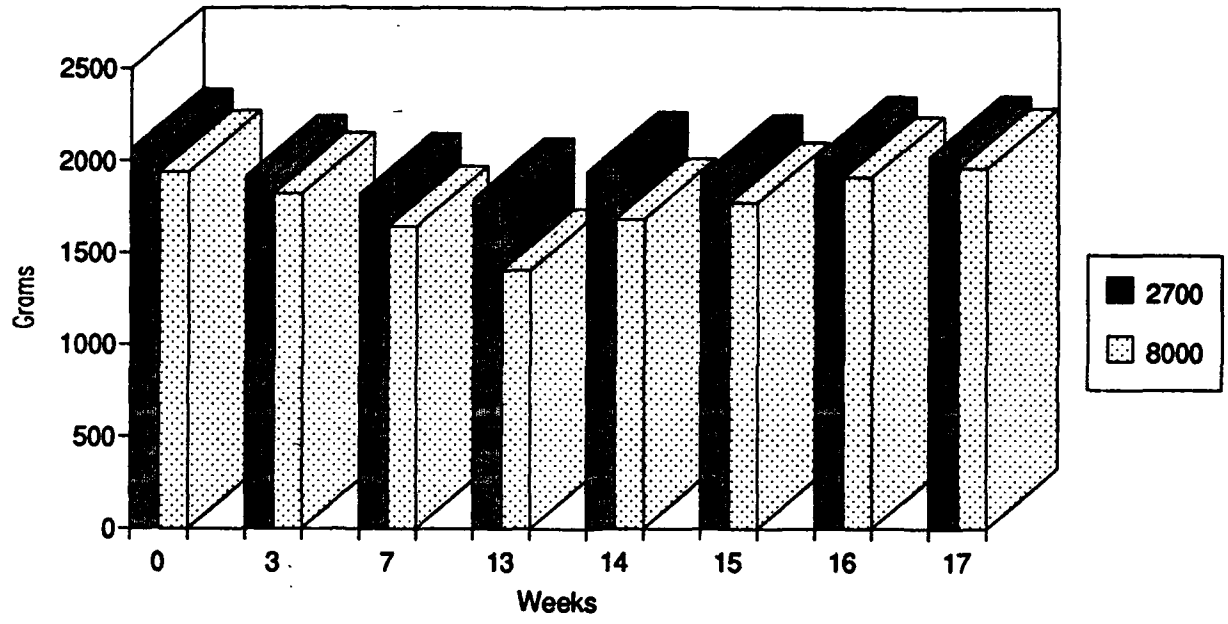
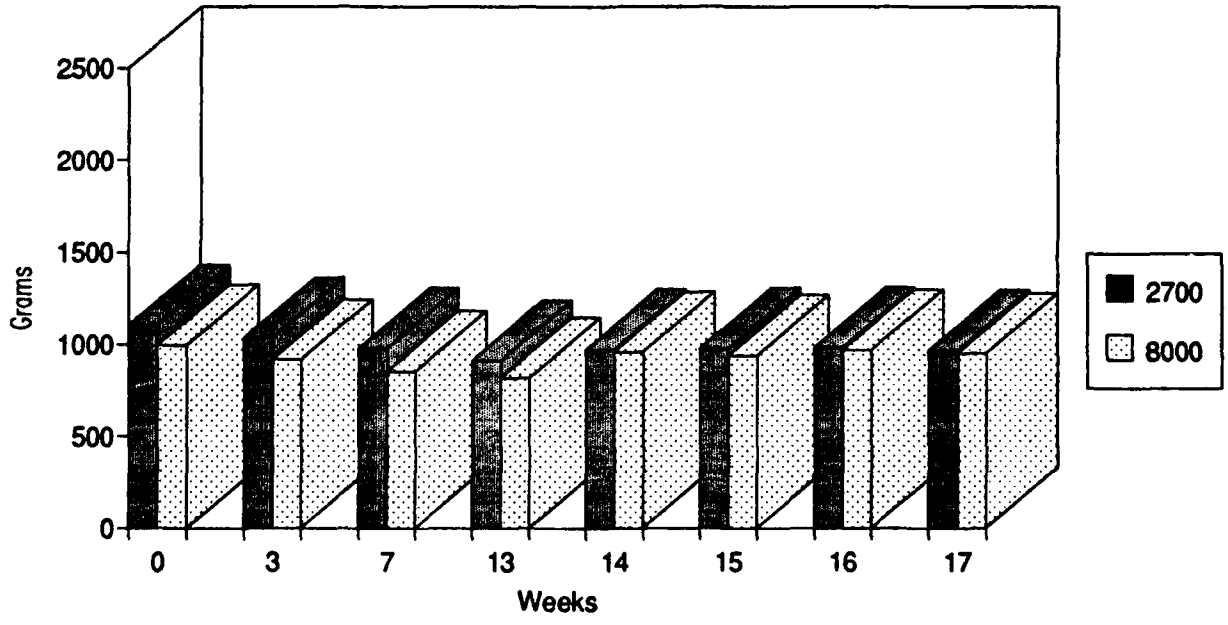


Figure B 3.4.1b Body Weight, Recovery, Weeks 14-17
(2700 and 8000ppm, N=2/sex)

Males



Females



3.4.2 Hematology and Clinical Chemistry

The pattern for all of the erythrocyte-related effects except the reticulocyte count was similar, with complete resolution occurring by the second or third week post-treatment. The reticulocyte count increased in all groups at the first post-treatment week to 5-8% from Week 13 values of 2-5%. Thereafter the values returned nearly to pre-treatment values. The plasma cholinesterase values returned to normal after the first week on untreated feed.

The hematocrit, Heinz body and reticulocyte counts for the two specific animals of each sex from the recovery groups are tabulated in Tables B 3.4.2 a-c, respectively. The tables include the Week 0 and Week 13 counts as well as the four weeks of recovery. Figures B 3.4.2 a-c, respectively, contain the same data except Weeks 0 - 13 contain group means for all animals, not solely the recovery animals.

Table B 3.4.2a Hematocrit Recovery, Weeks 14-17
(2700 and 8000 ppm, N=2/sex)

Dose Level	Sex	Week 0	Week 13	Week 14	Week 15	Week 16	Week 17
2700	Male	45	49	46	52	49	46
2700	Female	51	44	51	47	49	46
8000	Male	48	44	45	49	49	50
8000	Female	48	45	47	45	46	46

Table B 3.4.2b Heinz Bodies (% x RBC) Recovery, Weeks 14-17
(2700 and 8000 ppm, N=2/sex)

Dose Level	Sex	Week 0	Week 13	Week 14	Week 15	Week 16	Week 17
2700	Male	0	1.55	1.2	0.6	0	0
2700	Female	0	1.05	0.2	0.8	0	0
8000	Male	0	12.1	3.1	0.75	0	0
8000	Female	0	14.5	4.45	1.95	0	0

Table B 3.4.2c Reticulocytes (% x RBC) Recovery, Weeks 14-17
(2700 and 8000 ppm, N=2/sex)

Dose Level	Sex	Week 0	Week 13	Week 14	Week 15	Week 16	Week 17
2700	Male	2.2	1.2	2.15	2.45	2.05	2.15
2700	Female	1.75	1.4	8.5	2.9	2.15	2.45
8000	Male	2.1	2.95	5.3	2.85	2.95	2.65
8000	Female	1.35	2.3	8.4	2.0	2.2	2.0

Figure B 3.4.2a Hematocrit Recovery, Weeks 14-17
(2700 and 8000ppm, N=2/sex)

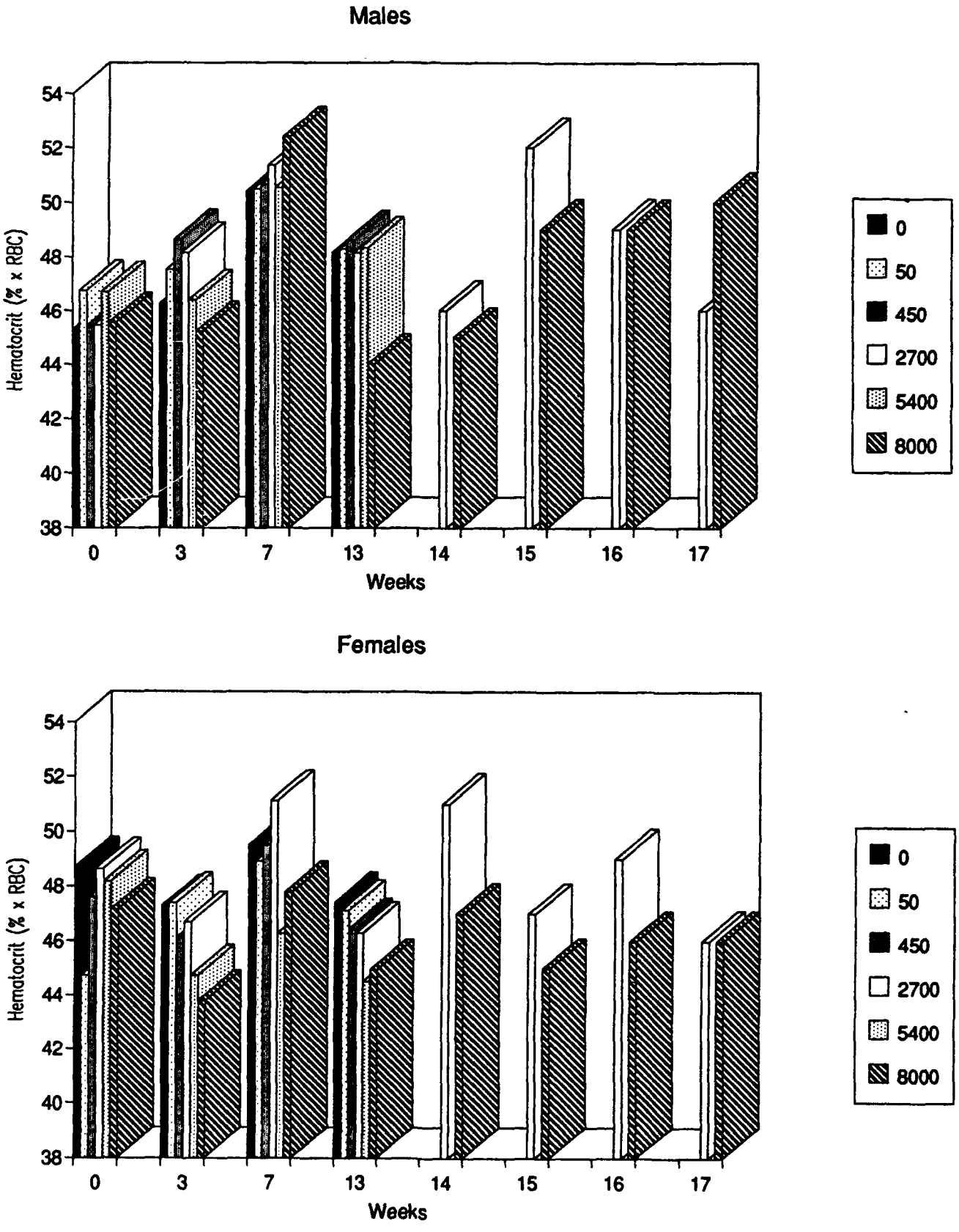


Figure B 3.4.2b Heinz Bodies (% x RBC) Recovery, Weeks 14-17
(2700 and 8000ppm, N=2/sex)

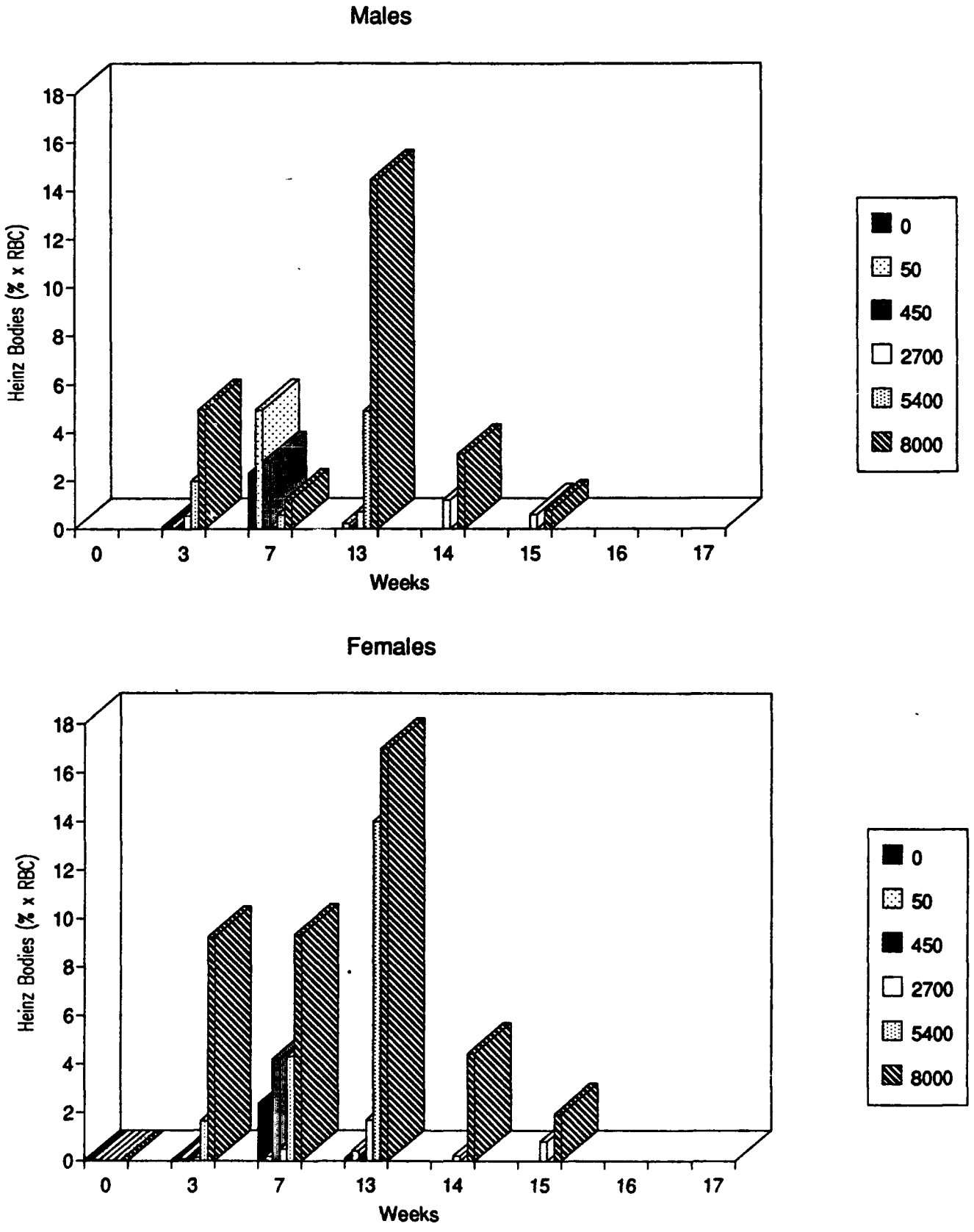
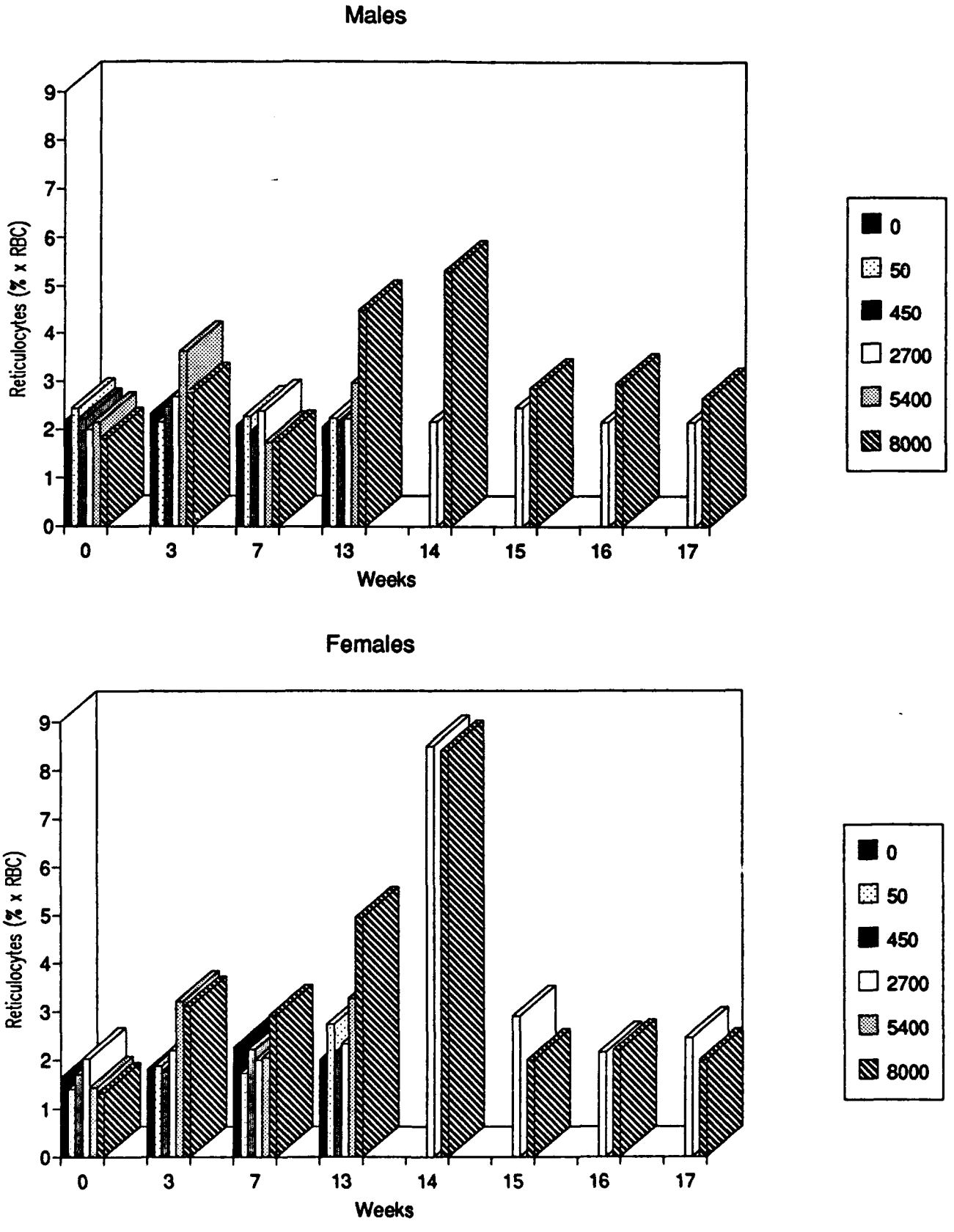


Figure B 3.4.2c Reticulocytes (% x RBC) Recovery, Weeks 14-17
(2700 and 8000ppm, N=2/sex)

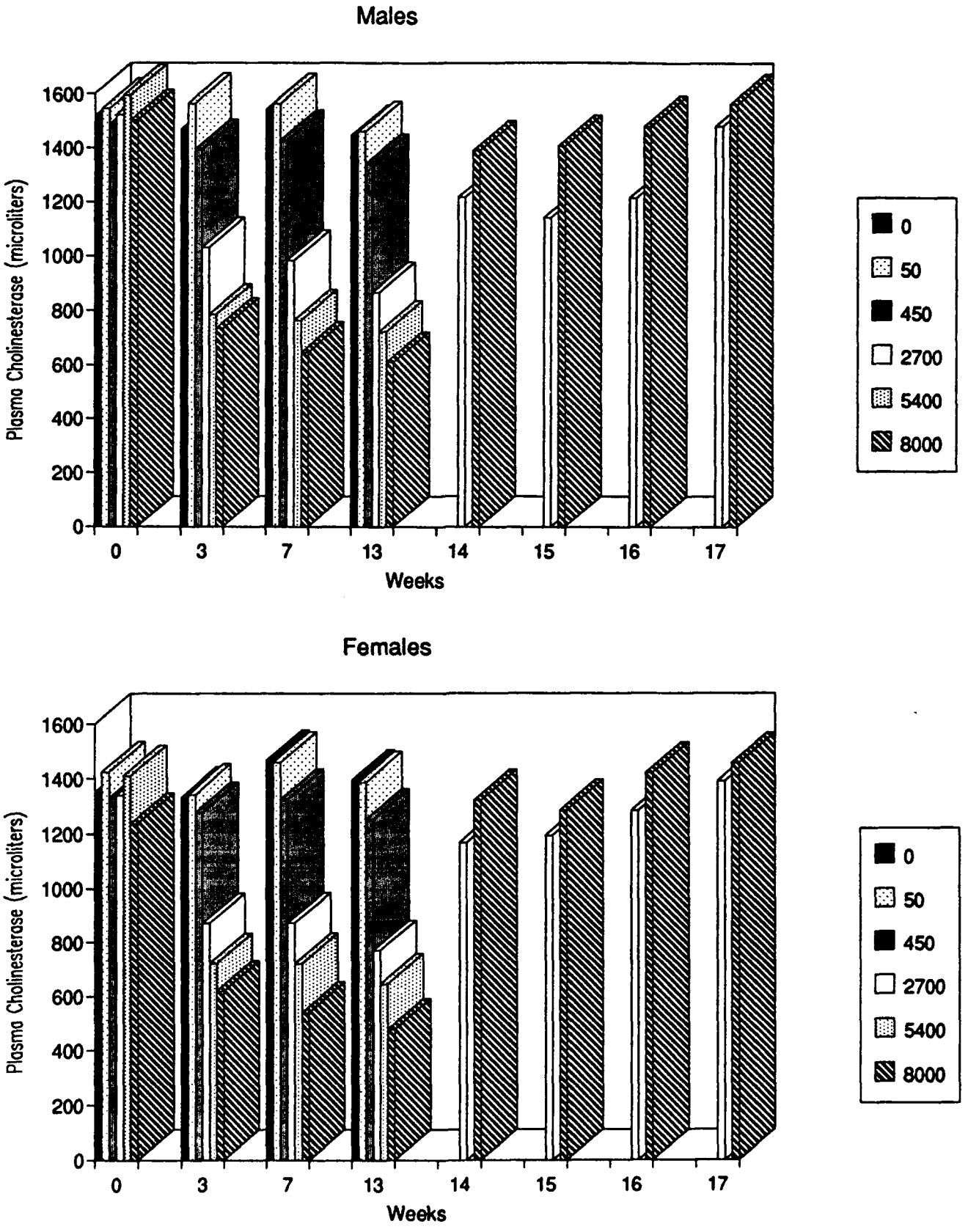


Plasma cholinesterase is tabulated for the recovery animals for Weeks 0, 13 and the recovery weeks in Table B 3.4.2d and plotted (all groups from Weeks 0-13 plus recovery animals for Weeks 14-17) in Figure B 3.4.2d.

Table B 3.4.2d Plasma Cholinesterase (PChE) Recovery, Weeks 14-17
(2700 and 8000 ppm, N=2/sex)

Dose Level	Sex	Week 0	Week 13	Week 14	Week 15	Week 16	Week 17
2700	Male	1150	422	1216	1141	1215	1482
2700	Female	1214	644	1169	1194	1284	1393
8000	Male	1444	656	1388	1408	1480	1457
8000	Female	1237	480	1321	1285	1422	1457

Figure B 3.4.2d Plasma Cholinesterase (PChE) Recovery, Weeks 14-17
(2700 and 8000ppm, N=2/sex)



3.5 Necropsy and Organ Weights

3.5.1 Necropsy

No animals died early or had to be removed from the study before the date of the scheduled necropsy. At necropsy, there were no treatment-related tissue abnormalities although the reduction in body weight of the 8000 ppm group was apparent. The several random necropsy findings and their microscopic correlates are tabulated by individual animal in the Pathology Report, Appendix 10.

3.5.2 Organ Weights

There were no changes in organ weight as a result of treatment. The weight of individual organs and group mean weight with standard deviation, tabulated separately by sex, are listed by treatment group in Appendix 11. The organs were also compared as percent of brain weight as well as percent of body weight. Since there were no differences in mean absolute weight of individual organs among treatment groups, there were also none when their ratios to brain weight were compared. However, when organ weights were expressed as a percent of body weight, some organs of the 8000 ppm group appeared significantly larger than the control group values because the mean body weight in the 8000 ppm group was significantly less. The mean liver weight as a percent of body weight increased in the females in the 5400 ppm dose group, although the group's mean body weight did not decrease significantly nor did the mean weight of the liver increase compared to that of the control. There was no change in the liver weight as a percent of brain weight among any of the groups.

3.6 Histopathology

The complete set of organs and tissues specified in the protocol was examined microscopically from all animals in the 8000 ppm dose group and in the untreated controls. In the 8000 ppm group, only the spleen of both sexes and the thymus and stomach of males had a microscopic alteration that was marginally increased in incidence or severity compared to the 0 ppm group. These organs were then examined in all groups that received DIMP, as well as in the 8000 ppm pair-fed control group (same sex only). The 8000 and 2700 ppm groups were represented by 8 animals each; two from each group were held one month longer on untreated feed, and they were tabulated separately. As indicated by the tabulations below, the spleen was the only organ revealed to have a mild change that could be considered treatment-related. The findings are tabulated as number positive/number examined (average severity grade, 1-4).

3.6.1 Spleen: Increased Hematopoietic Cell Proliferation

PPM	0	50	450	2700	5400	8000	0PF2
Male	6/10 (1.0)	8/10 (1.0)	7/10 (1.0)	7/8 (1.0)	9/10 (1.2)	7/8 (1.7)	6/10 (1.0)
Female	4/10 (1.5)	5/10 (1.2)	4/10 (1.3)	5/8 (1.0)	8/10 (1.5)	7/8 (1.6)	4/10 (1.0)

The incidence and severity were marginally increased in the 5400 and 8000 ppm groups.

3.6.2 Thymus: Cortical Atrophy (Reduction in Width of the Zone of Thymic Lymphocytes)

PPM	0	50	450	2700	5400	8000	0PF2
Male	0/10	0/10	0/10	0/8	1/10 (1.0)	2/8 (1.0)	1/10 (1.0)

Presence of the alteration in the 0PF2 untreated pair-fed group was evidence that it was not caused by treatment.

3.6.3 Stomach: Focal Inflammation of Mucosa

PPM	0	50	450	2700	5400	8000	0PF2
Male	1/10 (2.0)	1/10 (1.0)	2/10 (2.5)	2/8 (2.0)	1/10 (2.0)	2/8 (2.0)	2/10 (1.5)

The low incidence and comparable severity in all groups suggested this change to be common to all the animals, and not related to treatment.

3.6.4 Recovery Animals

The recovery animals were examined at necropsy in the same manner as the other 152 mink, and all 44 tissues were examined microscopically. No gross or microscopic lesions were detected in any organ from these animals.

3.6.5 Pathologist's Conclusion, 90-Day Study

The pathologist concluded, "No clear compound-related lesion was detected in male or female mink administered the compound. A marginally increased incidence or severity of hematopoietic cell proliferation of the splenic red pulp of male or female mink administered 8000 ppm was considered a probable compound effect."

The pathologist's report with data tabulations of gross and microscopic findings is included in Appendix 10.

4.0 DISCUSSION

4.1 Variances from Protocol

There were relatively few variances from the protocol, for a study that encompassed a number of facets that were not standardized beforehand. Given the realities of availability of personnel, funding, facilities and animals, we strove to incorporate practices that would enable us to obtain reliable data. To our knowledge, none of the variances from protocol resulted in biased or compromised data.

4.1.1 Target versus Actual Concentrations of DIMP in Feed

The protocol specified the target doses to be 0, 50, 450, 2700, 5400 and 8000 ppm in feed. Our Standard Operating Procedures stipulated that we would not accept the doses that varied from these concentrations by more than 10%. As noted in Section B 2.4.1, at the time the DIMP was incorporated into the feed, we had no data about its stability in feed, although we knew that 'neat' DIMP was stable. To prevent underdosing, we deliberately added 10% more than the target dose, anticipating that exceeding the target doses by 10% would not compromise the study. The target doses were arbitrary, as explained in Section B 1.2. The overage that we did achieve, 8-14% (Table B 2.4.2) was consistent. We accepted it and believe it did not affect the study outcome adversely.

4.1.2 Analysis of Feed Contaminants

The protocol specified that analysis of the ration for contaminants would include determination of deoxynevalenol (DON), among several mycotoxins incriminated as a cause of morbidity in mink. The laboratory that provided analyses of contaminants and nutrients of the mink ration was the best-qualified laboratory of those responding to our solicitation, but it did not perform this specific analysis. Instead it included analyses for a greater number of other mycotoxins (Appendix 3). We accepted their analysis. There was no morbidity in our animals, and none attributable to mycotoxins on their ranch of origin, where the same ration was being fed to thousands of animals. Significantly with respect to DIMP, organophosphate insecticides were below detectible limits in the feed.

The Standard Plate Count of aerobic bacteria (19,000,000/gm) was higher than expected. Counts of this magnitude (exceeding five million per gram) are often associated in mink with diarrhea and mild elevations of liver enzyme concentrations in blood. Our animals had no unusual experience with either. We surmise that the elevated counts resulted from bacterial replication during the sampling procedure and did not truly reflect the condition of the food consumed (Wustenberg letter, Appendix 3).

4.1.3 Room Temperature

A cooling malfunction affected the temperature in all rooms, 2 - 6 June, 1991 (Week 3 of the study). Room temperatures exceeded the maximum specified (28°C) by 10% (28 versus 31°C). There was no discernible effect on food consumption or other observable characteristics.

4.1.4 Light:Dark Schedule for Room Lights

The protocol specified that room lights would be controlled automatically to match the ambient outdoor ratio of light:dark hours current at the start of the study, determined to be 12.5 hours light and 11.5 hours dark. During Week 7 of the study, the timers were discovered to differ among the seven rooms housing these mink; three rooms were correct, but the other four had been set for 13.5 hours light and 10.5 hours dark.

In nature, the mink's annual reproductive cycle and annual "molt" (shedding of winter fur and growth of summer pelage) are strongly influenced by photoperiod. Our animals began their molt within the first two weeks of the study, and no differences among the rooms were apparent. Review of the blood chemistry and hematology to that point also showed no systematic room effect.

The photoperiods were not changed. Rooms 345b, 345m, 345l and 345k were on a 13.5:10.5 light:dark cycle for the full study, while rooms 114, 116, and 118 were on 12.5:11.5 hours. No systematic room effect was noted in any evaluation at the end of the study.

4.1.5 Pair Feeding

The plan to feed untreated ration to cohorts in quantities to match the amount eaten by the two highest-dosed groups was successful only for the first nine weeks and only in the 8000 ppm males. We allowed the pair-fed groups ad libitum feeding during any week following one in which their treated cohort consumed 90% or more of that eaten by the 0 ppm controls. This was to avoid fasting them when it might not be warranted. We did not anticipate the degree to which the animals would compensate for previous deprivation. In retrospect, obviously, the pair-fed group should have been offered exactly the amounts eaten by the treated groups, even if they exceeded that eaten by the 0 ppm group.

The male 5400 ppm group consumed as much as the 0 ppm group throughout the study, so pair feeding was not invoked on their behalf. The male 8000 ppm group refused some of their food through the first nine weeks of the study, and their pair-fed cohort was fed in parallel, one week later. The food consumption and body weight performance of these groups was similar over that period, confirming that reduction in body weight was caused by decreased food intake (Section B 3.1.1 and B 3.1.2) Both

female pair-fed groups had several opportunities to "refeed"; they finished similar to the 0 ppm control in food consumption and body weight.

The female animals (mean body weight about 1 kg) consumed approximately 150 gm food/kg BW. The males (2 kg in weight) consumed about 115 gm/kg BW. The food intake was remarkably constant across dose groups, on the basis of body weight. The sex difference in food consumption has been recognized by mink ranchers (Joergensen, 1985).

The male 0PF1 group was given untreated food ad libitum throughout the study, and their data could be used as representing a second 0 ppm group of males. The female groups "escaped" rigorous pair feeding so their weights and food consumption did not match their respective treated groups. However, because their food had been restricted at intervals, we did not consider them 0 ppm controls either. Consequently, we did not use blood data from the pair-fed groups for comparison with the treated animals.

4.1.6 Examination of Cecum and Preputial/Clitoral Glands

The protocol specified that organs listed in the National Toxicology Program Statement of Work and the PAI Necropsy SOP, would be examined grossly and microscopically. Those lists, originally designed specifically for rodents, included preputial/clitoral gland in males and females, respectively, and cecum in both sexes. The mink has neither structure, but the organs were inadvertently not deleted from the lists. This oversight did not compromise the data.

4.2 Interpretation of Results

The food consumption and body weight performance of the 8000 ppm group and its pair-fed control group during the first nine weeks of the study provide persuasive evidence that the weight loss experienced by the higher dose groups was caused by reduction in food intake. This effect was also recorded by Aulerich et al., (1976) in their 21-day study at 10,000 ppm. There was a downward pattern in all body weights over the course of our study. This is part of a normal annual phenomenon in mink physiology, which includes increasing accumulation of body fat in the Fall preparing for winter months, followed by weight loss as breeding, reproduction and shedding occur in the Spring.

There was relatively little toxicity, even at the high doses, over the 90-day period studied. Except for the food refused and weight loss, the animals appeared healthy throughout.

The reduction in plasma cholinesterase was not unexpected, having been reported by Hart (1980) in his 90-day study in beagle dogs given 75 mg DIMP/kg BW/day. In Hart's study, the effect was inconsistent at that dose, and RBC acetylcholinesterase was also affected sporadically. Reduced plasma cholinesterase was clearly dose related at 2700

ppm (400 mg/kg BW/day) and above, in our mink. The RBC acetylcholinesterase was not affected. The physiologic role of plasma cholinesterase is unknown, although it does not reflect the status of acetylcholinesterase in the nervous system, while RBC acetylcholinesterase does (Chatonnet, 1989; Munro, 1991). Our 450 ppm mink ingested DIMP at approximately 73 mg/kg BW/day (average for both sexes combined). At Week 13, the plasma cholinesterase in 450 ppm females was 1252±126 units/L, compared with 1395±237 for the 0 ppm group at Week 13, and also compared with their own pretreatment (Week 0) value of 1331±214. In males, the Week 13 value was 1340±227 compared with 1447±134 or 1486±298, respectively. These reduced values at Week 13 were statistically significant in the range of $p = 0.05 - 0.07$, compared with the Week 13 value of the 0 ppm groups. But the with-in group reduction between Week 0 and Week 13 for each sex of the 450 ppm group, was not significant, and was only 6% for females and 11% for males. We believe that plasma cholinesterase values are sufficiently labile and sufficiently unrelated to nervous system cholinesterase that we regarded these changes as biologically unimportant in this dose group. In the higher dose groups, even with plasma cholinesterase reduction of 61% (8000 ppm females, Week 0 versus Week 13), there was no reduction in RBC acetylcholinesterase and no clinical signs of inhibition of acetylcholinesterase. Further, the plasma ChE returned to normal in one week in all eight animals placed on untreated feed at the end of the 90 days of treatment.

The increase in Heinz bodies, seen in the animals in the two highest dose groups, indicated that DIMP at those doses caused oxidative injury to hemoglobin, configurational change in the molecule's tertiary structure, and formation of the Heinz body. Affected erythrocytes have a reduced lifespan, eliciting compensatory hematopoiesis. The pattern of reduced RBC count, increased cell volume (immature RBCs in circulation) and increased reticulocyte count were all consistent with the hematopoietic response to shortened RBC survival. The pathologist's observation of marginally increased extramedullary hematopoiesis (splenic) was also consistent. These changes were mild, and resolved within two weeks when treatment was stopped. Only the reticulocyte count was somewhat anomalous. Healthy mink should probably have less than 1% of their RBCs as reticulocytes, although our "normal" value was approximately 2%. This is attributed to excitement attendant to collection of samples as demonstrated in cats by Fan (1978). By Week 13, the 8000 ppm group mean was approximately 5% reticulocytes, which we anticipated would resolve promptly with cessation of DIMP exposure. However, after one week on untreated feed, the reticulocyte count in all four former 8000 ppm animals increased from 2 - 3% at Week 13 to 4-5%; and in the two female former 2700 ppm animals, the count increased even more, from 2% at Week 13 to 8% at Week 13 + 1. We interpreted this increase as a response to our blood sampling procedure. At the end of Week 13, the final 6 ml blood sample was taken from all animals; from 24 of them, including the eight recovery animals, an additional 4 ml was taken for PAI to analyze for RBC acetylcholinesterase and to confirm any change in plasma ChE. The reticulocyte response noted one week later most probably was a response to the blood sampling, not to the DIMP exposure.

Blood urea nitrogen values were elevated slightly in the 5400 and 8000 ppm animals beginning at Week 7 and increased slightly again by Week 13. While the differences achieved statistical significance, the absolute changes were not noteworthy clinically (maximum 17 mg/dL in the female 8000 ppm group versus 12 mg/dL in the 0 ppm female at Week 13). We regard the change as secondary to reduced food intake, or weight loss, and not as a primary response to DIMP.

4.3 Comparisons with the Aulerich et al. (1979) Study in Mink

Aulerich et al. exposed groups of 6 male and 24 female pastel mink to DIMP at dietary levels of 0, 50, 150, or 450 ppm to study the effect on mink reproduction. The animals were three months old at the start, were mated, and young were born and nursed during the 49-week exposure. The study was not designed to obtain data for risk assessment in humans. Body weights were determined twice monthly until mating, again at whelping and one month later. The animals were housed individually in outdoor sheds. There was no report of verification of target dose in feed. Food consumption was measured 18 times during the first 20 weeks but not during gestation and lactation so accurate exposure data are uncertain. The available data on food consumption permitted estimation of daily DIMP intake to be 0, 11, 37, and 95 mg/kg BW in the respective dose groups.

There were no adverse effects of DIMP noted in any of the measurements taken, including the food consumption, body weight, and hematologic and chemical analyses. All reproductive indices were also unaffected, e.g., viability, number and size of offspring. A negative outcome was the report of excess mortality in 450 ppm females (5/24). The cause of death was not determined. Aulerich described an earlier 21-day study in the same report, in which 8-month old female mink were fed doses up to 10,000 ppm. The 10,000 ppm group ate less than the controls and lost weight. In the 1-year study, the 450 ppm females died without prodromal signs of any kind. Further, Aulerich was conducting an identical study with dicyclopentadiene (DCPD) concurrently with the DIMP study, using animals of the same lot and in the same facility. In that study, described in the same report, the mortality in the untreated animals was the same as for the 450 ppm DIMP and the high dose DCPD groups. We concluded that DIMP was not the cause of death in the 450 ppm female mink in Aulerich's study. If this were so, then 95 mg/kg BW/day, the highest dose, would be the No Observed Adverse Effect Level for mink in that study.

Hart reported a 90-day dosed-feed study of DIMP in dogs (1980) in which there were no adverse effects at the highest dose, 75 mg/kg BW/day. Hart also reported 90-day studies in rats and mice (1976) in which the highest doses (rats 150 mg/kg, mice 315 mg/kg) produced no adverse effect.

Our study emerges as the first 90-day study of DIMP to report a NOAEL lower than the highest dose, i.e., we were able to identify a toxic range and demonstrate both

a NOAEL and LOAEL. The respective doses were 73 mg/kg BW/day, and 400 mg/kg BW/day. The effects noted were mild. Had we used a dose between 73 and 400, the NOAEL might have been higher, i.e., 73 mg/kg BW/day is a conservative NOAEL.

5.0 SUMMARY AND CONCLUSIONS, 90-DAY STUDY

Diisopropyl methylphosphonate (DIMP) appears to be only slightly toxic to mink at the higher doses employed in this study. The changes noted decrease in plasma cholinesterase and injury to erythrocytes were rapidly reversible upon cessation of exposure. The NOAEL for DIMP was 73/mg/kg BW/day, with 400 mg/kg the LOAEL, in this study.

C. METABOLIC/PHARMACOKINETIC STUDY

The principal objectives were to determine the rate of excretion of DIMP by mink and the identity and relative abundance of any metabolites produced. Hart (1976) reported a study of the fate and excretion of ^{14}C-labeled DIMP in mice, rats, and dogs; and Ivies (1980) reported metabolism of DIMP for a single cow. To establish a point of comparison with the Hart report, we studied rats in addition to mink.

1.0 STUDY DESIGN

1.1 Overview

The protocol followed EPA's Health Effects Testing Guidelines (U.S. EPA 1985). For both rats and mink, eight animals of each sex were housed individually in metabolism cages, anesthetized and administered either a high dose or a low dose of radioactive DIMP by oral gavage, or were given the low dose intravenously (IV). All animals were fasted overnight before treatment and were permitted untreated food and water ad libitum thereafter. Blood, urine and feces were collected at intervals during the 120 hours following dosing. Aliquots of all specimens were prepared and counted at UMN for total radioactivity. Matching samples were submitted to SwRI, where the identity and relative abundance of the parent compound (DIMP) and metabolites were determined and quantified as a function of time after dosing.

1.2 Rationale for Dose Selection

The data on which to base selection of doses for this study were not readily available. To make the doses for mink and rats comparable on some biologic basis, we began with the single dose oral LD_{50} study reported by Hart (1980) for female rats, and that reported by Aulerich (1979) for female mink. Each used a different method to calculate the LD_{50}. We used a proprietary SAS program to recalculate the probability of lethality from their respective reported outcomes.

Hart (1980) reported the following observations at 24 hours in female rats:

DIMP (mg/kg)	Response (dead/exposed)
430	0/10
632	0/10
928	5/10
1362	10/10
2000	9/10

Hart (1980) calculated the LD_{50} to be 826 mg/kg. Our program produced 986 mg/kg as the LD_{50}, from these data. We selected the LD_{10} from our program, 660 mg/kg, as the oral high dose for rats in this study, hopeful that the true LD_{10} would represent a dose that offered some toxic or pharmacologic effect. We set 10% of this amount (66 mg/kg) to be the low oral dose and low IV doses.

Approaching the mink data in the same way, Aulerich's 24-hour results were:

DIMP (mg/kg)	Response (dead/exposed)
0	0/3
75	0/2
150	0/4
300	1/4
450	1/4
500	4/6
550	2/5
600	4/4

Aulerich reported an LD_{50} of 503 mg/kg. Our program yielded 464 mg/kg from these data. The LD_{10} from our program was 267 mg/kg. We set 270 mg/kg for the high dose and 27 mg/kg for the low dose in mink.

2.0 MATERIALS AND METHODS

2.1 Radioactive DIMP

2.1.1 Source and Characterization

Radioactive DIMP (^{14}C-DIMP), labelled on the methyl carbon, was obtained from Dupont NEN Products, Boston, MA. The specific activity was 5 millicuries/millimole, and the supplier stipulated the radiochemical purity to be 97%.

2.1.2 Dose Formulation

The mink and rats were each given 40 μCi/kg body weight, except the mink dosed intravenously were given 20 μCi/kg to preclude high levels of radioactivity in early urine specimens. The radioactive DIMP was diluted with "cold" DIMP from the same stock as that used in the 90-day study to achieve the following total doses (mg DIMP/kg body weight):

	High Oral Dose	Low Oral Dose	IV Dose
Rats	660	66	66
Mink	270	27	27

The administered volumes were standardized to 4.0 ml/kg BW with distilled water for the oral doses and with physiological saline for the IV doses.

2.2 Mink

2.2.1 Animals and Dose Administration

Mink from the same stock as those in the 90-Day Study were used; they were 14-16 months of age at the start of the metabolic study. The animals were identified by cage cards. Eight animals of each sex were given 27 or 270 mg/kg by oral gavage or 27 mg/kg intravenously. For each dose, four animals of each sex were studied concurrently, with the second set of four begun on the following day.

The mink were fasted overnight, anesthetized with the same ketamine/xylazine mixture used to obtain the blood samples in the 90-Day Study, and were administered their respective dose of radioactive DIMP by oral gavage or intravenous (jugular vein) injection. Most mink remained anesthetized for 20-30 minutes, except those given the high dose did not recover fully for 30-60 minutes. Untreated food and water were available ad libitum after dosing.

Blood samples were taken at 1, 2.5, 5, 10, and 30 minutes, and at 1, 2, 3, 4, 6, 7 and 24 hours after administration of DIMP. The initial samples, taken while the animals were still under anesthesia, were obtained from the jugular vein with syringe and needle. The later samples, usually beginning with the hourly ones, were obtained initially by toe nail clip, with the awake animal restrained manually. This approach proved to be unsatisfactory for several reasons. Collecting the sample and achieving hemostasis required 10 - 20 minutes for each animal for each toe nail, too long for even experienced mink handlers to control the animals. Also, the animals became restive, resulting in urination and defecation with consequent potential contamination and loss of radioactivity.

Four animals of each sex and dose group were added, which were allowed to remain in their cages without being bled. Urine and fecal collections were more complete from these animals, and the counts from these animals were used as the basis for recovery of radioactivity from urine and feces.

The original eight animals of each sex and dose group were used for the data on radioactivity in blood. The restraint procedure was modified by the use of a stainless steel restraint device that controlled the animal's head and neck, allowing the handler to cradle the animal in one arm and hand while extending the mink's head and neck with the other, permitting sampling by jugular venipuncture. The device was Y-shaped, with two bars across the open end. One was placed in the animal's mouth as a gag, and the more distal one was fitted on the dorsum of the neck. Gentle upward leverage on the handle achieved safe and effective control of the neck and head. The jugular sampling minimized the need for hemostasis. This procedure was carried out while holding the animal above the wire mesh metabolism cage to minimize loss of urine that was still voided. Contamination and loss of radioactivity were greatly reduced, blood sampling was easily accomplished and restraint time and stress were minimized.

The blood that was obtained through the toe nail clip was collected directly into heparinized snap-cap vials. That from venipuncture was collected with heparinized syringes and discharged into the vials. Before subsampling, the blood was mixed and triplicate 10 µL aliquots of whole blood were pipetted into separate scintillation vials for counting. Whole blood was used because preliminary data indicated that there was no statistically significant difference in radioactivity in plasma versus whole blood (Appendix 12). The residual samples were centrifuged, and plasma was removed with a transfer pipette into snap cap vials; plasma and packed cell samples were stored at -20°C. These plasma samples were used to determine relative concentrations of DIMP and metabolites.

2.2.2 Metabolism Cages

The standard mink "pelting" cages used for the 90-Day Study were modified by the addition of a fine-meshed wire screen fitted into metal channels attached beneath the wire mesh cage bottom. A galvanized metal funnel was suspended below the screen to direct urine into a 600 ml plastic beaker on the floor under the funnel outlet. The beaker was set in a styrofoam box that contained pelleted dry ice. Feces accumulated on the cage bottom and on the screen beneath.

Urine and feces were collected at 4, 8, 12 and 24 hours, and at 2, 3, 4 and 5 days. At collection times, the urine beakers were removed and weighed. the urine was allowed to thaw and was transferred to plastic storage vials and refrozen at -20°C until analysis. The cage and funnel were rinsed at each collection with 20-40 ml of water, which was collected, weighed and stored at -20°C until analyzed. The urine collection beaker was replaced with a clean tared beaker under the funnel and the dry ice was replenished. To collect feces, the animal was confined to the nest box with a removable grate, and feces

were scraped from the cage bottom onto the screen, then removed from the screen by spatula. The feces from each collection were stored in a -20°C freezer and weighed at completion of the study.

2.3 Rats

2.3.1 Animals and Dose Administration

Groups of eight 10-week old male and female Sprague-Dawley rats were used. After quarantine, they were weighed, and their tails were marked with a color code. They were fasted overnight prior to dosing. To more closely match the procedure used for the mink, the rats were also anesthetized for dosing and collection of the initial blood samples, using the same anesthetic and dose rate. As in the mink, anesthesia was significantly prolonged in the rats given the high dose of DIMP. One rat died under anesthesia and was replaced. The sample collection schedule for the rats was the same as that for mink.

The intravenous DIMP was administered via tail vein in the rat, the relative volume equal to that used in the mink (4.0 ml/kg). Blood samples were obtained from the orbital sinus via heparinized capillary tubes into heparinized snap-cap vials.

After the animals had recovered from anesthesia, they had to be removed from their metabolism cage and restrained for collection of subsequent blood samples. Urine and feces were again lost (although less than with the mink), and adequate blood could not always be obtained on schedule. We were unable to obtain satisfactory blood samples from the high dose males after 2 hours post-treatment without risking unacceptable loss of urine and feces.

The sampling procedure was changed to improve collections and reduce need for restraint. The rats were anesthetized with inhalant isoflurane gas, administered through an anesthesia machine with a cone placed over the rats' heads. Induction was very rapid and recovery was full, 1-2 minutes after the cone was removed. All subsequent blood samples were taken after anesthetizing the animals in their metabolism cages to preclude possible contamination and loss of urine and feces.

2.3.2 Metabolism Cages

The rats were housed in commercial polycarbonate metabolism cages (Nalgene,™ Nalge Company, Rochester, NY). Food (NIH 31) and water were available ad libitum. Urine and fecal specimens were removed per protocol, and the cage was rinsed with 10-30 ml water with each urine collection. Handling of specimens and data were the same as for the mink except the rat urine could not be collected over dry ice because the plastic cage precluded its use.

2.4 Specimen Preparation; Determination of Total Radioactivity

2.4.1 Blood and Urine

In a preliminary test in mink, a comparison of counts between whole blood and plasma by single-factor analysis of variance revealed no statistical difference between the two in samples taken during the first 24 hours (Appendix 12). Counts from whole blood were therefore used to monitor radioactivity in blood.

The blood and urine were processed similarly. Urine and cage rinse water from the same cage collection were weighed, and 10 µL aliquots were pipetted into scintillation vials in triplicate. Counts of the radioactivity of each urine collection and its respective rinse water were added together. Whole blood (10 µL) was pipetted directly into the vials. One ml of a tissue and gel solubilizer (MEN Research Products, Boston, MA) was added to each sample. The samples were capped, mixed, and shaken in a 60°C water bath for one hour, and 0.25 ml of 30% hydrogen peroxide was added to each. The vials were loosely capped and replaced into the shaking water bath for 30 minutes. The samples were again cooled 10 minutes, and 15 ml of biodegradable scintillation fluid, Ecoscint A, (National Diagnostics, Manville, CO) was added and mixed, followed by 0.5 ml of 0.5 N hydrochloric acid. After mixing again, vials were placed into a Beckman Scintillation Counter (Packard Instruments, Downer's Grove, IL) with each animal's samples separated by a blank that contained all the reagents. Acceptable background for the blanks was 200 disintegrations per minute (DPM), or less, at the beginning of the run. The samples and blanks were counted automatically.

2.4.2 Feces

Fecal specimens were oxidized with an oxidizer (Packard Instruments, Downer's Grove, IL) before being counted. The feces from each animal were thawed at room temperature and mixed in a Stomacher blender (Tekman Co, Cincinnati, OH) for up to 2 minutes. Triplicate aliquots of 150-300 mg were removed from different areas of the sample and placed into tared combustion cones containing sample pads. The combustion cones were placed onto numbered steel trays and transported in plastic boxes. After weighing, the samples were dried at 65°C for four hours. Blanks and calibrated vials and combustion cones (Spec-Check, Packard Instruments, Meridan, CT) were run with each animal's specimen to check recovery of ^{14}C.

The Packard Oxidizer was operated according to the manufacturer's instructions. Carbosorb and Permafluor V, Packard reagents, were used to trap CO_2 and to act as a scintillator, respectively. The samples were combusted into prenumbered scintillation vials and counted by the same instrument used for urine and blood specimens. The Ecoscint A biodegradable scintillator fluid used for the blood and urine specimens had inherent autofluorescence, resulting in background counts up to 200 DPM. The toluene-based Permafluor V used for the fecal samples had background count under 100 DPM.

The percent recovery of Spec-Check controls for feces was 100%±2% for weekly runs.

2.5 Specimen Preparation; Determination of Parent Compound and Metabolites

Specimens of plasma, urine and feces were collected and prepared as described in Section 2.4, and shipped frozen to SwRI for identification and quantification of radiolabelled compounds. Following is a brief description of their preparatory procedure. Details of the methods and instrumentation are described in the SwRI report, Appendix 2.

2.5.1 Plasma

Cold acetronile was added to aliquots of plasma, mixed and centrifuged. The supernatant was separated and fractionated by reverse-phase high performance liquid chromatography. Fractions were subsequently identified by gas chromatography/mass spectrometry and proton nuclear magnetic resonance.

2.5.2 Urine

Urine specimens were analyzed in the same manner as plasma, except an additional set of aliquots of urine specimens from the 0-24 hour collections was also subjected to enzyme hydrolysis (beta hyaluronidase and limpet sulfatase) before analysis to detect possible conjugated DIMP or metabolites.

2.5.3 Feces

Fecal samples were homogenized with distilled water before addition of the acetonitrile. The supernatant was filtered and processed in the same manner as were the plasma and urine.

2.6 Statistical Analyses

Counts of radioactivity in blood , urine and feces at various times, as percent of administered dose, were analyzed as repeated measures (Winer, 1971). Contrasts were used to compare values among the species, sexes and dose routes, and to analyze for possible polynomial effects of time. The analyses were performed with the "Repeated" option of PROC GLM of SAS (SAS, 1987).

Cumulative and total recovery of radioactivity in urine, feces and combination of urine and feces, as percent of administered dose, were analyzed statistically with a completely random design. Contrasts were used to compare species, sexes and dose routes. The analyses were conducted with the PROC GLM program of SAS.

The detailed statistical report is included as Appendix 4B.

3.0 RESULTS - METABOLIC STUDY

3.1 Rats - Total Radioactivity in Blood, Urine and Feces (N = 8 Rats)

The radioactivity detected in whole blood, plasma, urine and feces from each animal per collection is tabulated in Appendix 12.

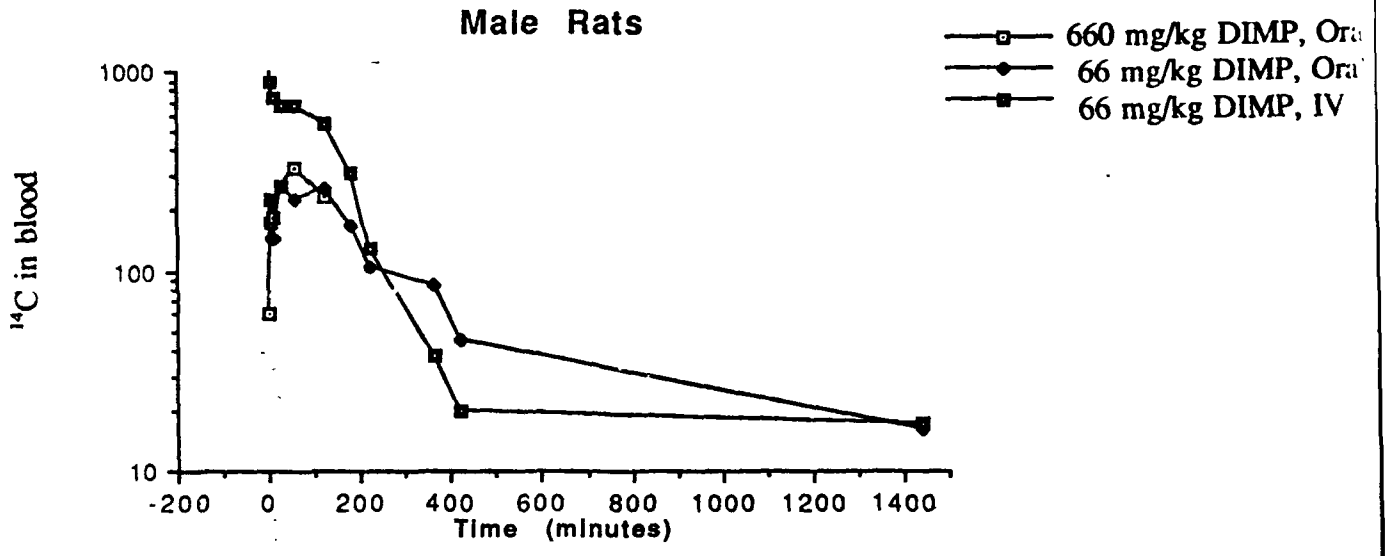
3.1.1 Total Radioactivity in Whole Blood

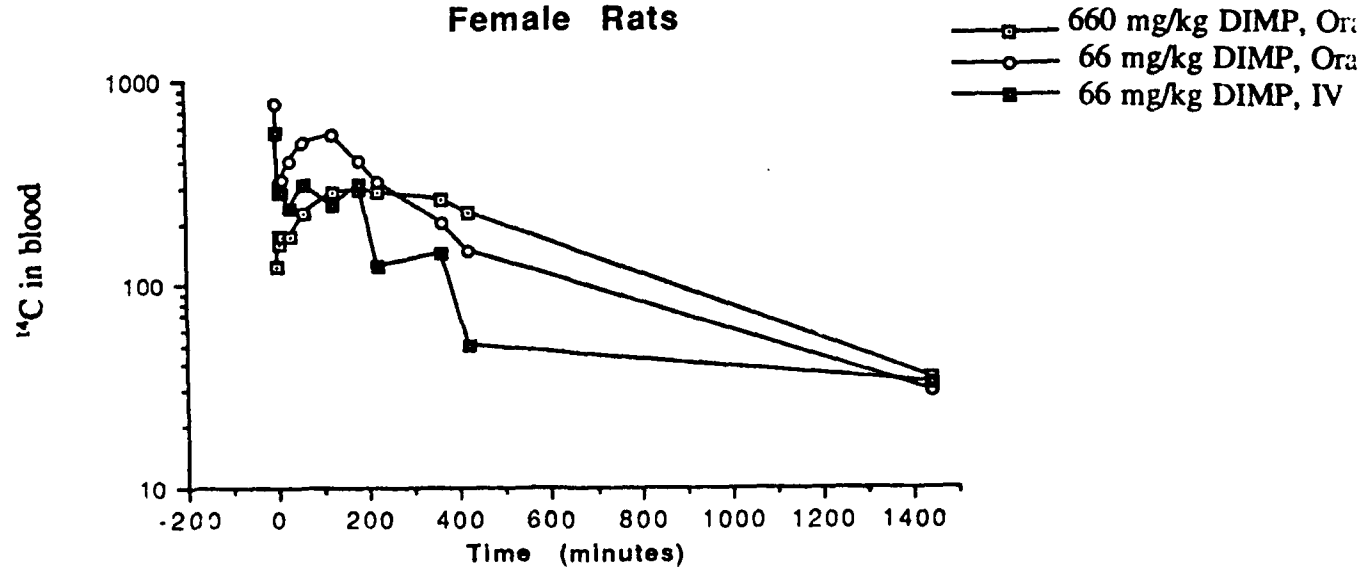
Group mean counts detected during the first 24 hours are summarized in Table C 3.1.1 and plotted in Figure C 3.1.1

Table C 3.1.1 Rat ^{14}C Radioactivity in Whole Blood
(cpm per 10μL/40μCi/kg BW; N = 8)
NS = Non Sufficient

Dose Level	Sex		1 min	2.5 min	5 min	10 min	30 min	1 hr	2 hr	3 hr	4 hr	6 hr	7 hr	24 hr
660 Oral	Male	Mean	61.67	172.97	228.40	188.42	288.13	331.47	236.00	NS	NS	NS	NS	NS
		SD	79.31	57.69	122.48	128.49	92.63	101.63	210.57	NS	NS	NS	NS	NS
66 Oral	Male	Mean	NS	232.46	146.58	144.04	288.44	228.38	258.58	170.21	106.88	88.63	45.21	16.43
		SD	NS	152.56	39.85	29.96	237.30	63.72	79.33	64.83	34.07	80.91	20.71	13.01
66 IV	Male	Mean	1559.72	1069.67	875.21	745.15	684.73	686.81	550.38	309.38	128.29	38.10	20.67	17.71
		SD	351.31	149.97	72.51	113.53	82.64	60.91	33.58	47.24	20.14	25.28	3.50	20.58
660 Oral	Female	Mean	122.24	174.00	159.46	175.88	175.29	228.92	288.90	294.29	285.98	262.15	223.13	34.38
		SD	115.64	73.79	43.01	39.15	58.71	95.31	125.5	91.45	102.80	137.75	87.00	78.98
66 Oral	Female	Mean	767.80	322.05	301.29	328.58	403.83	503.92	535.75	406.17	318.04	203.21	148.96	29.21
		SD	1207.95	242.04	126.77	100.20	109.78	97.59	50.97	51.46	48.55	41.84	43.13	20.79
66 IV	Female	Mean	564.33	289.28	292.47	289.58	243.56	311.17	250.42	315.83	122.38	142.25	50.46	32.88
		SD	233.88	225.92	82.52	194.31	113.09	130.57	131.27	314.37	67.41	191.69	68.77	38.40

Figure C 3.1.1 Rat ^{14}C Radioactivity in Whole Blood





Radioactivity units are counts per minute (cpm) per 10 μL whole blood/40 μCi of ^{14}C administered.

When administered orally to rats, DIMP was absorbed rapidly and cleared rapidly from blood. Radioactivity in blood peaked in 2 -3 hours and returned to background in 4-7 hours. Figure C 3.1.1 is a plot of the logarithm of the counts. The curves resulting from monitoring over the 24-hour period are generally similar in shape in both sexes and all doses although blood levels were higher initially in males and then decreased more rapidly, in comparison with females. The female high-dose oral group retained radioactivity longest in blood (blood samples were not obtained from high dose males after 2 hours, as explained in Section 2.3.1).

The half-time for the slower-disappearing component cannot be calculated accurately because the 'break' in the curve occurred at an undetermined time between the 7-and 24-hour sampling. Best estimates from the data available suggest a half-time for this component to be 11-35 hours in males and 6-24 hours in females. The fast-disappearing component has a half-time of approximately 1 hour in males and 1-2 hours in females.

3.1.2 Total Radioactivity in Urine and Feces

The recovery of total radioactivity in urine and feces over the 120-hour collection period is listed in Table C 3.1.2a and plotted in Figure C 3.1.2a. The cumulative ^{14}C excretion in urine is summarized in Table C 3.1.2b and plotted in Figure C 3.1.2b, and the fractional excretion of urine and feces are plotted in Figures C 3.1.2c-e and C 3.1.2f-g, respectively.

Table C 3.1.2a Rat 120-Hour Total Recovery of ^{14}C
(Percent of Administered Dose)

Dose	Sex	Urine	Feces	Total
660 mg/kg Oral	Male	80.3	8.8	89.1
	Female	59.1	8.7	67.8
66 mg/kg Oral	Male	91.0	6.3	97.3
	Female	63.0	9.4	72.4
66 mg/kg IV	Male	79.2	6.7	85.9
	Female	59.8	7.1	66.9

Figure C 3.1.2a Rat 120-Hour Total Recovery of ^{14}C
(Percent of Administered Dose)

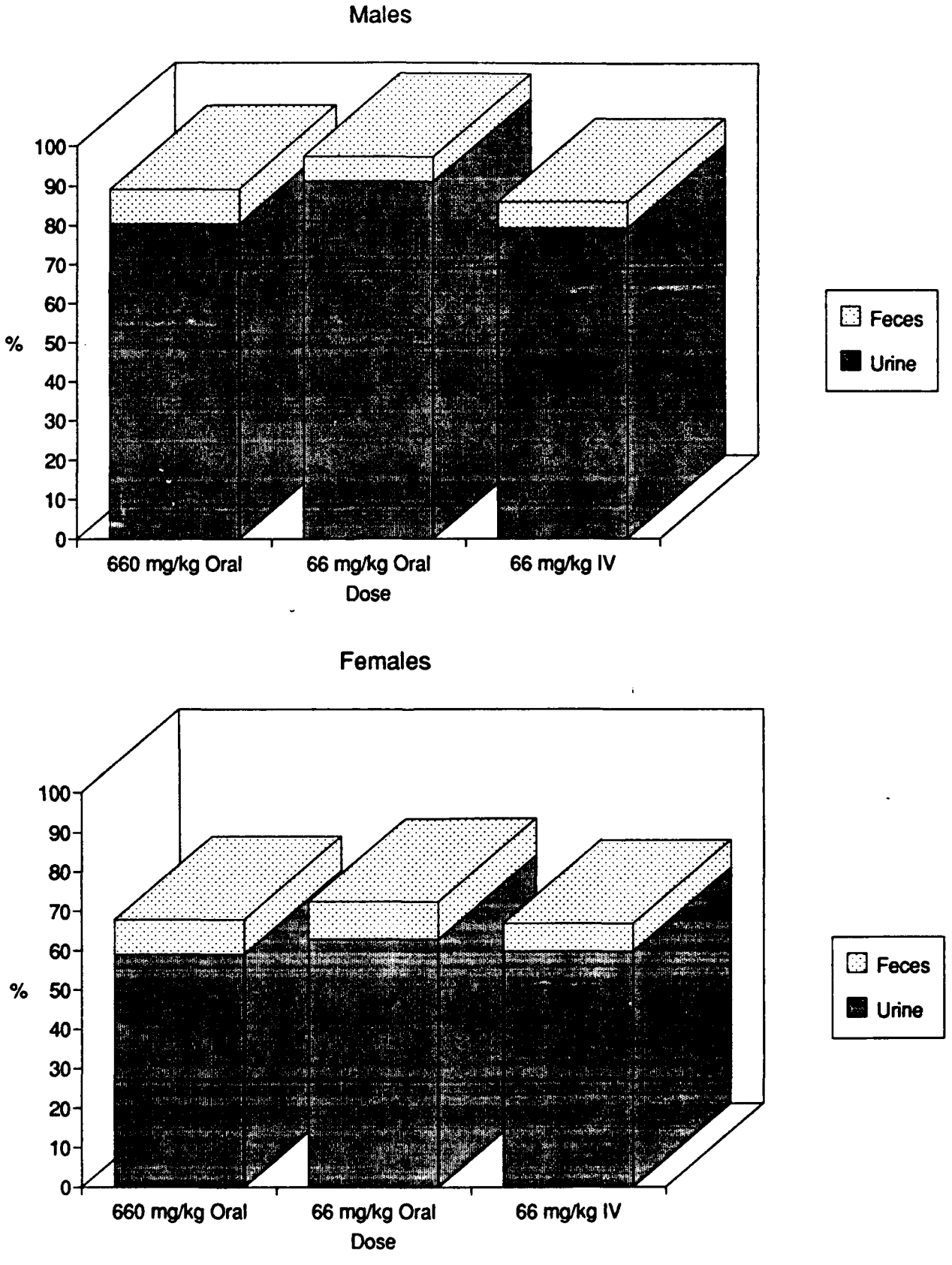
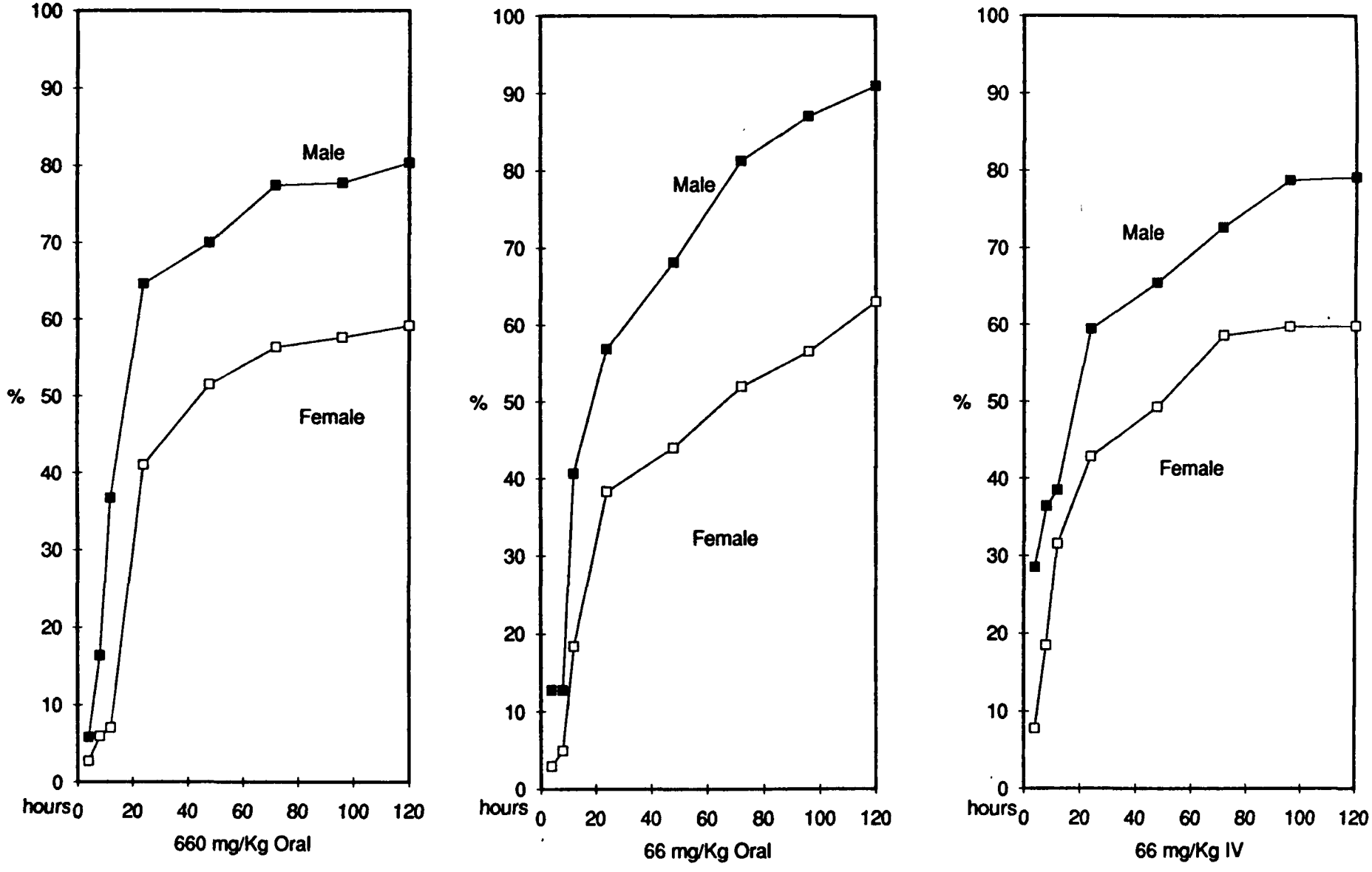


Table C 3.1.2b Rat Cumulative ^{14}C Excretion in Urine
(Percent of Administered Dose)

Hours Post-Treatment		4	8	12	24	48	72	96	120
660 mg/kg Oral	Male	5.8	16.3	36.7	64.6	69.9	77.4	77.7	80.3
	Female	2.7	5.9	7.0	41.0	51.5	56.3	57.6	59.1
66 mg/kg Oral	Male	12.7	12.7	40.6	56.8	68.1	81.3	87.1	91.0
	Female	2.9	4.9	18.3	38.3	44.0	52.0	56.6	63.0
66 mg/kg IV	Male	28.5	36.4	38.4	59.4	65.4	72.6	78.7	79.1
	Female	7.7	18.4	31.5	42.8	49.3	58.6	59.7	59.8

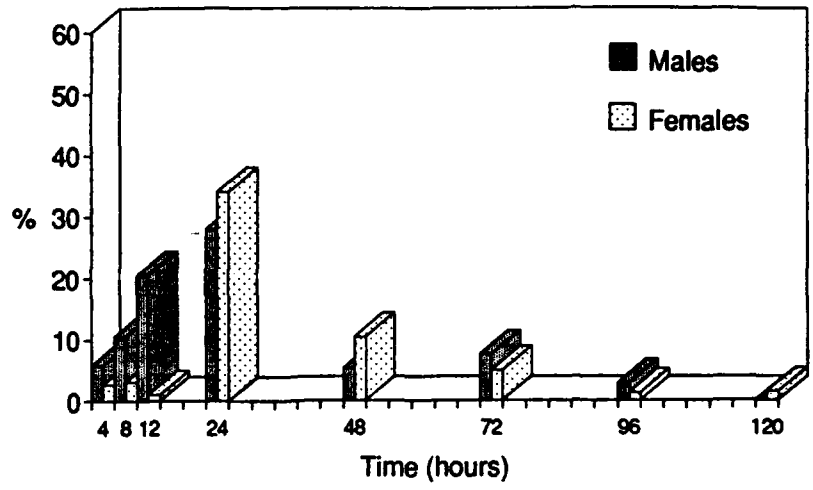
Figure C 3.1.2b Rat Cumulative ^{14}C Excretion in Urine
(Percent of Administered Dose)



Figures C 3.1.2 c-e Rat Fractional Excretion of ^{14}C - Urine
(Percent of Administered Dose)

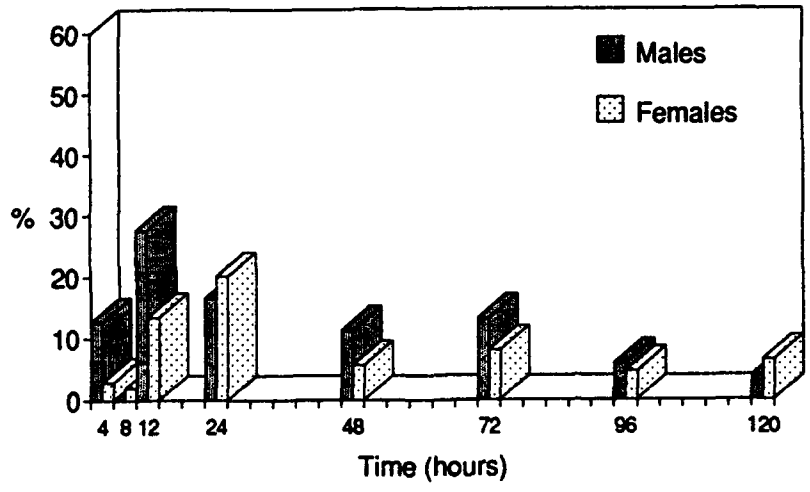
660 mg/kg Oral

c.



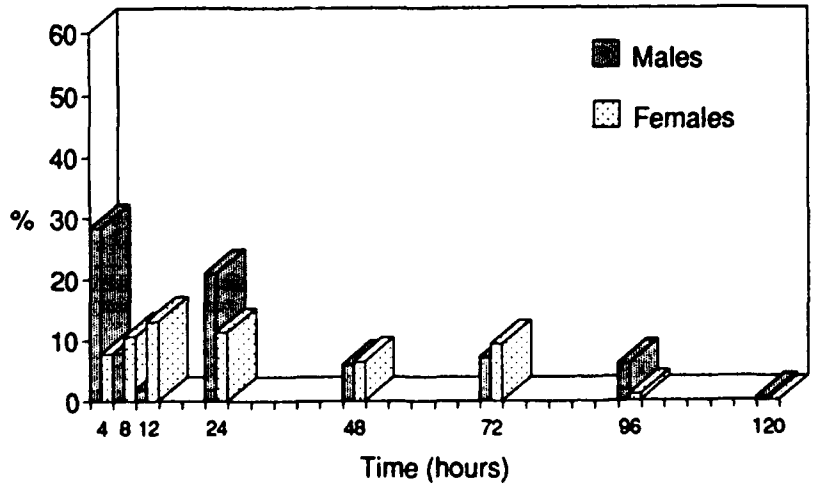
66 mg/kg Oral

d.



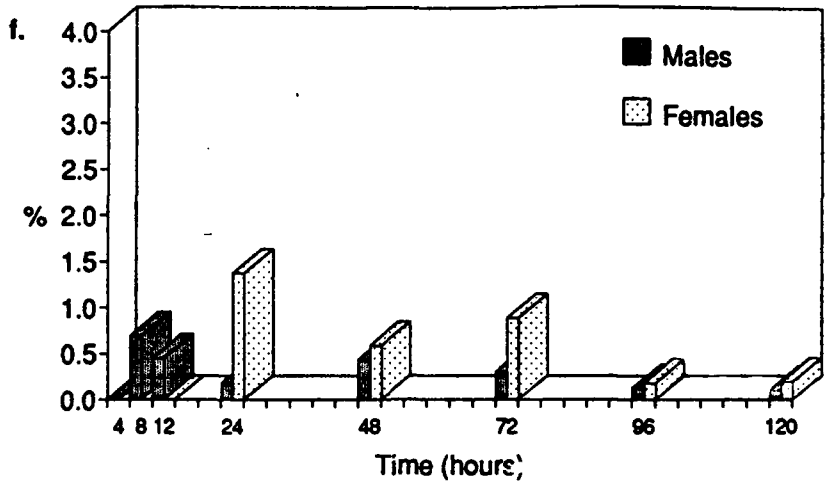
66 mg/kg IV

e.

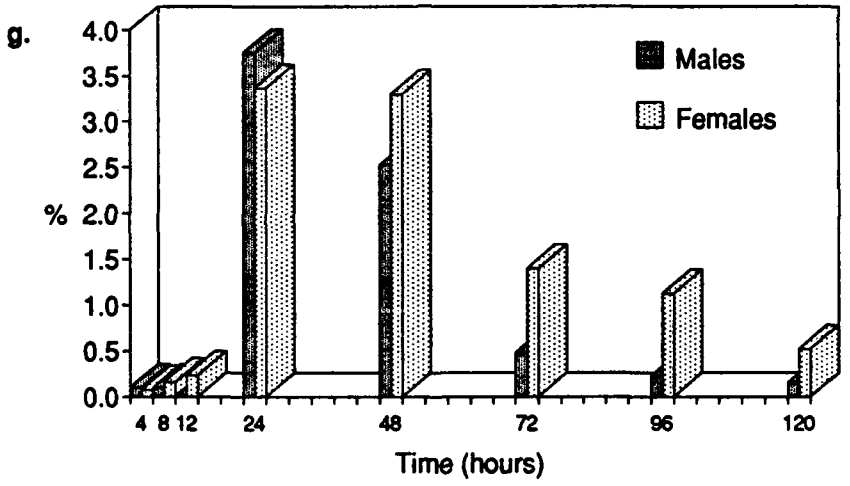


Figures C 3.1.2 f-h Rat Fractional Excretion of ^{14}C - Feces
(Percent of Administered Dose)

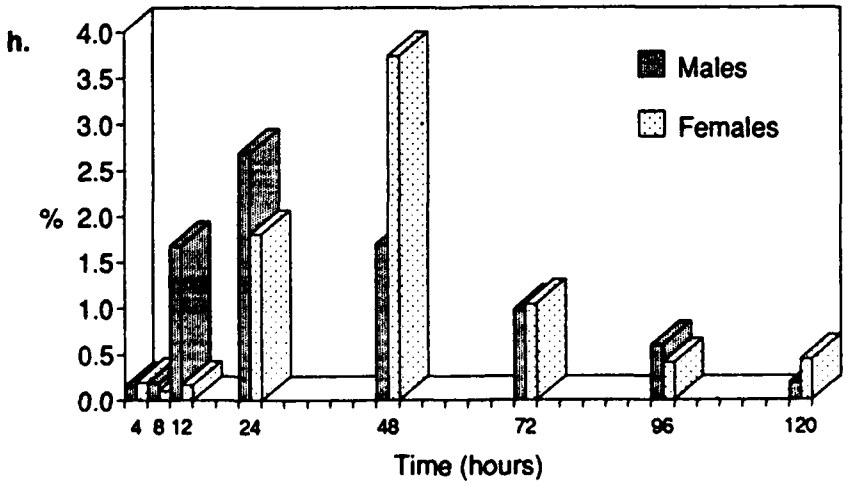
270 mg/kg Oral



66 mg/kg Oral



66 mg/kg IV



Excretion of ^{14}C by female rats lagged that of males in both rate and total at all time points. Elimination of radioactivity was rapid for 24-48 hours, then slowed. In females of the high oral dose group there was a notable 12-24 hour delay in excretion of radioactivity.

Radioactivity in feces (Table C 3.1.2a) was of similar magnitude and rate of appearance with peak at 24-48 hours irrespective of dose or route of administration (Appendix 12).

In males, 86-97% of administered radioactivity was recovered by 120 hours, whereas only 67-72% was accounted for in females (Table C 3.1.2a).

3.1.3 ^{14}C-DIMP and Metabolites in Urine and Feces

Fractions associated with ^{14}C radioactivity were resolved by isocratic reverse-phase high performance liquid chromatography. The urine from rats contained three radioactive components, as listed in Table C 3.1.3.

Table C 3.1.3 Rat Percent ^{14}C Radioactivity Associated with Different Peaks Resolved by Isocratic Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) of 0-48 Hour Urine

Dose	Dose Route	DIMP	Metab1*	Metab2
660 mg/kg Oral	Male	1.6	98.2	0.2
660 mg/kg Oral	Female	0.9	99.1	ND
66 mg/kg Oral	Male	ND	99.6	0.4
66 mg/kg Oral	Female	ND	98.8	1.2
66 mg/kg IV	Male	<0.1	99.9	ND
66 mg/kg IV	Female	1.0	97.8	1.2

* IMPA (isopropyl methylphosphonic acid)
ND = not detectable

The primary component designated "Metab 1" contained 97-100% of the radioactivity and had a retention time of 2.5 minutes. It was identified by nuclear magnetic resonance spectroscopy to be **isopropyl methylphosphonate (IMPA)**. The other two components had retention times of 4 and 11 minutes. The retention time of ^{14}C-DIMP under identical conditions was 11 minutes (Appendix 2). Thus virtually all of

the administered DIMP was being excreted in urine as the primary metabolite, IMPA, with traces of a second, unidentified product. All radioactivity in feces was associated with IMPA; there was no DIMP in rat feces.

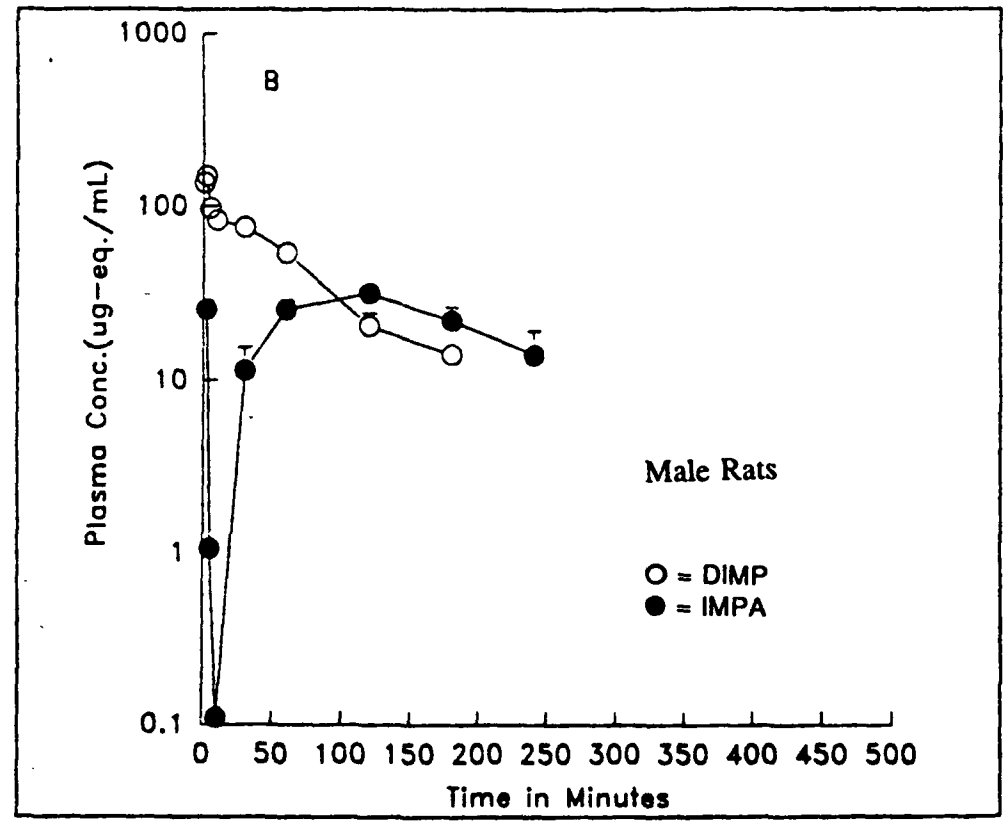
Animals given either sized dose, by either route, produced the same result, except less parent compound appeared in urine after the low oral dose.

When subsamples of the 0-48 hour urine specimens were first treated with either beta-glucuronidase or limpet sulfatase before analysis, there was no evidence that either the parent compound or the radioactive metabolites had been conjugated (Appendix 2).

3.1.4 ^{14}C-DIMP and Metabolites in Plasma

Profiles of the concentrations ^{14}C-DIMP and its primary metabolite (^{14}C-IMPA) are plotted versus time in Figures 3.1.4a-c for intravenous dose, low oral dose, and high oral dose, respectively.

Figure C 3.1.4a Rat 66 mg/kg IV DIMP and IMPA in Plasma



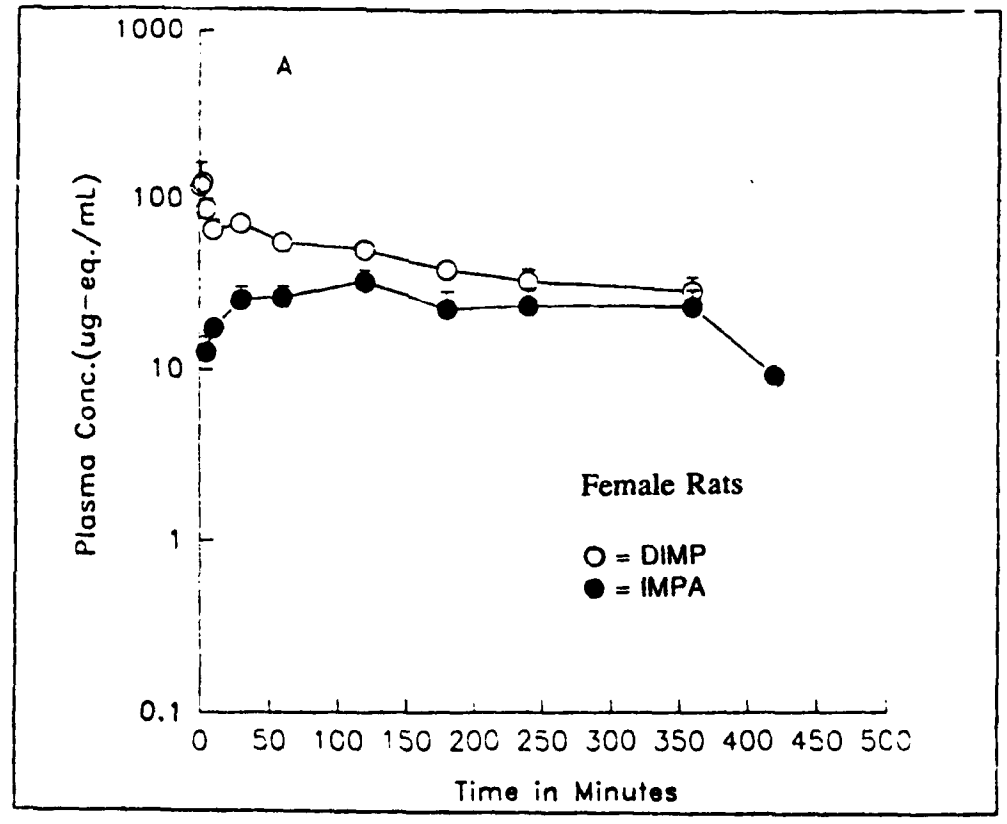
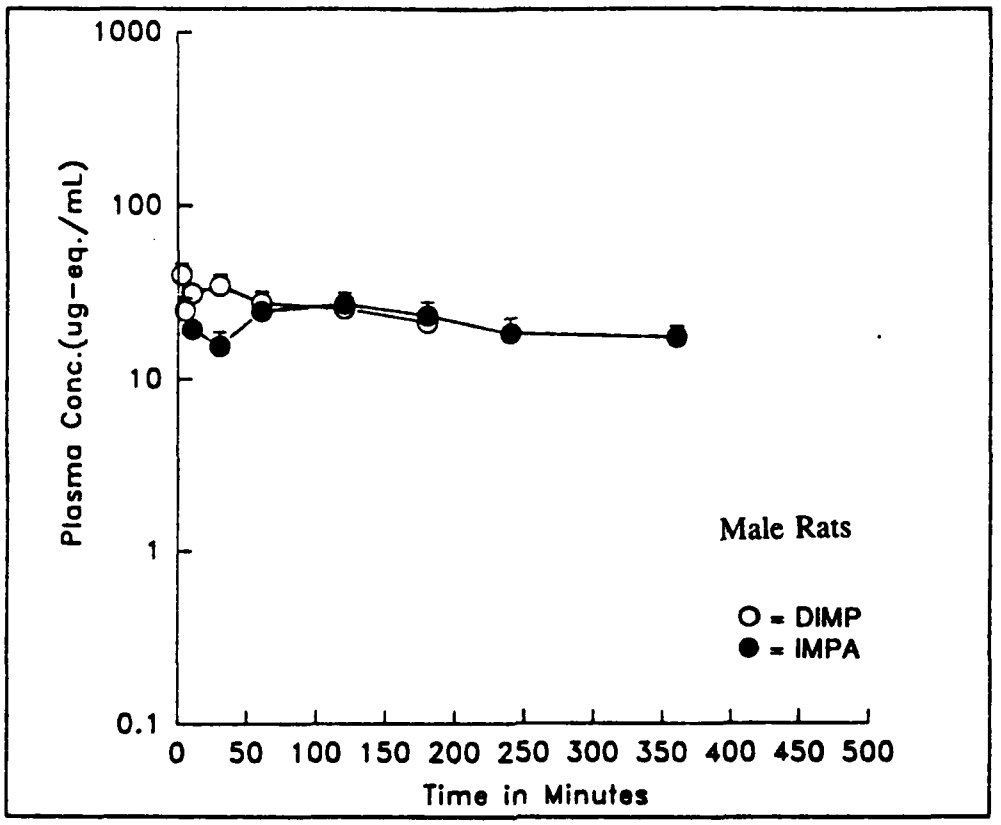


Figure C 3.1.4b Rat 66 mg/kg Oral DIMP and IMPA in Plasma



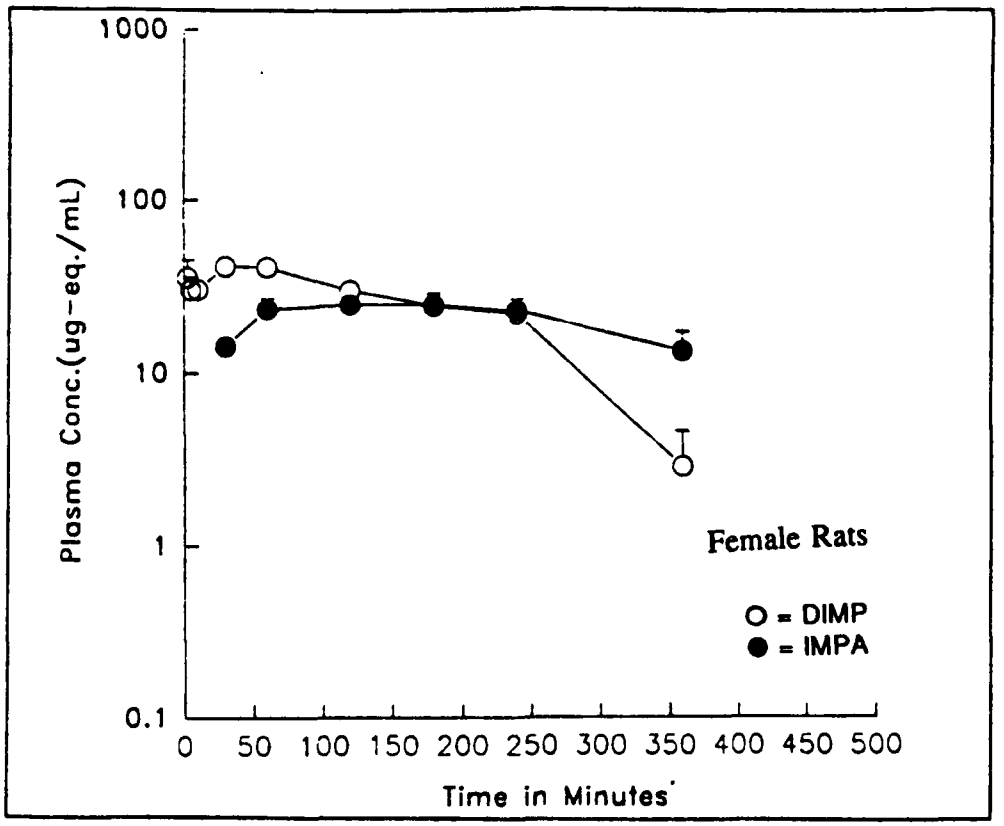
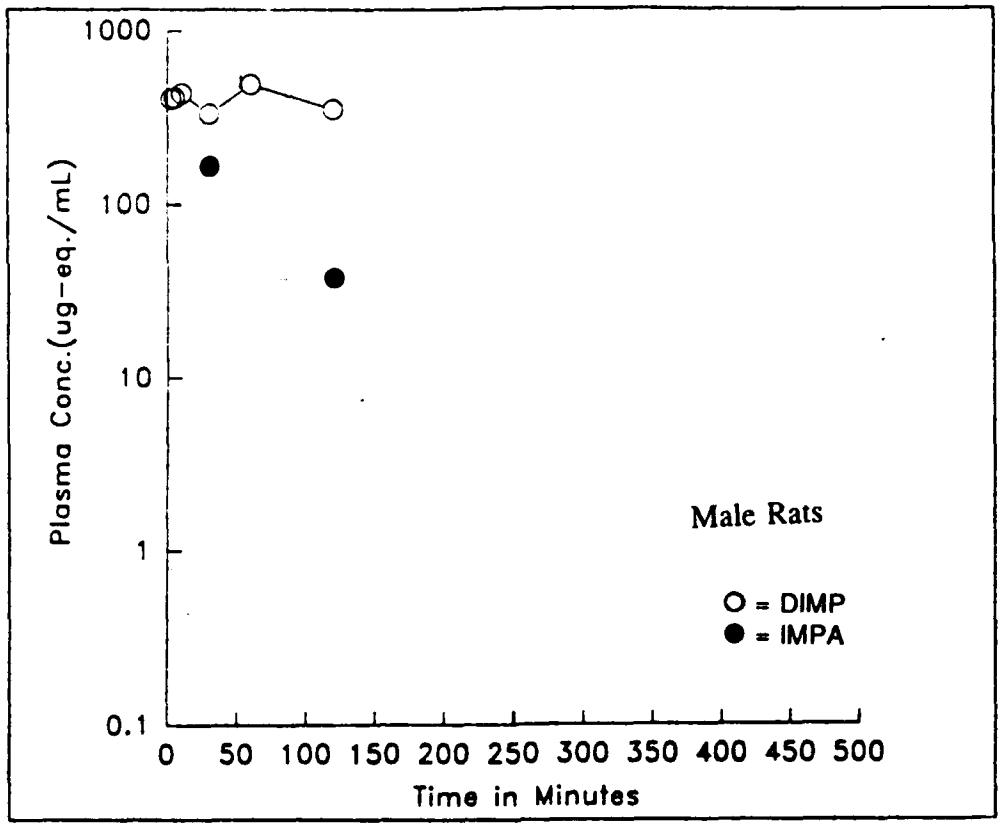
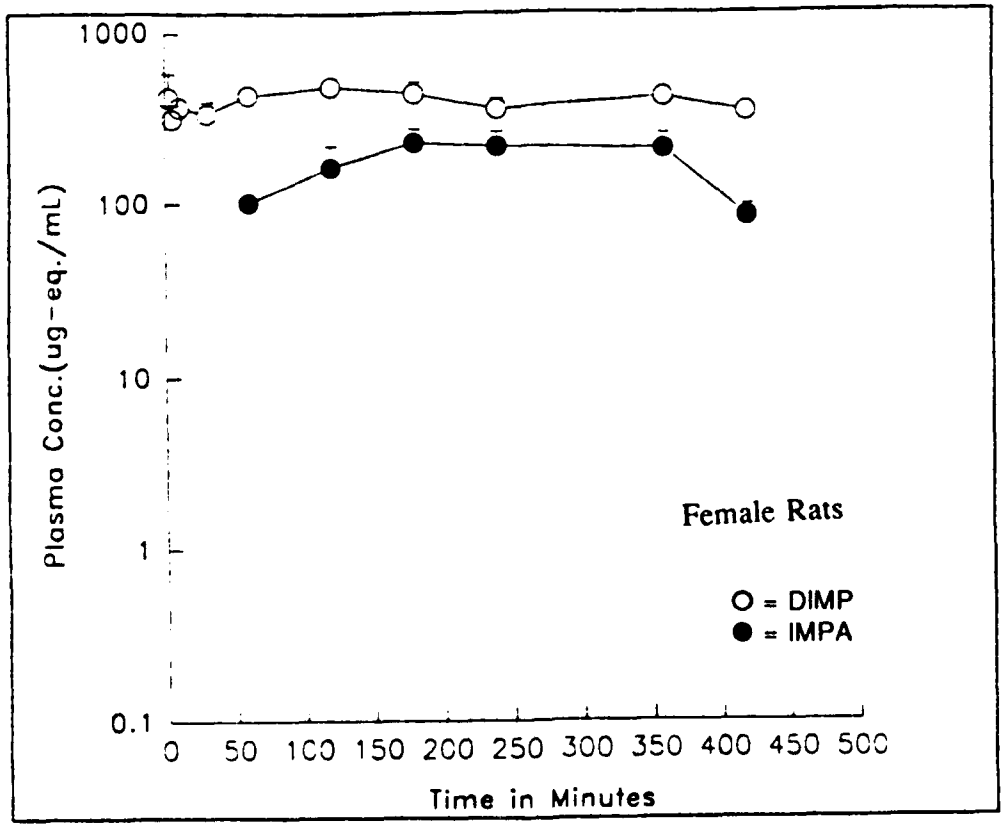


Figure C 3.1.4c Rat 660 mg/kg Oral DIMP and IMPA in Plasma





The parent compound (DIMP) was converted to its primary metabolite at somewhat different rates in males versus females after intravenous administration. The apparent plasma elimination half-life of DIMP was approximately 45 minutes in males and up to 250 minutes in females. The major metabolite reached peak concentration in about 120 minutes after intravenous administration of DIMP. The plasma concentration of DIMP was maximum by 30 minutes in both sexes after the low oral dose, indicating rapid absorption of this dose. However, maximum plasma concentrations were delayed to 60-120 minutes after the 660 mg dose, with females slower. By both routes, from the appearance of the profiles and by estimation of the area under the plasma concentration/time curve (AUC), the males appeared to convert DIMP to IMPA more actively than the females did, corroborating the lag in appearance of total radioactivity (IMPA) in the urine of the females.

Further, considering the profile and AUC for the high oral dose, it was clear that retention was not linear with dose, i.e., the high dose apparently saturated the primary pathway for conversion of DIMP to IMPA. The AUCs are compared quantitatively in Table C 3.1.4.

Table C 3.1.4 Rat Comparison of Mean AUC (μg-eq·min/ml)

Dose	Male		Female	
	^{14}C-DIMP	^{14}C-IMPA	^{14}C-DIMP	^{14}C-IMPA
66 mg/kg IV	8744	7216	15721	11999
66 mg/kg Oral	5422	7865	8992	10951
660 mg/kg Oral	NA	NA	486,969	83692

NA - Not Available

The similar values at equal doses (IV versus low dose) suggest that DIMP was well absorbed from the gastrointestinal tract and was efficiently extracted from plasma. The DIMP AUC following 66 mg/kg orally in rats was less than the DIMP AUC for the 66 mg/kg IV dose, yet the oral IMPA AUCs were equivalent with IV IMPA AUCs . This is strong evidence of a first-pass metabolism effect. At the high dose (tenfold greater than the low dose), the DIMP data in females were more than 10x the low dose value, i.e., the conversion pathway to the metabolite was not linear. This was further substantiated by the IMPA data at the high dose, showing that less than 10x was converted.

3.2 Mink - Total Radioactivity in Blood (N=8/sex), Urine and Feces (N=4/sex)

The radioactivity detected in blood, urine and feces from each animal per collection is tabulated in Appendix 12.

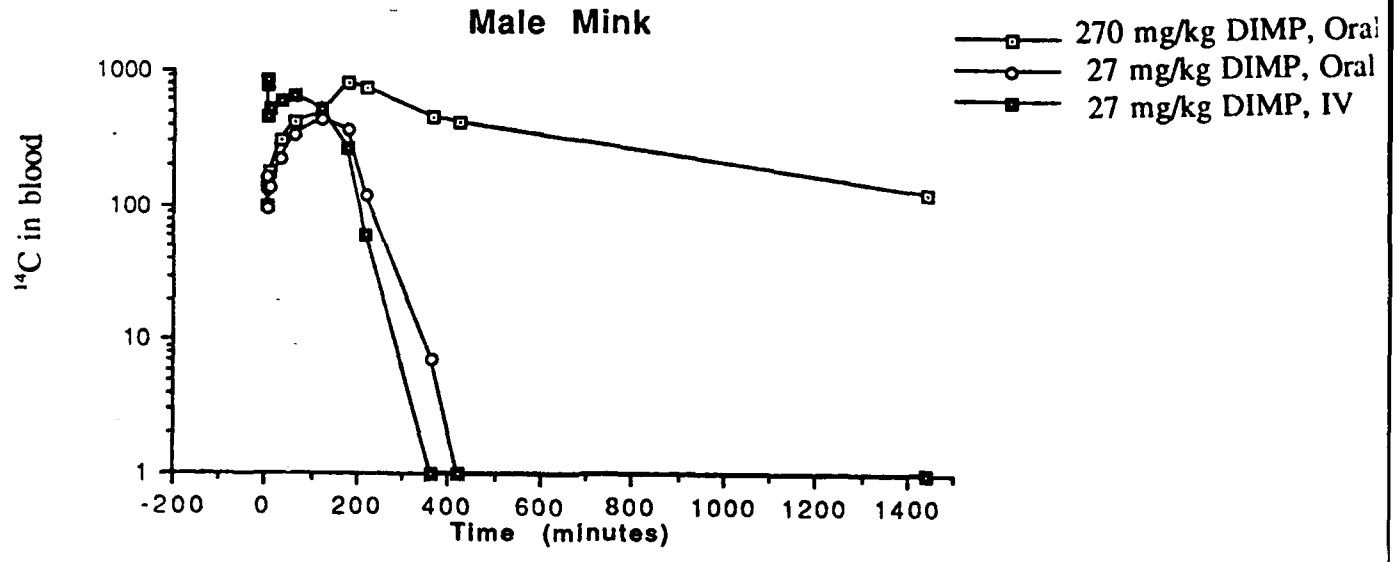
3.2.1 Total Radioactivity in Whole Blood

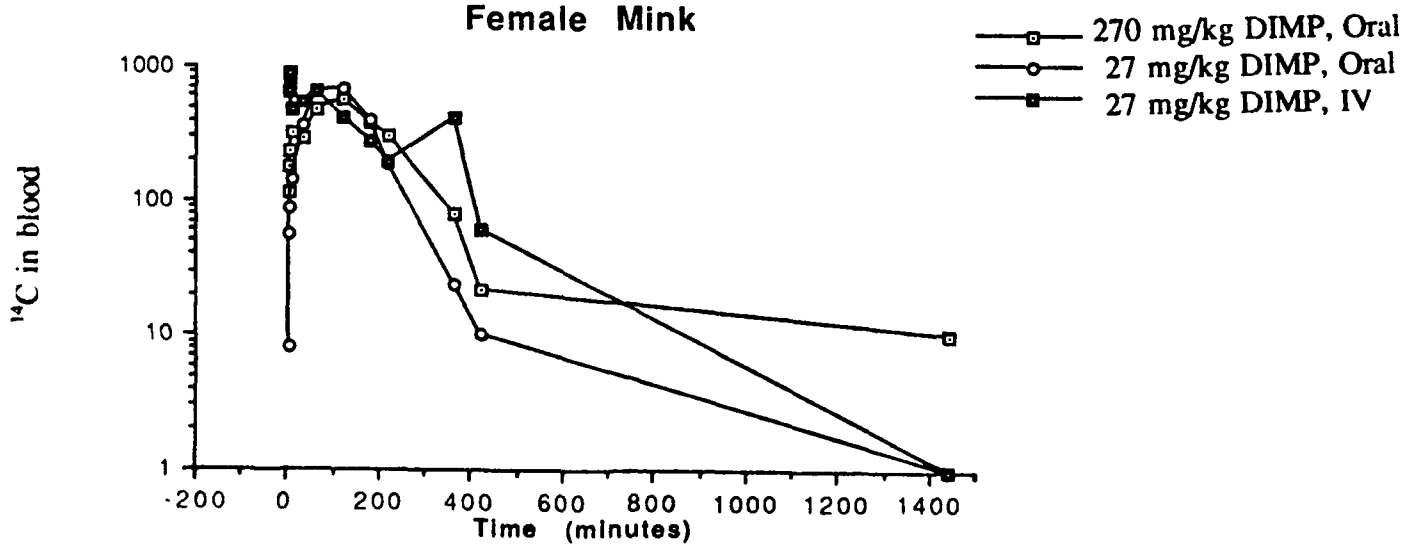
Group mean counts detected during the first 24 hours are summarized in Table C 3.2.1 and plotted in Figure C 3.2.1.

Table C 3.2.1 Mink ^{14}C Radioactivity in Whole Blood
(cpm per 10µL/40µCi/kg BW; N = 8)

Dose Level	Sex		1 min	2.5 min	5 min	10 min	30 min	1 hr	2 hr	3 hr	4 hr	6 hr	7 hr	24 hr
660 Oral	Male	Mean	97.42	142.54	148.21	176.46	298.04	416.50	470.29	812.29	728.88	443.04	414.33	121.00
		SD	130.83	149.14	133.33	98.74	102.61	193.37	358.59	753.59	370.99	255.67	485.03	150.41
66 Oral	Male	Mean	129.33	158.29	96.62	134.81	215.62	329.52	430.38	353.38	119.38	7.43	1.24	0.00
		SD	189.74	161.58	127.04	147.29	115.95	123.91	110.38	159.21	54.53	14.36	3.28	0.00
66 IV	Male	Mean	773.00	829.75	458.00	505.50	577.33	653.83	503.17	262.17	60.83	0.00	0.00	0.00
		SD	164.26	111.37	311.06	119.37	106.41	58.48	207.12	94.96	49.52	0.00	0.00	0.00
660 Oral	Female	Mean	111.08	177.83	228.42	307.92	287.27	468.54	554.25	372.44	302.85	80.83	22.25	10.54
		SD	136.89	142.28	141.02	181.33	77.48	115.09	161.39	146.07	154.83	88.44	35.63	23.31
66 Oral	Female	Mean	8.00	55.67	86.83	140.21	363.08	644.88	880.08	396.98	181.08	24.04	10.63	0.00
		SD	11.08	65.12	55.48	91.22	196.42	234.43	104.68	127.33	76.19	27.09	13.29	0.00
66 IV	Female	Mean	861.53	696.47	636.53	476.27	541.60	645.11	403.78	280.67	190.44	412.00	60.67	0.00
		SD	431.36	202.56	141.66	78.18	187.28	198.20	37.69	237.11	325.83	713.60	105.08	0.00

Figure C 3.2.1a Mink Radioactivity in Whole Blood
(cpm/μL/40μCi)





After oral administration, absorption of ^{14}C-DIMP was rapid, with peak blood values obtained in two hours (except high dose males peaked at four hours). Elimination from blood was also prompt, for all doses and all routes. Again, however, the high dose oral group lagged, males slightly more than females. The low oral dose and IV males had no detectable radioactivity in blood after seven hours; and in all female groups, there were 60 or fewer cpm at seven hours. The low doses reached zero at some undetermined point before 24 hours. The high dose males and females had detectable but low levels remaining at 24 hours, suggesting altered kinetics at this dose level.

The curves suggest a two-component model (at least); the faster component, for the low doses, had a half-time in the neighborhood of one hour, for both sexes. The half-time of the slower component was approximately 15 hours for the females treated orally with the high dose, and was 9-15 hours in the oral high dose males. The elimination curve cannot be determined with better accuracy since a change in elimination rate was probable during the period 7-24 hours after dosing, when samples were not obtained.

3.2.2 Total Radioactivity in Urine and Feces (N=4/sex)

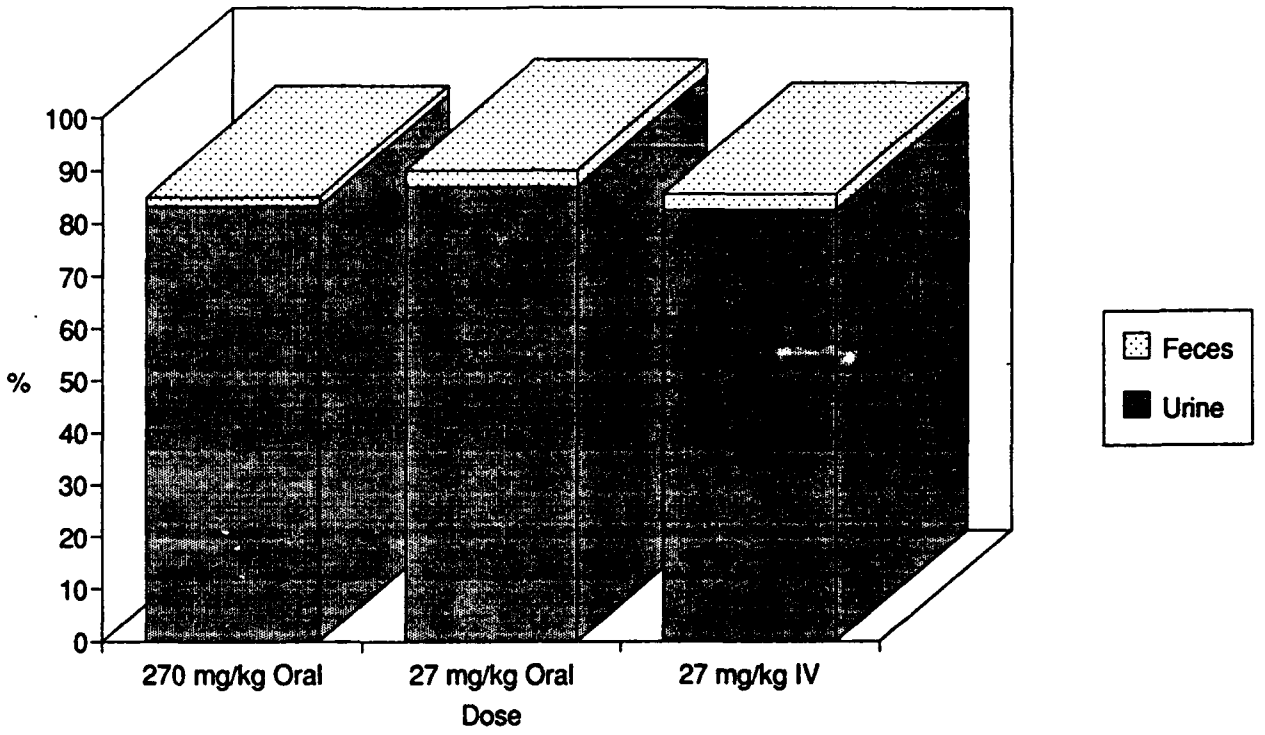
The recovery of total radioactivity in urine and feces over the 120-hour collection period is listed in Table C 3.2.2a and plotted in Figure C 3.2.2a and b; the cumulative excretion in urine is shown in Table C 3.2.2c, and plotted in Figure C 3.2.2c. The fractional excretion over time in urine and feces is plotted in Figures C 3.2.2c-e, and C 3.2.2f-h, respectively.

Table C 3.2.2a Mink 120-Hour Total Recovery of ^{14}C
(Percent of Administered Dose)

Dose	Sex	Urine	Feces	Total
270 mg/kg Oral	Male	83.3	1.7	85.0
	Female	83.6	2.5	86.1
27 mg/kg Oral	Male	86.9	3.1	90.0
	Female	91.5	3.7	95.2
27 mg/kg IV	Male	82.5	3.1	85.6
	Female	69.6	2.9	72.5

Figure C 3.2.2a Mink 120-Hour Total Recovery of ^{14}C
(Percent of Administered Dose)

Males



Females

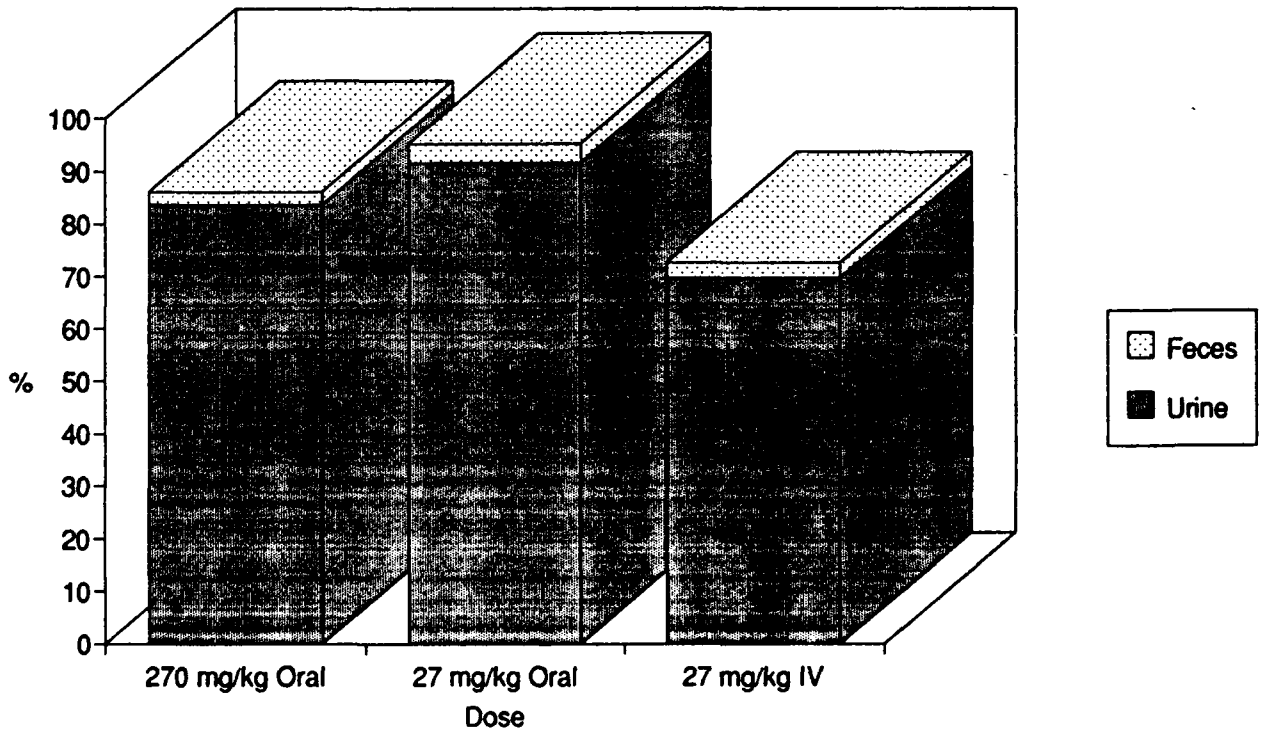


Figure C 3.2.2b Mink Cumulative ^{14}C Excretion in Urine
(Percent of Administered Dose)

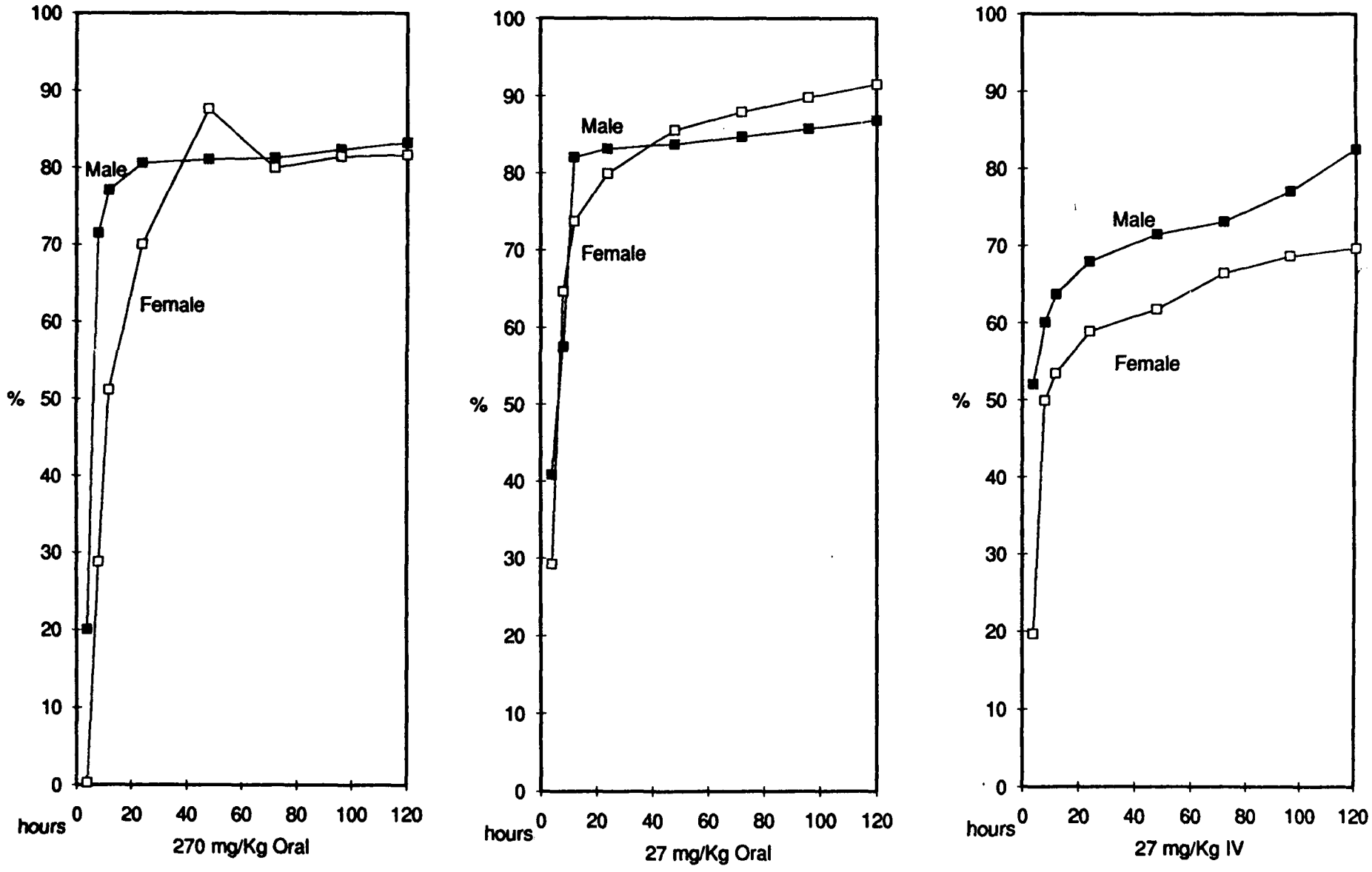
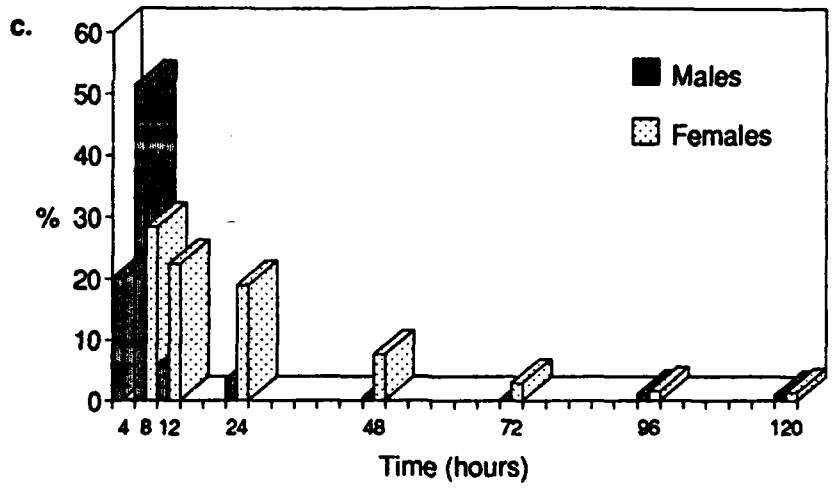


Table C 3.2.2c Mink Cumulative ^{14}C Excretion in Urine
(Percent of Administered Dose)

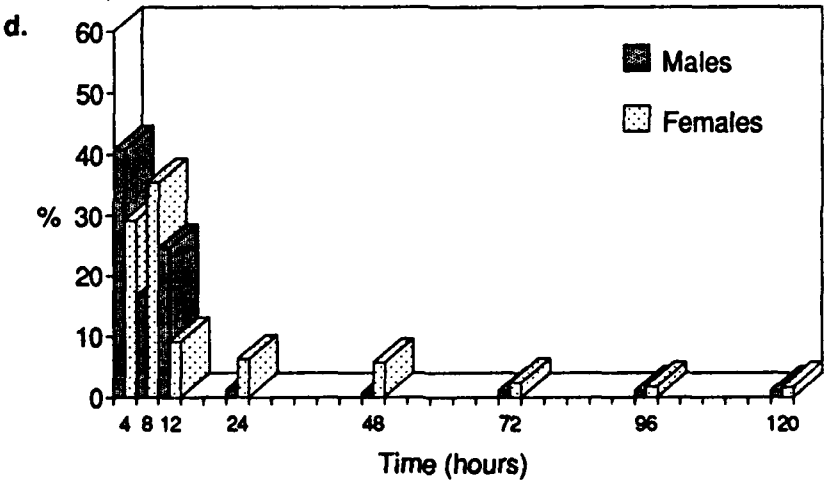
Hours Post-Treatment		4	8	12	24	48	72	96	120
270 mg/kg Oral	Male	20.0	71.4	77.0	80.5	81.0	81.2	82.3	83.2
	Female	0.3	28.7	51.1	70.0	87.6	79.9	81.4	81.6
27 mg/kg Oral	Male	40.8	57.4	82.0	83.1	83.7	84.7	85.7	86.8
	Female	29.2	64.6	73.7	79.9	85.5	87.9	89.8	91.5
27 mg/kg IV	Male	52.0	60.0	63.7	67.9	71.4	73.1	77.0	82.5
	Female	19.6	49.9	53.4	58.9	61.7	66.4	68.6	69.6

Figures C 3.2.2 c-e Mink 120-Hour Fractional Recovery of ^{14}C - Urine
(Percent of Administered Dose)

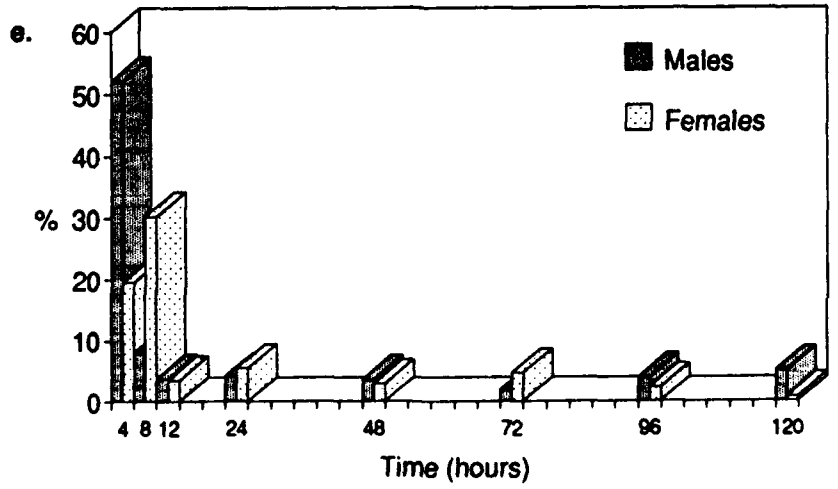
270 mg/kg Oral



27 mg/kg Oral

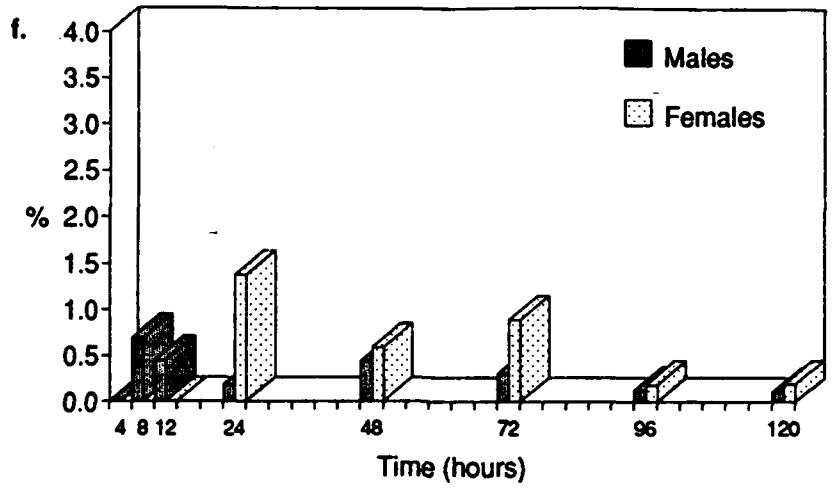


27 mg/kg IV

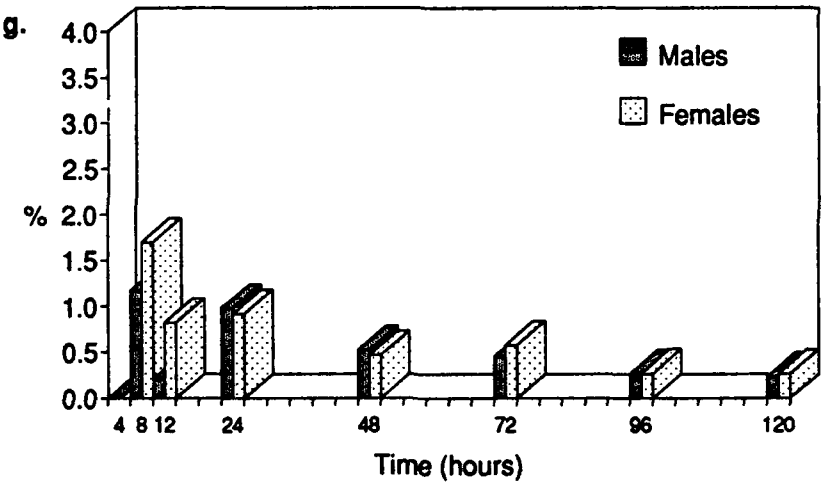


Figures C 3.2.2 f-h Mink 120-Hour Fractional Recovery of ^{14}C - Feces
(Percent of Administered Dose)

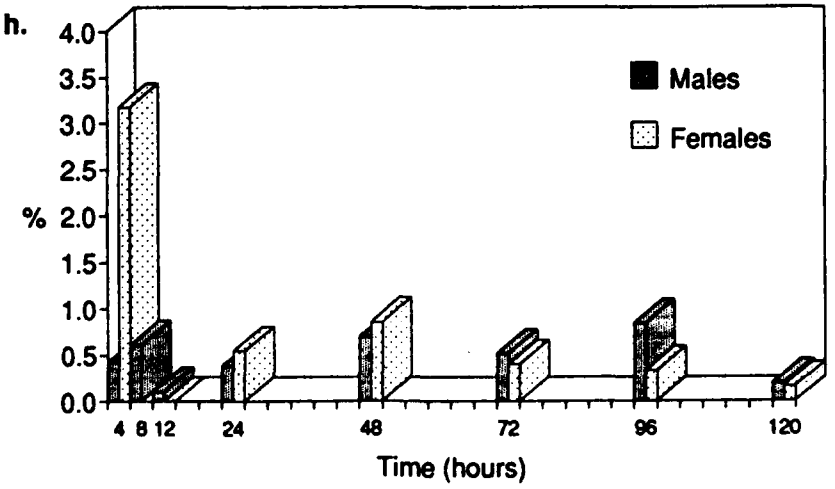
270 mg/kg Oral



27 mg/kg Oral



27 mg/kg IV



The concentration of DIMP in urine peaked at four to eight hours after administration in all routes and doses. The females appeared to lag slightly, especially after intravenous administration. Recovery of radioactivity in urine amounted to 70-91% of that administered. Output in feces brought the total to 72-95%. We have no measure of possible cross-contamination between urine and feces, although our results are consistent with those of Hart (1976) in other species. The patterns and general magnitude of the ^{14}C recovery are valid, but there is reasonable certainty that small amounts of radioactivity were not recovered from the mink cages (or mink fur). Thus the recovery reported here is probably conservative.

3.2.3 ^{14}C-DIMP and Metabolites in Urine and Feces (N=4/sex)

Reverse-phase HPLC revealed only DIMP and **one metabolite, IMPA,** in all samples of urine and feces; DIMP was found in very small concentration and only in the urine of the high-dose group (Table C 3.2.3). All radioactivity in feces was associated with IMPA; there was no DIMP in mink feces.

Table C 3.2.3 Mink Percent ^{14}C Radioactivity Associated with Different Peaks Resolved by Isocratic Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) of 0-48 Hour Urine

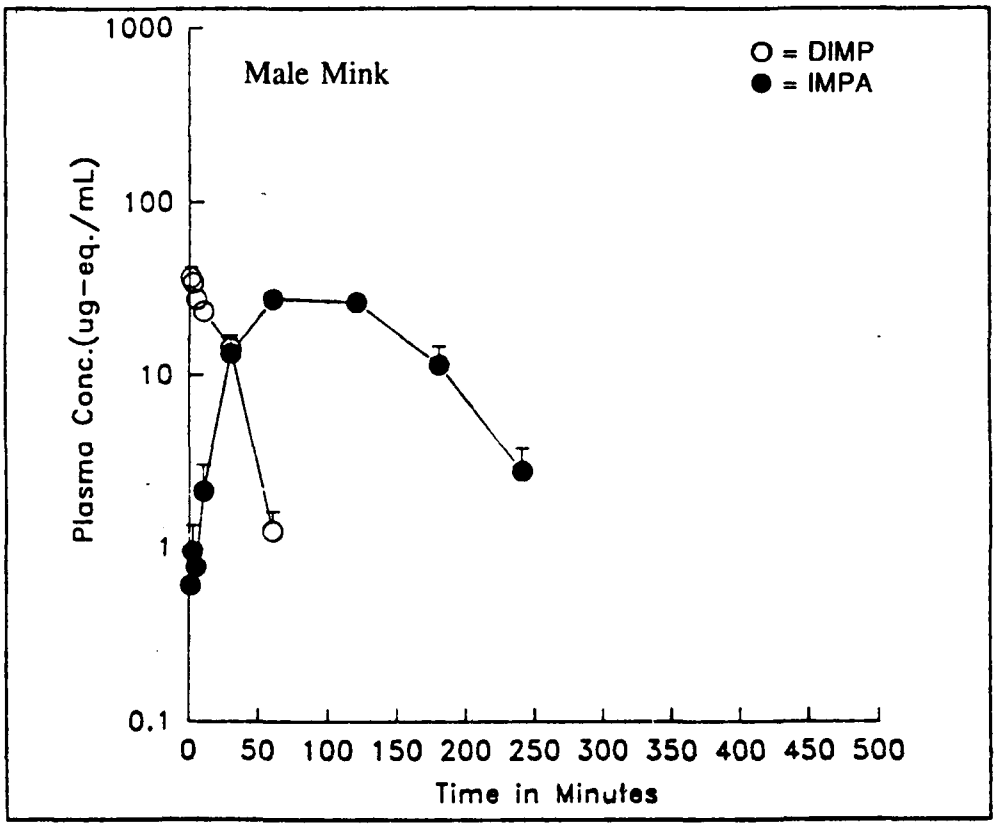
Dose	Dose Route	DIMP	METAB1*	METAB2
270 mg/kg Oral	Male	1.6	98.5	ND
270 mg/kg Oral	Female	0.04	99.9	ND
27 mg/kg Oral	Male	0.1	99.9	ND
27 mg/kg Oral	Female	ND	100	ND
27 mg/kg IV	Male	ND	100	ND
27 mg/kg IV	Female	ND	100	ND

* IMPA (isopropyl methylphosphonic acid)
ND = not detectable

3.2.4 ^{14}C-DIMP and Metabolites in Plasma

Profiles of the concentrations of ^{14}C-DIMP and its only metabolite ^{14}C-IMPA, in the mink are plotted in Figures C 3.2.4a-c for IV, low oral and high oral doses, respectively.

Figure C 3.2.4a Mink 27 mg/kg IV DIMP and IMPA in Plasma



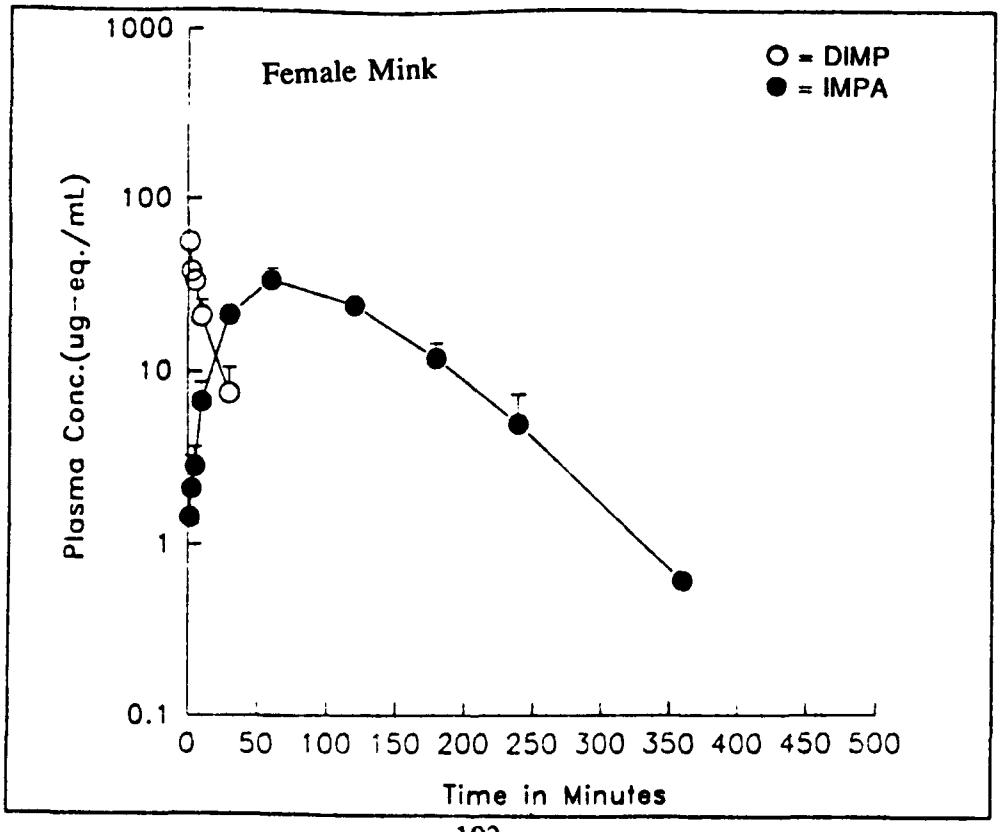
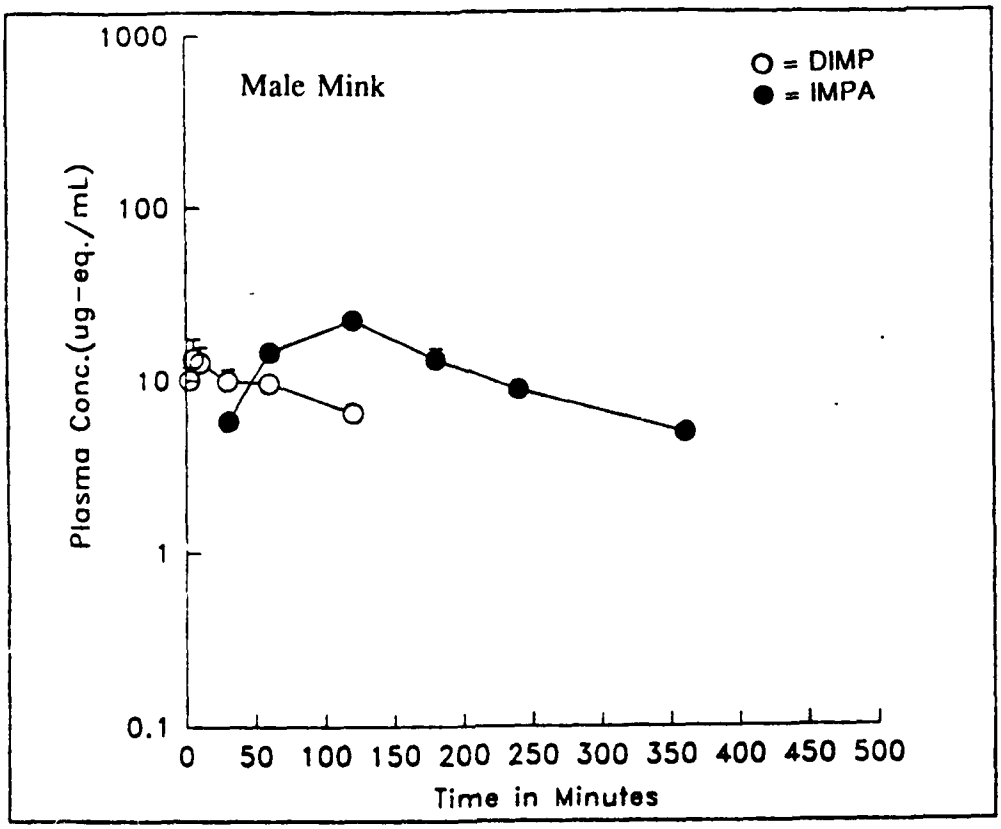


Figure C 3.2.4b Mink 27 mg/kg Oral DIMP and IMPA in Plasma



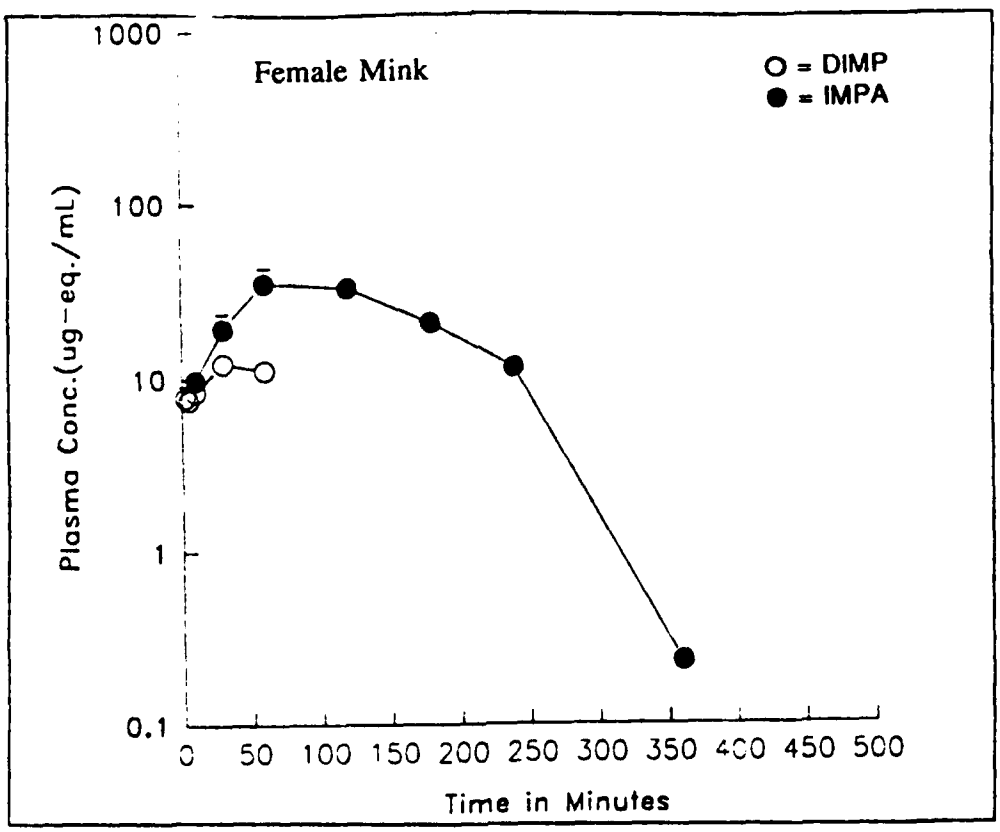
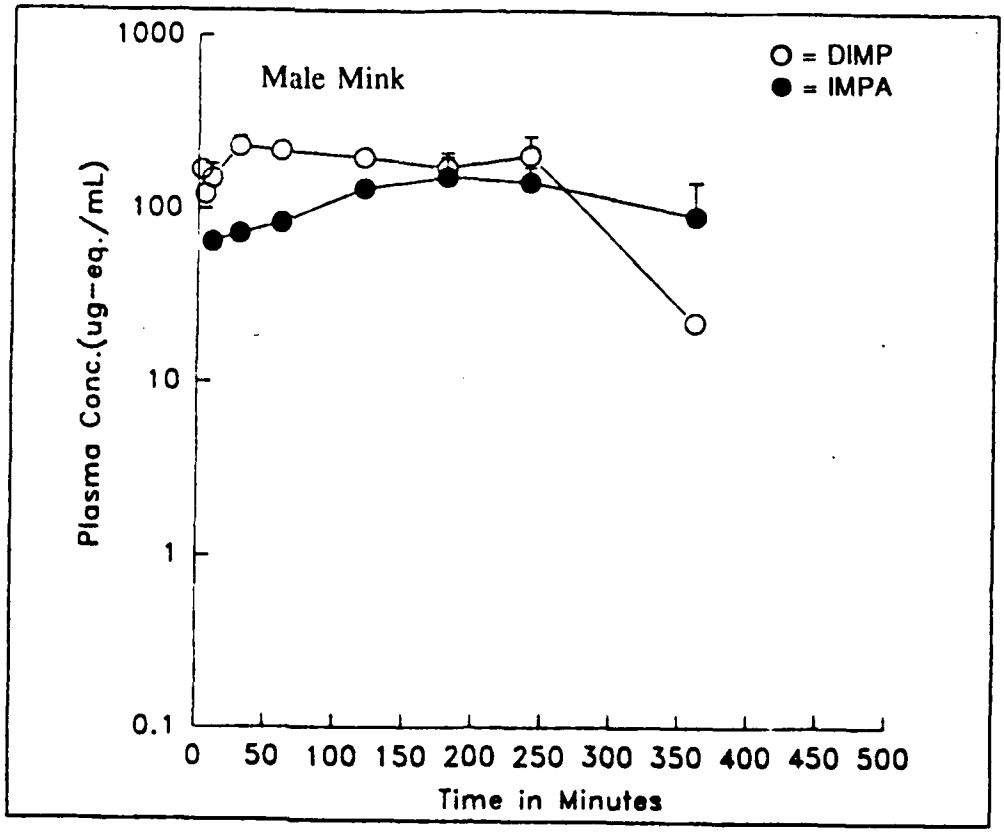
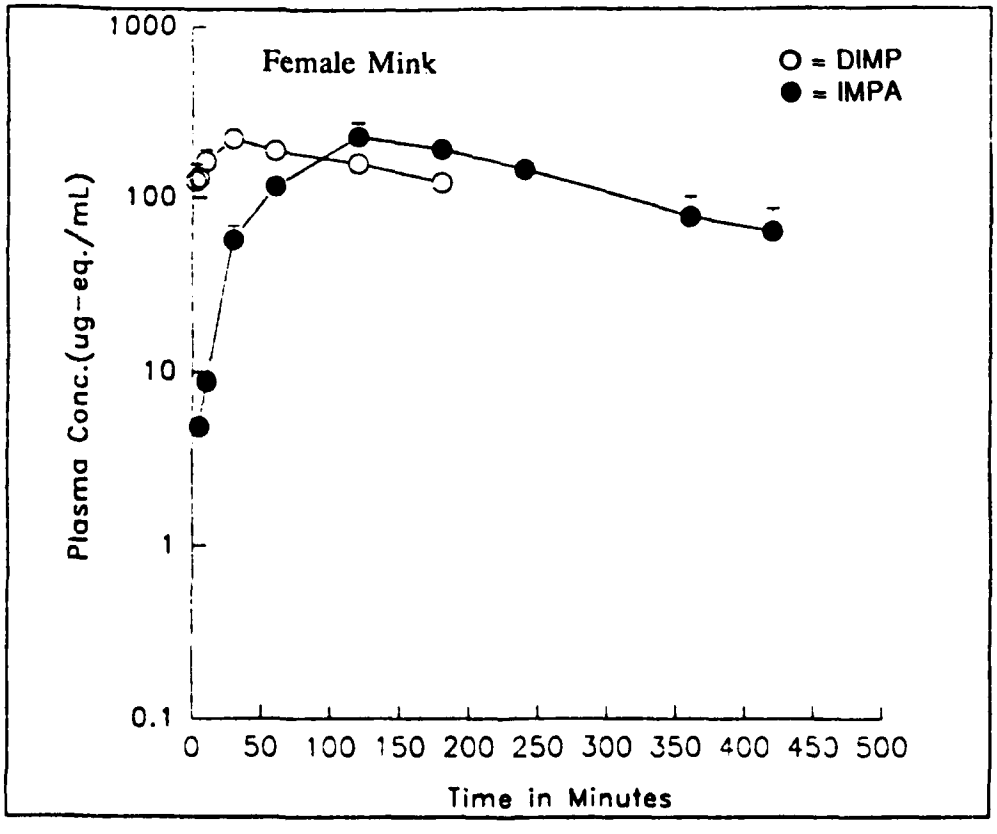


Figure C 5.2.4c Mink 270 mg/kg Oral DIMP and IMPA in Plasma





After intravenous administration in both sexes, conversion of DIMP to IMPA was very rapid. The apparent plasma half-life of DIMP was 12-14 minutes, with the metabolite achieving peak concentration by 60 minutes in both sexes. Following oral administration in both doses, the DIMP concentration peaked in plasma at 30 minutes in both sexes and the metabolite peaked between 60-180 minutes, with males somewhat slower than females. In male mink, conversion to the metabolite was consistently slower, with more parent compound and less metabolite at each dose and time.

The profiles of the elimination curves, and the estimates of the AUC suggest non-linear retention of the higher dose, i.e., saturation kinetics, as in the rat.

Table C 3.2.4 contains a comparison of the quantified AUC data between DIMP and IMPA in the different dose groups. Differences between male and female, while marked in rats, were less in mink.

Table C 3.2.4 Mink Comparison of Mean AUC (µg-eq·min/ml)

Dose	Male		Female	
	^{14}C-DIMP	^{14}C-IMPA	^{14}C-DIMP	^{14}C-IMPA
27 mg/kg IV	911	4107	752	4814
27 mg/kg Oral	1102	4917	622	6470
270 mg/kg Oral	62301	72940	34040	68683

In mink as in rats, comparable AUC values after similar doses by IV and oral routes indicated that DIMP was well absorbed from the gastrointestinal tract and was extracted efficiently from plasma. The oral doses differed by a factor of ten, but the animals receiving the high dose retained DIMP in plasma for greater than tenfold increase above the low dose concentration, thus the conversion pathway to IMPA was apparently saturated. The corollary was also evident: the ratio of IMPA between the low and high dose was less than ten, corroborating the non-linear conversion at the higher dose. There was no apparent first-pass metabolism effect after the low oral dose in the mink.

4.0 DISCUSSION

4.1 Variances from Protocol

We were unable to find any reports of previous use of mink in metabolic/pharmacokinetic studies, nor did we find a satisfactory metabolism cage available commercially. An adaptation of the cages used for the 90-day study was improvised by equipping them with a metal screen and funnel that were suspended beneath the cage floor. A brief pilot study was conducted to test the dose volumes, route

and timing of dose administration, and ability of the cages to collect urine and feces separately. We had anticipated that control of urine from the mink might be uncertain; this was the reason the IV-dosed mink were given 20 μCi/kg rather than 40 μCi to minimize potential loss of highly radioactive urine during the initial period after dosing. We had reasoned that this precaution would not be necessary in the rats since their commercial metabolism cages were efficient. The outcome of the pilot study suggested that the protocol could be carried out as written. A few more days' experience with the actual study revealed that unacceptable losses of urine and sometimes feces occurred during bleeding of the animal, and that hemostasis could not always be achieved promptly enough to enable all blood samples to be collected on schedule, when mink were bled from the toe nail. The protocol was subsequently amended to incorporate corrective measures as described in Section C 2.2.1 and C 2.3.1 of this report.

4.1.1 Changes in Procedures to Obtain Blood, Urine and Fecal Samples

As described in Sections C 2.2.1 and 2.3.1, to preclude loss of radioactive urine and feces, replacement groups of four mink of each sex were given the respective treatments and were left undisturbed in their cages, from which urine and fecal samples were collected. These animals were not used for blood samples; the original sets of eight were. The replacement animals were from a group of 20 animals being held in reserve, fully acclimated to the laboratory conditions from the start of the 90-day study.

This change had the effect of reducing the scheduled eight animals per group to four for the data on radioactivity in urine and feces. Four animals per group is the minimum recommended by EPA.

To facilitate collection of blood from the sets of eight mink, we employed judicious application of a device designed for euthanasia of mink by cervical dislocation. Described in Section C 2.3.1, this device permitted the handler positive control of the animal head with minimum force and allowed the phlebotomist quick and efficient access to the jugular veins. The overlying fur was clipped beforehand. These samples were obtained easily, seemingly with minimal stress to the animals.

The rats required more restraint than we preferred to use during orbital bleeding. We adopted a rapid-acting inhalant anesthetic (isoflurane) to anesthetize them in their metabolism cages, then removed them to an absorbent-covered pad for orbital bleeding while they were still anesthetized. They recovered fully in 1-2 minutes after the inhalant cone was removed. Use of anesthetic introduces a variable: the possible interaction with DIMP metabolism. Our data are similar enough to those reported for male rats by Hart (1976) that serious compromise of our results appears improbable.

We did observe apparent potentiation of the anesthetic duration, however, in both species, in animals given the high dose of DIMP. Mink and rats given the high oral dose remained anesthetized nearly twice as long as those given lower doses. They also showed

a delay in appearance of radioactivity in blood, i.e., in absorption from the gastrointestinal tract. In the 90-day study, the 8000 ppm mink also had prolonged anesthesia during the blood sampling. Jones (1991) described an anesthetic-like state in Mallard ducks produced by high doses of DIMP and demonstrated that an LD_{50} dose produced reductions in systolic/diastolic and mean arterial pressures in the range of 40-50% in each of six male and female ducks. This hypotension developed during the first 30-45 minutes after dosing and was still present at 60 minutes. We speculate that a similar mechanism might account for our observations.

The rats in the metabolic study were anesthetized repeatedly with isoflurane, but they did not experience prolonged effect after the initial dose period. In the 90-day study with dosed feed, the mink had constant exposure to DIMP and were anesthetized with ketamine/xylazine on four occasions. Significantly, there was no shortening of "sleep time" with the repeat anesthetization, as might be expected if DIMP were inducing enzymes that also metabolized the anesthetic. Hart (1976) had reported that rats given DIMP for several days had decreased sleep time after hexane anesthesia, suggesting that DIMP induced biotransformation enzymes that were shared by hexane. We did not see this effect with respect to ketamine/xylazine, suggesting that the prolonged anesthesia on co-exposure with DIMP was probably due to a physiologic response such as hypotension rather than competition for the same deactivating enzyme system.

4.1.2 Collection of Urine and Feces for Five Days

The final major change regarding sample collection was the decision to collect urine and feces for five days instead of the original seven. The change was considered desirable because it would ensure that the metabolic series could be completed in all sets of animals using the same experienced personnel throughout, some of whom would not be available after the beginning of Fall classroom schedule. The seven-day period was to comply with EPA's test rules for metabolism studies, which specifies collection until 95% of the administered radioactivity is accounted for, or seven days, whichever occurs first. While we did not recover 95% within five days for most groups, it was clear that excretion had fallen to a very low rate by 120 hours, and that collection for 48 additional hours would increase the resources required but would not change the conclusion substantially (e.g., See Figures C 3.1.2c-h and C 3.2.2 c-h). The data from Hart's (1976) work in dogs, rats, and mice substantiated our observation. Hart reported that each species excreted 95% of the administered radioactivity in urine during the initial 72 hours. All of his animals were male. Our male rats excreted 75-80% in urine by 72 hours, plus 6 - 8% in feces, depending upon the administered dose of DIMP. Male mink excreted similar total amounts. The tradeoff, complete five-day collections from all animals by experienced personnel, was judged more desirable than documenting the final 48 hours of fractional collections and having to study the final sets of animals with new personnel.

4.2 Interpretation of Results

The data from both species were fitted to three potential pharmacokinetic models using a proprietary program (PC NON-LIN, Version 3.0, 1984. Statistical Consultants Inc., Lexington, KY).

The simplest model, describing first-order conversion of DIMP to its primary metabolite with excretion coefficients for both DIMP and IMPA, provided a good predictor for both DIMP and IMPA IV plasma data for both rat and mink. The estimated elimination rate (ke) for the parent compound, DIMP was slower that the estimated metabolic conversion rate constant rate (km), indicating rapid conversion of DIMP to IMPA. The estimated elimination rate constant (ke) may represent a tissue distribution rate rather than an excretion rate of DIMP since very little DIMP was actually excreted. Although some improvement in the fit of IMPA plasma levels was afforded by a more complex model that included saturable kinetic conversion to IMPA, the improvement may not be statistically supportable because of the increase in the number or iterable variables, and no improvement was noted in the fit of DIMP levels.

The metabolic and kinetic data resulting from administration of ^{14}C-DIMP to rats and mink in this study did demonstrate unequivocally that: 1) DIMP was absorbed rapidly by both species after oral administration; 2) DIMP was metabolized by a saturable pathway or pathways to a single primary metabolite, IMPA, that constituted 90+% of the excreted radioactivity; and 3) excretion was primarily via urine (90+%) and prompt. By 48 hours, mink excrete 60-87% of administered radioactivity as urinary IMPA, male rats 65-75% and female rats 44-50%. By 120 hours, fecal excretion accounted for an additional 6-8% of the administered dose in rats and 2-4% in mink. Percent of total radioactivity recovered from both routes by 120 hours was 72-95% in mink and 67-97% in rats. There was reason to believe some excreted radioactivity was not recovered from mink cages and mink fur, thus the recovery data for mink are considered conservative.

There was an obvious sex difference in rats in both rate and quantity of conversion of DIMP to IMPA and in excretion of IMPA, the female being considerably slower. This study did not elucidate the mechanism(s) for the difference.

Fecal excretion of IMPA after intravenous administration of DIMP was nearly equal to that after oral administration in both species. This suggests a hepatobiliary route of excretion for some of the circulating compound, or possibly direct transmural intestinal elimination.

4.3 Comparison with Previous Reports

Hart (1976) gave a single oral dose of 225 mg/kg ^{14}C-DIMP to male Swiss Webster mice, Sprague-Dawley rats and beagle dogs. Blood was analyzed at various times for radioactivity, as were urine and feces that were collected at 24-hour intervals

over a period of 72 hours. DIMP was absorbed rapidly in all species; peak plasma levels occurred in 15 minutes in mice and in two hours in rats and dogs. When radioactivity in plasma levels was plotted semilogarithmically versus time after dosing, the resulting curves suggest two components. The first component, presumably the parent compound, had half-time of 30 minutes in mice, 2.5 hours in rats and one hour in dogs. The second component, unidentified in Hart's (1976) study, accounted for 95%, 86% and 97% of the administered radioactivity by 72 hours, respectively, in mice, rats and dogs. Fecal excretion was in the 2-3% range. There was less than 0.5% in exhaled air at any time point.

Ivie (1980) gave one Jersey cow unlabelled DIMP orally for 5 days (10 mg/kg/day), followed 24 hours later by a similar dose of ^{14}C-DIMP. Peak plasma level occurred in two hours. By four hours, 30% of the radioactivity had been recovered in urine, with 84% accounted for in urine by 96 hours; feces contained 7%.

Hart found three radioactive moieties in the urine of his rats and mice, two in dogs. Only 1-3% was the parent compound, with the second component accounting for 93-99% of the radioactivity. Enzyme hydrolysis of paired samples before analysis revealed no evidence of radioactive conjugates in urine.

In a later report (1980), Hart showed the major metabolite in the urine of all three species to be isopropyl methylphosphonic acid (IMPA). Ivie's cow also excreted IMPA as the sole urinary metabolite, and 97% of her fecal radioactivity was also IMPA.

5.0 SUMMARY AND CONCLUSIONS, METABOLISM/PHARMACOKINETIC STUDY IN MINK

The general pattern of our results, exclusive of the female rats, conforms quite well to the earlier reports. Allowing for differences among species and differing experimental settings and times, the findings are reassuringly similar. The rapid absorption, rapid excretion predominantly via urine, conversion to the one predominant metabolite, and even rates of conversion and elimination have proven to be reproducible. Mink appeared to metabolize DIMP more akin to dogs than rats, and the metabolism of female mink was quite similar to males.

Female rats clearly handled DIMP differently from males, with less efficient conversion to IMPA and slower excretion of the IMPA.

D. GENERAL SUMMARY AND CONCLUSIONS

The 90-day dosed-feed study revealed that diisopropyl methylphosphonate was mildly toxic to mink of both sexes in doses of 400 mg/kg BW/day and higher (LOAEL) but caused no adverse effects at 73 mg/kg (NOAEL). No deaths or clinical morbidity occurred at doses between 400 and 1135 mg/kg BW/day, and toxicity was limited to reduction in erythrocyte

lifespan (production of Heinz bodies) and reduction in plasma cholinesterase activity. Both changes were mild to moderate and reverted to normal within 1-3 weeks after cessation of treatment. There were no treatment-related histopathologic changes except equivocal splenic hematopoiesis in the highest dose group.

In the metabolic study, mink absorbed and metabolized DIMP in a manner quite similar to that reported for dogs, rats, mice and bovines. Gastrointestinal absorption was prompt, DIMP was rapidly converted by a saturable pathway to a single metabolite, isopropyl methylphosphoric acid (IMPA), which was eliminated promptly, primarily via urine. Between 65 and 95% of administered DIMP was excreted in urine and feces 120 hours after administration.

Taken together, these results indicate that mink (Mustela vison) should have no unique species susceptibility to toxicity from DIMP.

E. ADDENDUM: Examination of Tissues of Rats for Residual ^{14}C, 120 Hours After A Single Oral or Intravenous Dose of ^{14}C-DIMP

1.0 INTRODUCTION

As part of the Metabolism/Pharmacokinetic (M/P) Study of DIMP, ^{14}C-diisopropyl methylphosphonate (^{14}C-DIMP) was given to 48 ten-week-old Sprague Dawley rats. Eight animals of each sex were given 660 mg/kg or 66 mg/kg DIMP in a single oral dose, or 66 mg/kg intravenously. Each dose contained 40 Ci/kg of ^{14}C-DIMP. The animals were housed in commercial metabolism cages. Blood, urine and fecal specimens were collected at intervals for 120 hours. The specimens were analyzed for total radioactivity and for identity and quantification of radioactive parent compound and radioactive metabolites. Only one metabolite, isopropyl methylphosphonic acid (IMPA) was produced in quantity greater than 0.1% of the parent compound.

Radioactivity collected in urine and feces for 120 hours after dosing accounted for 80-95% of the dose administered to males, but only 60% in females. In the pharmacokinetic studies, the females converted DIMP to IMPA at a slower rate than the males did. This difference between male and female rats in rate of metabolism and excretion of DIMP has not been reported previously. To our knowledge, the only previous metabolic/pharmacokinetic study of DIMP in rats was conducted in males only (Hart, 1976).

This study was undertaken to identify tissues that may contain residual radioactivity, and to quantify the parent and/or metabolite compound in those tissues, in both sexes. Identification and quantification of the radioactive compounds was performed at the Southwest Research Institute, San Antonio, TX.

Hart (1976) administered 225 mg/kg DIMP to 14 male 180-280 gram Sprague Dawley rats. The doses contained 30 mg of ^{14}C-DIMP/ml with specific activity of

0.20 Ci/mg (45 Ci/kg). Two rats were sacrificed at intervals after treatment, for quantification of radioactivity in blood, urine, feces and various tissues.

The residual radioactivity in tissues of Hart's two rats, examined after one hour and after 72 hours, is tabulated below, as g ^{14}C/gm of tissue.

Table E 1.0 Tissue Radioactivity (Hart, 1976)

Tissue	1 Hour	72 Hrs
Kidney	312.34	0.79
Urinary Bladder (empty)	253.97	0.74
Spleen	119.29	0.70
Liver	476.06	0.63
Fat	100.82	0.63
Lung	471.82	0.26
Skin	117.83	6.31

Several other tissues were measured also, with 1-hour values less than 120, and 72-hour values less than 0.27. The skin value at 72 hours appears anomalous, especially relative to the 1-hour value. Hart studied dogs and mice similarly; in the mice, skin values were high at 72 hours also, but they were not in dogs. We suspect the high 72-hour value may represent external contamination from urine and feces in rodents.

Our animals received approximately the same dose of radioactivity per kg as Hart's, however, our animals were not sacrificed until 120 hours post-dosing. Our total doses of DIMP, 660 mg/kg and 66 mg/kg, were approximately three-fold and one-third of that used by Hart.

In our pharmacokinetic study, DIMP was converted to IMPA at different rates in males versus females. After intravenous administration, the apparent plasma elimination half-life of DIMP was approximately 45 minutes in males, and up to 250 minutes in females (N=8). Analyses of the area under the plasma concentration/time curves (AUC) revealed that the pathway of conversion of DIMP to IMPA was saturated at the higher dose, causing non-linear kinetics. (The 10-fold higher dose resulted in a 50-fold larger AUC for DIMP, but only seven-fold higher for IMPA). Females converted a smaller proportion than males and at a slower rate. These data are consistent with less complete elimination of the administered dose in urine and feces of females, and prompted the current study, to compare residual activity in tissues of the females versus males, and to compare our results with those of Hart.

2.0 MATERIAL AND METHODS

2.1 Counts of Residual ^{14}C Radioactivity in Tissue

In our M/P study, groups of eight rats of each sex were given a single dose of ^{14}C-DIMP orally by gavage (660 mg/kg or 66 mg/kg) or intravenously (66 mg/kg). The specific activity of the ^{14}C-DIMP was 5 millicuries/millimole, and it was diluted with "cold" DIMP and distilled water (oral) or saline (IV) to deliver 40 Ci/kg body weight in 4.0 ml doses. The animals were sacrificed 120 hours after dosing, following overnight fast.

Frozen bodies of four of these animals of each sex from the groups given 660 mg/kg (Males No. 01, 02, 03, 06; Females No. 01, 02, 03, 05) were thawed and dissected, and combined into pools of the following organs and tissues:

Lung - with trachea removed

Kidney

Liver

Urinary Bladder - transected at the neck, opened and rinsed in deionized water.

Skin - shaven, from two locations, mid-dorsum and mid-ventrum.

Adipose tissue - abdominal and pelvic

Heart

Skeletal muscle - biceps femoris and quadriceps, left rear leg

Small intestine - from pylorus to cecum, with contents

Large intestine - cecum, colon, rectum, with contents

Remaining tissues and carcass

Weights were recorded for the undissected bodies and also for each pool. Similar pools of negative control tissue were prepared from four untreated rats. The pooled tissues were sliced into 1-cm cubes and stored at -20°C in plastic bags until prepared for analysis.

The carcasses minus the listed tissues were thawed and homogenized in an 8-liter commercial Waring blender. Approximately 50 grams of this homogenate, and each of the listed tissues, were ground in sequence, in ascending order of expected residual radioactivity, based on the results published by Hart (1976). He reported skin (site unspecified) to contain approximately 6.3 g ^{14}C/gm, far more than other tissues.

For grinding, each pool of frozen tissue, in sequence, was placed between layers of dry ice chips (solid CO_2) in a Universahlmuhle M20 grinder (Stalfen, W. Germany) and processed to a fine powder. The work was performed in a -20°C freezer. Urinary bladders were not powdered because of their small size. The dry ice was allowed to sublimate, and the powdered tissues were stored at -20°C until analyzed.

Weighed triplicate subsamples of the powdered tissues were oxidized, and their ^{14}C activity was measured by the same methods used to determine ^{14}C activity in feces for the M/P study (Section C 2.4.2. of the basic report). For these tissues, the "burn" time of the oxidizer was 0.8 minutes per sample, and 10 ml each of Permafluor and Carbasorb were used. Tissue aliquots were between 70 and 600 mg.

Tissue from untreated animals was prepared similarly. For calibration of efficiency, a 10 l aliquot of ^{14}C "Spec Check" (Packard Instruments, Meridian, CT, 8.57 x 10^5 dpm/ml 3%) was added to each triplicate sample of tissue from the control pools, to one triplicate set of tissue-free oxidizer cones with pads, and into a triplicate set of empty scintillation vials. Efficiency of recovery of counts from each tissue was estimated by comparing the counts from the Spec Check added directly to the empty vials with counts from the respective tissue samples. Tables of raw counts for bothmale and females are in Appendix 14. On this basis, the percent efficiency for the control tissues (average of triplicate counts) was as listed in Table E 2.1.

Table E 2.1 Counting Efficiency for Detection of Radioactivity in Control Tissue

SAMPLE	% RECOVERY
Blank Cone	119%, 99%, 100%
Ventral Skin	112%
Dorsal Skin	93%
Lung	100%
Heart	99%
Skeletal Muscle	102%
Small Intestine	103%
Large Intestine	101%
Remaining Carcass	102%
Adipose Tissue	37%**
Liver	94%
Urinary Bladder	98%
Kidney	99%

** These samples of control adipose tissue oxidized at a very rapid rate and were not completely oxidized to CO_2. The scintillation vials showed a marked dark to black color from the unoxidized carbon. This interfered with the recovery. For the treated animals, addition of a damp piece of Kim Wipe tissue paper to the samples of adipose tissue prevented rapid oxidation and most adipose samples were clear. Any dark color was noted and those samples were excluded from the calculation of percent recovery.

Background scintillation counts were determined on blank cones oxidized without the addition of ^{14}C. Background was 76 cpm during analysis of tissues from high dose females, and 93 cpm for the high dose males.

2.2 Identification of Radioactive Compounds in Tissue

Only those tissue pools exceeding 1000 cpm/gm had sufficient specific activity to be analyzed, i.e., urinary bladder, small intestine and large intestine, but the quantity of tissue available from urinary bladders was insufficient for definitive analysis.

Pooled tissue homogenate samples (small and large intestine and urinary bladder) were shipped from UMN to SwRI by overnight express delivery, on dry ice and were transferred to storage at -20°C until analysis.

The low activity of the samples precluded the use of high performance liquid chromatography. Intestine samples were fractionated chromatographically by thin layer chromatography (TLC).

All solvents used for TLC studies were analytical grade reagents. The chromatographic system consisted of silica gel-coated (250 m) glass TLC plates (Analtech, Lot No. 01590) with a glass TLC developing chamber. Detection of metabolites was conducted by liquid scintillation counting (LSC) of scraped sections of the gel. Gel sections were carefully scraped into 20 ml LSC vials followed by addition of 0.5 ml methanol to allow dissolution of the radioactive compounds. Fifteen milliliters of ScintiVerse TM LC (Fisher Chemical Co.) was then added. Samples were capped and vigorously mixed. Prior to counting, the samples were stored overnight, protected from light. They were counted on a Packard Tri-Carb 4530 LSC. A radiolabeled standard of diisopropyl methylphosphonate (DIMP) was run simultaneously with each batch of samples.

Sample preparation involved ultrafiltration of the homogenates using Ultrafree-MC (Millipore) filter assemblies. Approximately 0.5 ml of the homogenate was placed in the upper chamber of the filter assembly and centrifuged on a Marathon 21K/R refrigerated centrifuge at 13,300 rpm for 15 minutes and 10°C. The resulting ultrafiltrate was used to spot the TLC plates. The plates were dried in ambient air prior to development in 100% HPLC-grade acetone at the mobile phase.

Preliminary studies were conducted to determine the retention characteristics of DIMP and isopropyl methylphosphonate (IMPA) on this system. DIMP exhibited a consistent Rf value of 0.60 (Rf = distance from origin to compound spot/distance from origin to solvent front) whereas IMPA and MPA remain at the origin. Detailed description of these methods are included in Appendix 14.

3.0 RESULTS AND DISCUSSION

3.1 Counts of Residual ^{14}C Radioactivity in Tissue

The total counts for the total tissue pool, the mean counts per gram of tissue and percent of total administered dose for each pool are summarized in Tables E 3.1.1 and E 3.1.2, for males and females respectively. The net cpm/gram and percent of administered dose represented are tabulated for each individual tissue sample in Appendix 14.

Table E 3.1.1 Residual Radioactivity in Tissues from Male Rats (660 mg/kg)

Tissue	Total CPM	CPM/ Gram	% Admin Dose
Ventral Skin	153	958	0.000795
Dorsal Skin	NS*	NS*	0.0
Lung	NS*	NS*	0.0
Heart	NS*	NS*	0.0
Skeletal Muscle	2,547	108	0.002113
Small Intestine	28,938	770	0.024007
Large Intestine	44,691	1,618	0.037075
Carcass	207,449	210	0.1721
Adipose Tissue	2,586	185	0.002146
Liver	1,876	41	0.001556
Urinary Bladder	702	853	0.000582
Kidney	1171	110	0.000972
Total Oral Dose	120,539,428		
Total % Recovered All Tissues			0.241346

* NS - denotes counts below background for that tissue.

Table E 3.1.2 Residual Radioactivity in Tissues from Female Rats (660 mg/kg)

Tissue	Total CPM	CPM/ Gram	% Admin. Dose
Ventral Skin	3,507	728	0.00395
Dorsal Skin	335	27	0.000377
Lung	NS*	NS*	0.0
Heart	55	14	0.000062
Skeletal Muscle	636	30	0.000716
Small Intestine	42,943	1,370	0.048368
Large Intestine	68,943	2,549	0.077653
Carcass	320,713	466	0.361228
Adipose Tissue	2,575	287	0.0029
Liver	6,210	209	0.006995
Urinary Bladder	768	1,914	0.000865
Kidney	2,945	442	0.003318
Total Oral Dose	88,784,227		
Total % Recovered All Tissues			0.506431

* NS - denotes counts below background for that tissue.

Because of the low recovery of radioactivity in all tissues from both sexes given the high oral dose (660 mg/kg), recovery of ^{14}C from the tissues of animals given only 66 mg/kg was not attempted.

The results presented above for male rats, documenting recovery of only an additional 0.24% of the administered dose from the animals killed 120 hours after treatment are plausible, considering that 85% - 90% had been recovered in urine and feces. These data are also compatible with those of Hart (except for his data on skin). Hart reported 0.70%, in carcass and GI tract, at 72 hours.

Recovery of 0.5% residual radioactivity from the tissues of females also is consistent, in comparison to the male data, in view of the slightly slower metabolism and excretion of DIMP in females. But recovery of only 0.5% of the administered dose still leaves nearly 40% of the dose unaccounted for.

One potential route of excretion of ^{14}C is exhalation of $^{14}CO_2$. We did not monitor this; exhalation of $^{14}CO_2$ was trivial in Hart's work, with 0.05 - 0.53% of the administered dose recovered from this route at various times up to 72 hours in male rats (N = 2 animals per time point).

Ivie (1980) reported that DIMP was quite volatile, while IMPA was not. The half-time of disappearance of DIMP from plasma of male rats in our M/P study was about 45 minutes, while in females it was up to 250 minutes (N = 8 per sex). The resulting increased area under the time/concentration curve for DIMP in plasma in females (approximately 160% of the male value, Appendix 14) would theoretically permit a greater proportion of DIMP to be exhaled by females than males, but the differential could not account for 40% of the administered dose. The DIMP was entirely converted to IMPA by 12-14 hours (females required about 15% more time than males). The plasma half-life for IMPA was 2-3 hours in both sexes. On a theoretical basis, given these kinetics, the females should not retain 40% of the dose.

We believe the most plausible explanation for the unaccounted radioactivity is loss of urine while the animals were being caught and restrained, for repeated blood samples during the first day of the study. Female rats commonly urinate during restraint much more than males do. The P/M study protocol specified collection of blood at 1, 2.5, 5.0, 10, 30, and 60 minutes, then at 2, 3, 4, 6, 7, and 24 hours after the single dose of ^{14}C-DIMP. The animals were anesthetized initially with isoflourane for dosing, and the initial 4-5 blood samples were obtained while the animals were under the influence of that anesthetic. For subsequent blood samples, the animals were anesthetized with isoflurane via nose cone, while still in their metabolism cage. They were then placed on a nearby surface for us to obtain blood. The anesthesia was used in an attempt to minimize loss of urine, but it was clear from obvious voids of urine that the strategy was not completely successful. (With the mink, we modified the protocol and added a cohort of animals that were not bled, but were left undisturbed in metabolism cages for quantitative recovery of urine and feces). Even small quantities of urine lost to counting during the first 24 hours would represent significant decrements, since urinary excretion of ^{14}C was most abundant during this period. Approximately 60-80% of the total 120-hour recovery from urine occurred during the first 24 hours.

Hart (1976) reported nearly ten times more ^{14}C in "skin" than in an any other tissue, in two male rats analyzed at 72 hours after administration of ^{14}C-DIMP. We shaved the hair from samples of both dorsal and ventral skin of our animals, and counts recovered from that skin (at 120 hours) were among the lowest that we recorded. We believe this supports our hypothesis that the "skin" counts in Hart's study were the result

of external contamination of hair by urine and feces, and therefore that skin is not an important tissue depot for DIMP. We did not quantify radioactivity on the shaven hair.

3.2 Identification of Radioactive Compounds in Tissue

Our M/P study, like those of Hart (1976) and Ivie (1980), demonstrated rapid disappearance of DIMP from plasma, rapid accumulation of IMPA in plasma and relatively rapid urinary excretion of IMPA as the major (+99%) metabolite of DIMP. It was of interest therefore, to determine whether residual ^{14}C radioactivity in tissue would also be identified as IMPA, 120 hours after administration of ^{14}C-DIMP.

Figures 1 and 2 contain illustrations of the TLC results for the homogenates of the small and large intestine. The radioactive component remaining in these tissues was retained at the origin for all samples, consistent with the mobility of IMPA in this system. DIMP standards that were run with the samples exhibited an Rf value of 0.60. No other major peaks (exceeding 10 dpm above background) were noted in either the standard or any of the intestine homogenates.

Figure E.1.a TLC Separation, Large Intestine Homogenate and Added DIMP, Male Rat

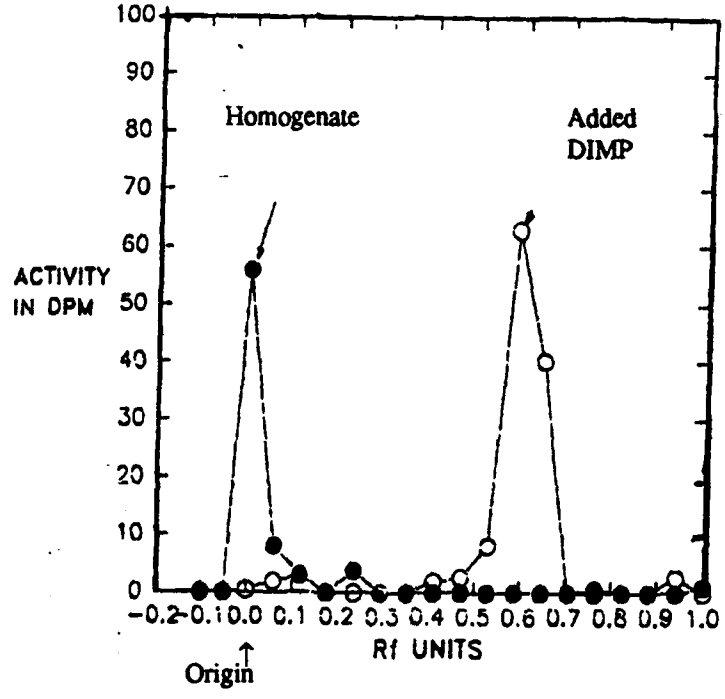


Figure E.1.b TLC Separation, Small Intestine Homogenate and Added DIMP, Female Rat

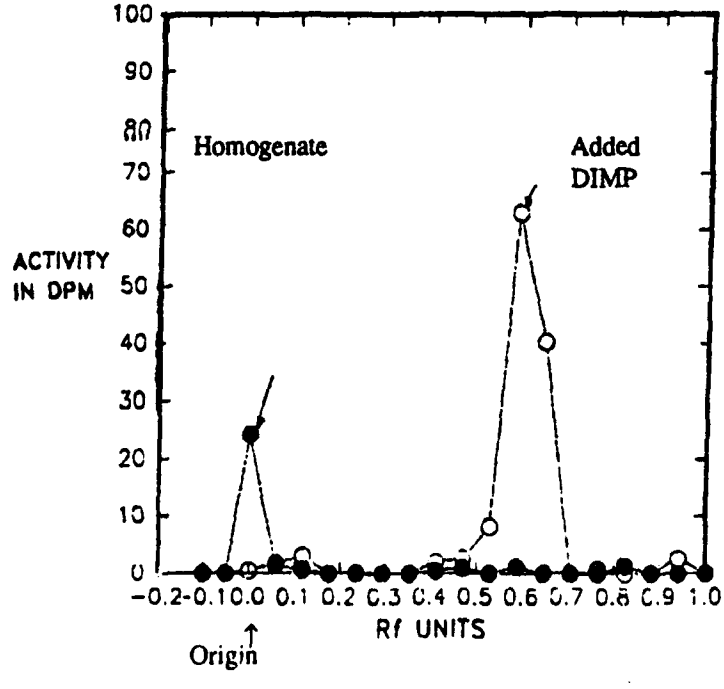


Figure E.2.a TLC Separation, Large Intestine Homogenate and Added DIMP, Male Rat

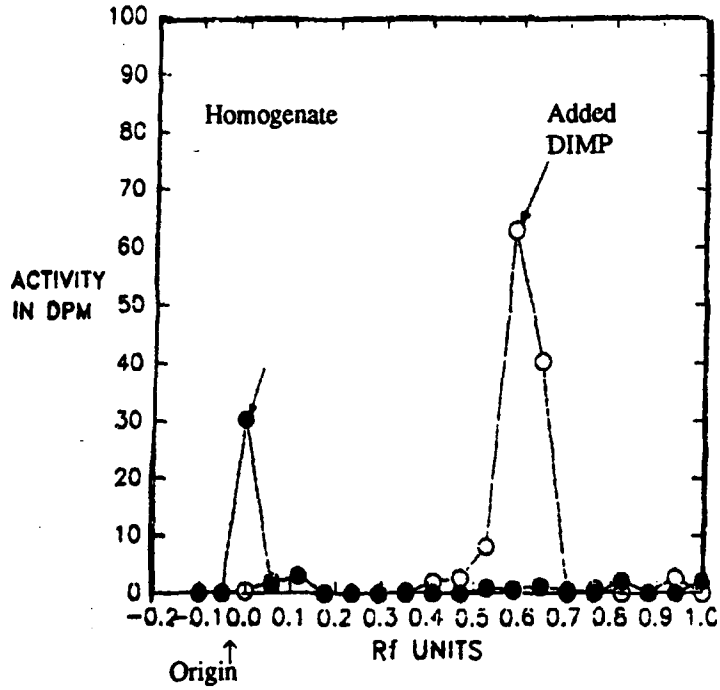
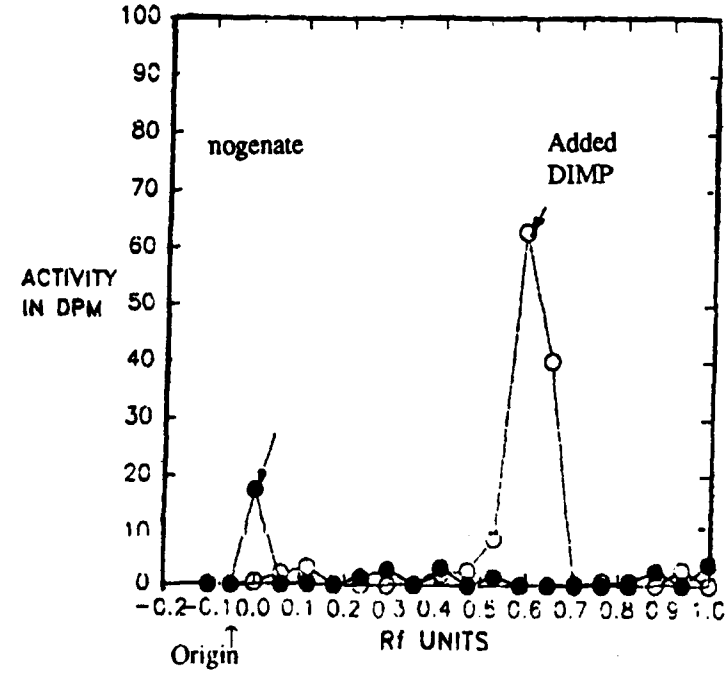


Figure E.2.b TLC Separation, Small Intestine Homogenate and Added DIMP, Male Rat



4.0 SUMMARY ADDENDUM

In summary, with the thin-layer chromatography system employed, all ^{14}C radioactivity was associated with IMPA, in the intestine samples from male and female rats. We believe that all radioactivity remaining in the tissues at 120 hours would be IMPA, and that there was no significant tissue depot of either DIMP or IMPA in the female rat to account for the unrecovered radioactivity. We believe that the most plausible explanation for the unaccounted ^{14}C is loss of urine to counting, associated with collection of blood samples during the first 24 hours.

F. REFERENCES

Aulerich, R.J., Coleman, T.H., Polin D., Ringer, R.K., Howell, K.S., Jones, R.E. and Kavanagh, T.J. (1979). Toxicology study of diisopropyl methylphosphonate and dicyclopentadiene in mallard ducks, bobwhite quail and mink. Michigan State University, East Lansing, MI. Contract No. DAMD17-75-C-0054. AD-AO87 257/2.

Bessis,L. "Living Blood Cells and Their Ultrastructure",(1973). Springer-Verlag, New York pp.,200-201.

Biskup, R.K., Manthei, J.H., Malloy, J.C., Wiles, J.S. and McKinley, E.R. (1978). Toxicity study in rats dosed with diisopropyl methylphosphonate (DIMP) in their drinking water for 26 weeks. Technical Report ARCSL-TR-77073. Chemical Systems Laboratory, U.S. Army Armament Research and Development Command, Aberdeen Proving Ground, MD. AD-AO54 733.

Calabrese, E. (1990). Health Effects Assessment of Diisopropyl Methylphosphonate, Draft Report, 15 February 1990.

Chatonnet, A. and Lochridge, O. (1989). Comparison of butyrylcholinesterase and acetylcholinesterase. Biochem J. 260: 635-634.

Doull, J.C. (12 February 1990). Letter from Dr. John Doull, Committee on Toxicology, National Academy of Sciences to COL Frederick Erdtmann, Office of the Surgeon General, Department of the Army. Review of the Aulerich Study and recommendations for future studies.

Fan L.C., Dorner, I.L., Hoffman, W.E. (1978). Reticulocyte response and maturation in experimental acute blood loss anemia in the cat. J Amer Anim Hosp Assoc. 14: 219-224.

Freeman, D. H., Jr. (1987). Applied Categorical Data Analysis. Marcel Dekker, Inc., New York.

"Guide for the Care and Use of Laboratory Animals" prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 85-23, Revised 1985).

Hardisity, J.F., Pellerin, R.J., Biskup, R.K. and Manthei, J.H., (1976). Reproductive studies with diisopropyl methylphosphonate in rats. Technical Report ARCSL-TR-77037. Chemical Systems Laboratory, U.S. Army Armament Research and Development Command, Aberdeen Proving Ground, MD. AD-AO40 454.

Hart, E.R., (1976). Mammalian toxicological evaluation of DIMP and DCPD. Final Report. Litton Bionetics, Inc. Kensington, MD. Contract No. DAMD17-75-C-5068.

AD-AO58 323.

Hart, E.R. (1980). Mammalian toxicological evaluation of DIMP and DCPD (Phase 2). Final Report. Litton Bionetics, Inc., Kensington, MD. Contract No. DAMD17-77-C-7003, AD-AO82 685.

Ivie, G.W. (1980). Fate of diisopropyl methylphosphonate (DIMP) in a lactating cow. Bull. Environm. Contam. Toxicol. 24(1):40-48.

Jones, R.E. Jr. (7 Feb 1991). Texas Instruments, Dallas TX. Personal Communication with TJ Bucci regarding Effect of an Environmental Contaminant, Diisopropyl Methylphosphonate (DIMP) on the Blood Pressure of the Mallard. (unpublished) Written by Jones in 1978 while at Michigan Agricultural Experiment Station, E. Lansing, MI.

Joergensen, G. (1985). Mink Production, Scientifur, Hilleroed, Denmark.

Jain, N.C. "Schalm's Veterinary Hematology", (1986). Lea and Feibiger, Philadelphia.

Koch, G. G., Landis, J.R., Freeman,J.L., Freeman, D.H., and Lehnen, R.G., 1977. A general methodology for the analysis of experiments with repeated measurement of categorical data. Biometrics. 33:133.

Munro, N.B., Lee, R.S., Watson, A.P., et.al. (1991). Cholinesterase activity in domestic animals as a potential biomonitor for nerve agent and other organophosphate exposure. JAVMA 199 (1) 103-115.

National Toxicology Program (NTP 1987) General Statement of Work for the Conduct of Toxicity and Carcinogenicity Studies in Laboratory Animals. National Institute for Environmental Health Sciences, Research Triangle Park, NC

National Research Council. (1982) Nutrient Requirements of Domestic Animals, Number 7, Nutrient Requirements of Mink and Foxes. National Academy Press: Washington, D.C.

Poumas, B.T. and Biggs, H.G. (1972). Standard Methods in Clinical Chemistry, 7:175

Perman, V., Weiss, D.(1991). "Diagnostic Implications of Red Cell Shape Changes" Proceedings of 58th Annual Meeting, Animal Hospital Association, Toronto, Canada.

SAS. 1987. SAS/STAT Guide for Personal Computers: Version 6 Edition. SAS Inst. Inc. Cary, NC.

Shell Chemical Company, (1988). Toxicology and Risk Assessment of DIMP.

Steel, R. G. D. and Torrie, J.H., 1980. Principles and Procedures of Statistics: A Biometrical Approach, 2nd Edition. McGraw Hill Book Co., New York.

"Standard Guidelines for the Operation of Mink Farms in the United States", (1989). Fur Farm Animal Welfare Coalition, St. Paul, MN., March.

"Style Manual for USABRDL Technical Reports and Contractor-prepared Reports", (1980). USABRDL, Fort Detrick, Frederick, MD 21701, December.

Schwab, M.L., and Lewis, A.E., (1969). An Improved Stain for Heinz Bodies, Tech Bulletin, 39, pp. 93-95.

U.S. EPA (1985) U S Environmental Protection Agency. Health Effects Testing Guidelines, Subpart H. Federal Register 50(188) 39470-39471.

U.S. EPA (1989) Office of Drinking water. Diisopropyl methylphosphonate (DIMP). Health Advisory. Washington, DC.

U.S. EPA (1989a) U S Environmental Protection Agency. Toxic Substance Control Act. Good Laboratory Practices Standards. Federal Register 54(158) 34034-34050.

Viskochil, K., Lofsness, K. and Korni, K. (1978). Laboratory Manual of Clinical Hematology Methodology. University of Minnesota.

Winer, B. J. 1971. Statistical Principles in Experimental Design, 2nd Edition. McGraw-Hill Book Co., New York.

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H. LOCATION OF SPECIMENS AND RAW DATA

All study records and computer printouts to include animal husbandry, dosing, clinical chemsitry, hematology, and pathology records, as well as pathology slides, blocks and wet tissues, are stored in the Pathology Associates, Inc. achive at Frederick, Maryland,until specified otherwise by US Army representatives. The point of contact for archival status is:

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I. ACKNOWLEDGEMENTS

This undertaking required a higher degree of both teamwork and individual resourcefulness than the authors have encountered in previous work. It was an ambitious plan, modified many times in its gestation; it required coordination with multiple agencies of the federal government as well as state, academic and private institutions. The differences among them in procedures for contracting and procurement alone were notable. The work was distributed from North Branch, MN, to San Antonio, TX, and from Frederick, MD, to Denver, CO. It involved fur-producing animals in medical research for the Department of Defense; it contained elements of research associated with chemical warfare and with pollution of the environment and drinking water, all emotional issues with various advocacy groups. Meeting and adhering to performance standards relating to care and use of laboratory animals and good laboratory practices required forethought and dedication.

There were many individuals whose personal commitment to high standards and whose determination were applied unstintingly to ensure successful completion of the work and without whose individual and collective efforts, the work might have failed. Among those, the authors are particularly grateful for the extraordinary contributions of the following persons.

Drs. Irving Baumel and Jack Dacre of the U.S. Army Biomedical Research and Development Laboratory, Ft. Detrick, Frederick, MD, for their guidance and empathy from the beginning. Betty Raiford, Becky Vilches, Lisa Wiley, Sheila Frazier and Gordon Myers of Pathology Associates, Inc. for their positive attitude, resourcefulness and dedication to detail in providing the administrative support associated with all of the procurement, technical work and publication; Paul Gosnell for his invaluable help in compiling data and tables.

Dr. Richard Geary of Southwest Research Institute, San Antonio, TX, and Dr. Roger Harris of Midwest Research Institute, Kansas City, MO, for willingly sharing their expertise and for their interest and consultation above and beyond the requirements of their respective subcontracts.

At the University of Minnesota, Dr. Victor Perman for his expertise in marshalling the appropriate resources of the university, his ingenuity in resolving the inevitable logistic challenges and his sage advice. More than any other individual, it was Dr. Perman's steady dependability that kept the project on course. Dr. Mark Brenner, Associate Dean of the Graduate School was consistently supportive. Dr. Douglas Weiss' thorough professionalism assured the integrity of collection and analysis of all clinical specimens. Dr. Steven Bistner capably provided ophthalmologic examinations on what may be the largest number of mink so examined. Dr. Vicky King was responsive and thorough on all of the statistical analyses. Tom Knier was exceptional in his management of the animals, training of animal care personnel and assuring integrity of husbandry data. Ruth Kraemer, Mary Wictor and Ann Hupperts provided key laboratory assistance with the metabolic study, as did Nancy Pappenfuss in hematology and Jean Archambeau in clinical chemistry for the 90-day study.

Kathy Logue provided notable animal care and laboratory assistance in the 90-day study, Judi Bumgardner contributed diligent laboratory assistance for both the studies, and Dave Plath was especially dependable in preparation of the feed for the 90-day study.

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