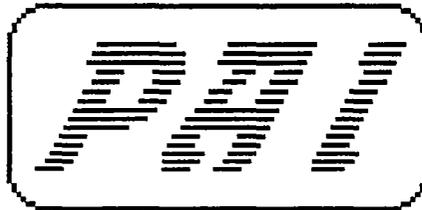


**Study No. TP-001
Two-Generation Reproductive Study in Mink Fed
Diisopropyl Methylphosphonate (DIMP)**

Final Report



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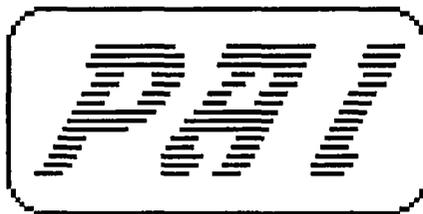


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Methylphosphonate (DIMP)

FINAL REPORT

Thomas J. Bucci
Michael D. Mercieca
Pathology Associates International
Frederick, Maryland 21701

and

Victor Perman
Douglas J. Weiss
College of Veterinary Medicine
University of Minnesota
St. Paul, Minnesota

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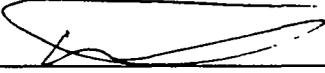
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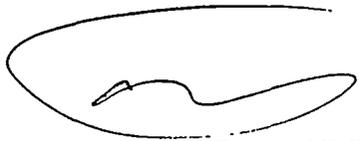


Thomas J. Bucci, VMD, PhD
Study Director

28 Aug 97
Date

Good Laboratory Practice Compliance Statement

All work conducted in support of this study (TP-001) was performed in accordance with the applicable EPA Toxic Substances Control Act (TSCA), Good Laboratory Practice Standards (40 *CFR* Part 792) except as noted in Appendix 13.



28 Dec 97

Thomas J. Bucci, VMD, PhD
Study Director,
Pathology Associates International



8/27/97

Gary L. Knutsen, DVM, MS
President,
Pathology Associates International

EXECUTIVE SUMMARY

Introduction

Diisopropyl methylphosphonate (DIMP) is a by-product of the manufacture of the military nerve agent Sarin, and is a groundwater contaminant at and near the Rocky Mountain Arsenal, Adams County, Colorado. There is long-standing disagreement between the State of Colorado Department of Health and the US Army concerning the degree of risk from this contamination to nearby citizens and the extent of remediation required.

This two-generation reproductive study was carried out in mink (*Mustela vison*) to reinvestigate the potential toxic effects of DIMP on female mink that undergo breeding, pregnancy, whelping and lactation while exposed to DIMP in their feed. There has been ongoing controversy about the toxicity of DIMP in this situation (CWQCC, 1993) since a similar one-generation study in mink reported that DIMP had no adverse effect on reproduction with doses of 50, 150 and 450 ppm DIMP in the diet. Approximately 10% of treated dams died during the study, but the cause of death of the animals was not determined (Aulerich *et al* 1979).

The US EPA and the National Academy of Sciences found the study by Aulerich to be inconclusive for the purpose of recommending a Health Advisory for lifetime exposure of humans to DIMP in drinking water. Based on other studies, the EPA currently recommends a human lifetime Health Advisory of 600 micrograms/liter (600 ppb) for DIMP in drinking water (EPA, 1989). The National Academy of Sciences concurs with this recommendation (Doull, 1990). In contrast, the Department of Health, State of Colorado and its consultant Dr. Edward Calabrese, accepted the early deaths of the mink in the Aulerich study as DIMP-induced and used the study to derive a ground water standard. Dr. Calabrese recommended 0.36 ppb (Calabrese, 1990) and the Colorado Water Quality Control Commission (CWQCC, 1993) selected 8 ppb for its standard.

In 1990, the US Army contracted with Pathology Associates International (PAI), Frederick, MD, to conduct a 90-day toxicity study of DIMP in mink and a 5-day pharmacokinetic study in mink and rats (Bucci *et al* 1992 and 1994; Weiss *et al* 1994). The mink were maintained under subcontract at the Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Minnesota (U of MN), St. Paul, MN. The university provided formulation of test rations, animal husbandry, clinical chemistry and hematology. Necropsy (at U of MN) and microscopic examination of the mink were performed by PAI. In the 90-day study, 10 mink of each sex were fed DIMP at one of six target doses ranging from 0 to 8000 ppm, including one group at 450 ppm

to match the Aulerich *et al* (1979) high dose. Food consumption and body weight were monitored, and a complete battery of hematologic and blood chemistry analyses and gross and microscopic examination of all major organs were performed. DIMP appeared to be only slightly toxic to the mink at the higher doses employed (the actual 90-day dose for the highest dose group was 8990 ppm). The only treatment-related changes noted, decrease in plasma cholinesterase and oxidative injury to erythrocytes, were mild and reversible upon cessation of exposure. The No Observed Adverse Effect Level (NOAEL) was 73 mg/kg BW/day, with 400 mg/kg BW/day the Lowest Observed Adverse Effect Level in the study. A dose of 75 mg/kg BW/day in a similar 90-day study of DIMP in dogs (the highest dose employed) was also a NOAEL in that study (Hart, 1976). The pharmacokinetic study demonstrated that mink metabolize and excrete DIMP in a manner very similar to the process in rats and dogs, i.e., mink were not uniquely susceptible to DIMP toxicity (Weiss *et al* 1994; Hart 1980). These studies did not replicate the reproductive toxicity test reported by Aulerich *et al* (1979), however, so in 1994 the US Army again contracted with PAI to conduct a study to re-examine the potential toxicity of DIMP fed to mink for at least one year, during which time the mink would breed and experience pregnancy, whelping and lactation. In the present study, the University of Minnesota again provided the animal care and clinical laboratory services.

Study Design

The study was designed to follow, to the extent permitted by the biology of the mink, the Health Effects Test Guidelines for Reproductive and Fertility Effects promulgated by the US EPA Office of Prevention, Pesticides and Toxic Substances (EPA, 1994). The Study Protocol was reviewed by US Army experts, the National Academy of Sciences Committee on Toxicology, and toxicologists in EPA and the Colorado Department of Health. The work was conducted in accordance with the "Guide for Care and Use of Laboratory Animals" (National Research Council, 1985) and the Good Laboratory Practice Standards of the EPA's Toxic Substances Control Act (EPA, 1985).

For the first (F_0) parental generation, groups of seven male and 35 female nine-month-old brown "Ranch Wild" mink were fed rations containing target doses of 0, 150, 450 or 2500 ppm DIMP beginning in February, 1995. Two control groups were used. The animals were housed indoors in individual cages, and their food consumption and body weight were recorded approximately weekly. They were harem-mated in March 1995, the dams whelped in April/May and the offspring (F_1 generation) were weaned in June, 1995. The males were sacrificed at the end of breeding in March and the dams after weaning in June. The F_1 kits not retained as parents for the F_2 generation were sacrificed in July, 1995. Blood was obtained from all F_0 animals at allocation in

February, 1995 and again at necropsy (March for males, June for females). The kits not retained as breeders were sampled at necropsy. All blood samples were obtained with animals under anesthesia. The blood was analyzed for a comprehensive set of chemical and hematological characteristics.

The reproductive endpoints measured included kits per litter, live kits per litter, litter biomass (weight) at birth and at 28 days, and sex ratio. Litters larger than six were standardized to six.

All parental animals and representative kits received gross necropsy and microscopic examination (limited to reproductive organs in the males and gross lesions in the kits). Acetylcholinesterase was measured in the brain (basal ganglia, striatum, and frontal cortex) of approximately 10 females per group. Males were examined for sperm count, morphology and motility.

The F₁ (2nd) generation was continued on the same DIMP dose that the dams received; these animals were allocated to individual cages in July, 1995, and blood samples were obtained from them (under anesthesia) in September and December, 1995 and again at their respective sacrifice dates (March 1996 for males, June 1996 for females and for all F₂ kits sampled). The number of F₁ males was increased to 13 per treatment group. The F₁ generation also mated in March (1996), whelped in April/May and weaned the offspring in June. The parental males were sacrificed in March after breeding, and the dams and all F₂ kits in June, 1996.

The allocation of animals to treatment groups is tabulated below:

Allocation of Mink to Treatment Groups

Dose (ppm)	F ₀		F ₁	
	Female	Male	Female	Male
0 (Control A)	35	7	35	13
0 (Control B)	35	7	35	13
150	35	7	35	13
450	35	7	35	13
2500	35	7	35	13

The test article DIMP was characterized for purity 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 purity of 97% (Batch 1) and 99% (Batch 2). Stability studies performed by the same contract laboratory on the DIMP used in the 1991 90-day study (different batch) revealed DIMP to be essentially unchanged after 24 days at -20°, 5°, 23° and 60°C (Bucci *et al* 1992,1994).

The basal ration for the mink was prepared by and obtained from the same source as the animals (North Branch Fur Farm, North Branch, MN). DIMP was incorporated into the basal ration to approximate the target dose and dispensed into individual-portion steel cans of 450-gm capacity (approximately 400 gm per can, calculated to be a slight 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 at room temperature before being placed in the cages.

The target dose groups were 0, 150, 450 and 2500 ppm. Dosed feed was prepared at approximately 3-week intervals, determined by the freezer storage space available. Test article concentration and homogeneity of mix were determined on the first nine sets of dosed rations prepared. The actual concentrations of DIMP varied between 106-111% of the target dose, and were accepted as effectively representing the target doses of interest. In the 90-day study, DIMP was demonstrated to be stable for up to 120 days at -20°C in the same basal ration (Bucci *et al* 1992,1994). Untreated control rations were analyzed for an extensive set of nutrients and contaminants. All were within acceptable limits, as was the result of analysis of the water used for food preparation and for drinking water in the cages.

RESULTS

Early Deaths

F₀ Generation

During the study period of this generation (February through June, 1995), six of the 175 females (3.4%) died before the scheduled sacrifice. All of the deaths except one were associated with a constellation of findings that represent a "final common path" in the demise of mink. Animals that undergo sufficient stress stop eating, experience dramatic lethargy and weight loss, develop fatty liver, atrophied thymus, kidney degeneration, and often gastrointestinal bleeding. They are usually severely dehydrated and have electrolyte imbalances. This syndrome appears to be secondary to a metabolic crisis precipitated by even short periods of reduced feed intake, given the mink's intensely nervous nature and high metabolic rate. With insufficient food intake, body fat is metabolized, but this requires a minimum carbohydrate intake to proceed normally. With insufficient carbohydrate available, the fat is only partially

metabolized, yielding ketones that further suppress appetite, and the stage is set for the crisis described above (Bruss, 1989).

The exceptional metabolic demand of heavy lactation makes mink uniquely susceptible; up to 10-15% of dams are not able to consume and metabolize sufficient energy to maintain themselves, and succumb to this syndrome, called "Nursing Disease" in these instances (Schneider and Hunter, 1993). The females in both generations of this study, like their counterparts on commercial mink farms, consumed increasing amounts of food yet gradually lost weight as lactation progressed. They attained greatest food consumption and greatest weight loss during the week preceding weaning. In the wild, mink dams spend increasing amounts of time away from the den as the kits grow, and are subject to less lactation demand.

In this report, we have elected to term the general case of this condition "stress syndrome", with a subset associated with nursing. Of the six early deaths, there were obvious precipitating causes in one control female (decomposing fetus in her uterus) and one from the 2500 ppm group (abscess in eye). Two females from the 150 ppm group had clear evidence of "Nursing Disease". The cause of death was less clear in a 450 ppm female that had the stress syndrome without evident cause and died after only one month on study, and in a second 450 ppm female that had whelped and appeared to be afflicted with Nursing Disease in that she was anorexic and lost weight dramatically, but at death did not have the fully-developed liver, kidney and thymic lesions.

Because of the clinical circumstances surrounding these deaths, the occurrence early in the study in two animals, the random distribution of the deaths among dose groups including an untreated female, and the association of the deaths with an identifiable condition that is a common malady in mink, the authors of this report are convinced that exposure to DIMP did not cause these deaths.

F₁ Generation

There were eight early deaths among the 175 females (4.6%) during the F₁ generation (July, 1995 through June, 1996). One of these was known to have seizures when excited by cage cleaning, restraint for weighing, etc. Her death at 3 months of age in August, 1995, was not anticipated because while seizure disorders are not uncommon in mink, they are not usually fatal. There were no causal microscopic findings. Five of these females succumbed to the stress syndrome, often a common precipitating cause. The animals were anesthetized in mid-December, 1995, for bleeding. They were slightly overweight and their respective anesthetic doses were based on their weight causing them to be overdosed slightly because body fat does not metabolize the anesthetic proportionately; several of the animals recovered abnormally

slowly from the anesthesia and then decreased their food intake. Most of the affected animals resumed normal feeding in a few days, but the five that did not went on to develop the signs of stress syndrome and died, including one from every treatment group, including both controls [one control (Control B) died as a result of the February bleed] and two from the 2500 ppm group. One male from the 450 ppm group also died in the same way. The eighth female, from the 2500 ppm group, showed all the signs of this stress syndrome late in her pregnancy, and upon her death she had severe peritonitis secondary to a ruptured (pregnant) uterus.

As in the F₀ generation, the distribution of these deaths among all groups, the timing and the association with an identified cause eliminate exposure to DIMP as the suspect cause of death.

Food Consumption and Body Weight

F₀ Generation

There was no meaningful difference in the amount of food consumed among the groups of F₀ females or males.

There was no difference statistically in overall mean body weight, among the controls and the treated groups of either sex.

F₁ Generation

Within sex, there was no appreciable difference among groups in the amount of food consumed, and there were no differences in body weight. The quantity of food consumed per 100 gm body weight was surprisingly consistent (note final columns in table that follows). The F₁ females consumed 11.5 grams of food/100 gm BW when all groups were averaged over the 11-month duration of exposure, compared with 17.2 for the F₀ females.

F₀ Group Mean Food Consumption, Body Weight, DIMP Ingested (DIMP at 97% Purity)

DIMP Dose Group (ppm)	DIMP Actual (ppm in diet)	Feed Consumed (gm/mink/day)		DIMP Consumed (mg/mink/day)		Mean Body Wt. (Kg)		DIMP Consumed (mg/kg/day)		gm/food consumed / 100 gm Body Wt.	
		male	female	male	female	male	female	male	female	male	female
	(μ g/gm)										
0	0	223.5	212.4	0	0	2.274	1.142	0	0	9.8	18.6
0	0	239.1	205.3	0	0	2.296	1.200	0	0	10.4	17.1
150	162	202.8	182.7	32.85	29.60	2.199	1.156	14.94	25.61	9.2	15.8
450	480	221.4	200.9	106.27	96.43	2.244	1.137	47.36	84.81	9.9	17.7
2500	2722	233.0	191.6	634.23	521.54	2.227	1.132	284.79	460.72	10.5	16.9

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F₁ Group Mean Food Consumption, Body Weight, DIMP Ingested (DIMP weighted for 97% and 99% purity)

DIMP Dose Group (ppm)	DIMP Actual (ppm in diet)	Feed Consumed (gm/mink/day)		DIMP Consumed (mg/mink/day)		Mean Body Wt. (Kg)		DIMP Consumed (mg/kg/day)		gm/food consumed / 100 gm Body Wt.	
		male	female	male	female	male	female	male	female	male	female
	(μ g/gm)										
0	0	225.6	139.5	0	0	2.400	1.208	0	0	9.4	11.5
0	0	235.0	144.0	0	0	2.521	1.292	0	0	9.3	11.1
150	168	218.6	142.4	36.72	23.92	2.343	1.212	15.67	19.74	9.3	11.7
450	490	222.6	139.5	109.07	68.36	2.425	1.210	45.00	56.50	9.2	11.5
2500	2774	217.2	141.1	602.51	391.41	2.302	1.188	261.73	329.47	9.4	11.9

Consumption of DIMP

The overall rate of DIMP consumption for each parental group is tabulated above. The F₀ females consumed approximately 50% more food and therefore 50% more DIMP/kg BW/day during their four-month exposure than did the F₁ females (food consumption measured during 11 months). The authors recommend the data from the F₁ generation for any potential extrapolation for use in humans. This generation was born and raised in the laboratory and their body weight and food consumption were monitored throughout; in contrast, their F₀ parents made the transition from outdoor farm conditions to the laboratory at nine months of age and were exposed to DIMP and monitored for only four months.

Clinical Observations

A variety of clinical observations was recorded, derived from daily "cageside" observations and others recorded weekly when the animals were caught for weighing. There were no clinical observations specifically related temporally or in number or severity with exposure to the test article. Overall, the animals remained vigorous and well, except as noted under "Early Deaths". The clinical observations were sporadic, affected all dose groups, and were of the type that occur in all routine mink husbandry.

Reproductive Endpoints

There were no treatment-related differences in average litter size, percent of live births, kit weight or sex distribution in either generation as a function of treatment, nor were treatment-related birth defects detected.

Hematology

The biologically significant adverse hematologic effects of exposure to DIMP that occurred in this study were mild and were limited to the groups given the highest dose (2500 ppm target dose). The actual concentration of DIMP received by the F₁ females in this group, averaged over the duration of the study, was 2774 ppm. For the F₀ generation, it was 2722 ppm. At this dose (equivalent to 461 mg DIMP/kg BW/day), the F₀ females sampled at necropsy in June, 1995, had decreased red blood cell count and increased reticulocytes, mean cell volume and Heinz body count. In the F₁ generation (329.5 mg/kg/day), only the Heinz bodies were affected with increases at both the December, 1995 sampling and at necropsy in June, 1996.

DIMP-Related Hematologic Changes
(2500 ppm Female/ Control A Female)

	RBC (x 10 ⁶ /μL)	Reticulocytes (% of RBC's)	MCV (um ³)	Heinz Bodies (% of RBC)
F ₀				
Feb 95	7.72/7.59	3.3/3.1	61/62	0.0/0.0
June 95	7.38*/7.79	4.1**/1.8	60*/58	2.8**/0.0
F ₁				
Sept 95	7.94/7.97	3.1/3.1	60/60	0.0/0.0
Dec 95	8.36/8.45	3.9/3.4	61/60	0.2*/0.0
June 96	8.20/8.29	2.0/1.7	61/61	1.3**/0.1

* Statistically different from Control A value with p < 0.05

** Statistically different from Control A value with p < 0.01

The above changes are of minimal biologic significance, and especially so in the F₁ generation, which appeared to adapt to the chronic exposure and to respond even less than the F₀ generation did.

The remaining twelve hematologic characteristics measured, including RBC indices, total and differential white blood cell counts, platelet count and hemoglobin concentration, were not affected by exposure to DIMP in these animals.

In the males, only the 2500 ppm group of F₁ animals were affected adversely; that was at one sample period (September, 1995, when they were five months old) and affected only the Heinz Body count, which was elevated to 0.1 % of RBC's, versus the control count of 0.0%. It should be noted that counts were also 0.1% of RBC's in controls for other treatment groups and at other times, i.e., quantitatively this count is of marginal biologic significance.

The F₁ kits not retained for breeding and sampled at necropsy when they were approximately 11 weeks old had no hematologic changes attributable to exposure to DIMP. The F₂ kits, sampled at approximately six weeks of age, had a statistically significant decrease in RBC count in the male 2500 ppm group (4.06 versus 4.51

million/cubic μL). All other hematologic characteristics were unaffected to any biologically meaningful degree, in both generations.

Clinical Chemistry

Seventeen analytes or derived values were measured. The only changes directly attributable to exposure to DIMP were decreases in plasma, whole blood and, rarely, red blood cell cholinesterase. Acetylcholinesterase in the brain was unaffected. Only reductions exceeding 20% below Control A values are considered biologically adverse (EPA 1989b, 1995); these occurred only in the 2500 ppm group. The relevant values are tabulated below:

Cholinesterase (2500 ppm Female/ Control A Female)

	Plasma (U/L)	% ↓	Whole Blood (U/L)	% ↓	RBC (U/L)	% ↓	Brain (Micromol/gm/min)
F₀							
Feb 95	1040/1109	—	4355/4378	—	8099/8118	—	—
June 95	860**/1426 ^a	-40	3115**/3725 ^b	-16	6041**/6479 ^c	-8	7.13/6.53
F₁							
Sept 95	999**/1279	-22	4285**/4589	-7	7859*/8207	-4	—
Dec 95	894**/1133	-21	4572**/4793	-7	8174/8301	—	—
June 96	905**/1310	-31	3966*/4335	-8.5	7103/7332	—	6.81/7.24

* Statistically different from Control A value with $p < 0.05$

**Statistically different from Control A value with $p < 0.01$

Not shown: Reductions at lower doses that were significant statistically but less than 20%

^a at 450 ppm (-15%) and 150 ppm (-12%)

^b at 450 ppm (-10%)

^c at 450 ppm (-13%)

In the males, plasma cholinesterase was marginally reduced in the Sept95 bleeding of the 2500 ppm group (1214*/1412 U/L, a 14% reduction), and whole blood cholinesterase was decreased by 6% in the same group at the Dec95 sampling (4544**/4835 U/L).

The 2500 ppm group of female F₁ kits had a 17% reduction in mean plasma cholinesterase at necropsy in Jul95 (1509*/1807 U/L).

There were no clinical signs referable to inhibition of acetylcholinesterase apparent at any time in these animals, and none of the other chemical analytes measured had changes attributable to DIMP exposure.

Necropsy and Microscopic Examination

The only gross observation that was considered DIMP-related was significantly increased weight of the spleen ($p < 0.01$) in the females of the F₀ generation given 2500 ppm DIMP. The average absolute weight of the spleen, as well as its ratio to body weight and brain weight were increased. This was anticipated, since it is a correlate of the shortened lifespan of Heinz body-containing red blood cells. The spleen has the dual role of removing such damaged red cells from circulation, and also of participating in production of replacement red cells.

Accordingly, there was a treatment-related increase in hematopoietic cell proliferation in the spleen of these same animals, upon microscopic examination, evidence of production of replacement red blood cells. There were no other microscopic findings referable to exposure to DIMP in the F₀ animals, and the spleen changes were not present in the F₁ animals.

Conclusion

Biologically significant adverse effects of exposure to DIMP in this study were mild and were limited to animals given the highest dose level (2500 ppm target dose). The actual concentration received by the F₁ females in this group was 2774 ppm, averaged over the duration of the study. The adverse effects measured chemically were reduction in plasma and whole blood cholinesterase (21-40% less than control values; there was a 4-7% reduction in RBC cholinesterase). Hematologically the 2500 ppm groups had significantly increased Heinz body counts in their red blood cells, which correlated with shortened red blood cell survival that was manifest also by lower RBC count, increased reticulocyte count and, inconsistently, increased cell volume. The hematologic change was evident only in the 2500 ppm group of the F₀ females at necropsy and microscopic examination, manifest as increased weight of spleen grossly and as proliferation of replacement red blood cells microscopically. There were no clinical signs of illness attributable to exposure to DIMP, and no other gross, microscopic or biochemical abnormalities associated with exposure. There were no treatment-related effects on reproductive efficiency, kit development or semen quality in males.

The study revealed that female mink during their reproductive cycle are not uniquely susceptible to DIMP toxicity, and no deaths resulted from exposure to DIMP. The two generations experienced 3.4% and 4.6% mortality in groups of 175 dams, respectively, distributed approximately equally among experiment groups including untreated controls and with an identified cause of death in all but one.

The No Observed Adverse Effect level, considering the 450 ppm target dose group of F₁ females, was 56.5 mg DIMP/kg/BW/day. The Lowest Observed Adverse Effect Level in the F₁ females was 329.5 mg DIMP/kg/BW/day.

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Request for copies of Appendices should be addressed to:

Mr. Tim Kilgannon
Remedial Planning and Monitoring Branch
Rocky Mountain Arsenal
Commerce City, CO 80022-1748

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FINAL REPORT

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 the Rocky Mountain Arsenal.

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 30 mg/L (30,000 ppb) was advised for *Longer-term (7-year) exposure* of adult humans to DIMP in drinking water and that 600 μ g/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*.

To derive the *Longer term* Advisory, the EPA considered several animal studies. These included 90-day feeding studies in rats, mice and dogs (Hart, 1976) and rats, mice and dogs, Phase II (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 disqualifying shortcomings in each of the latter two studies, and all of the first four failed to demonstrate 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 considered most appropriate to serve as the basis for the *Lifetime Health Advisory*, by virtue of their duration, were the 90-day feeding studies listed above 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 $\mu\text{g/L}$ (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.

Pathology Associates International (PAI), Frederick, MD, 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 completely replicate the Aulerich work, however, which had included pregnant and lactating mink exposed to DIMP in feed for 12 months.

The Army awarded a contract to PAI, to develop protocols and begin to conduct studies consistent with the recommendations of the NAS. In 1991, PAI carried out a 90-day toxicity study with DIMP-dosed feed in 160 mink and also completed a 5-day pharmacokinetic/metabolic study in mink and rats (Bucci *et al* 1992 and 1994, Weiss *et al* 1994). Both studies were conducted following EPA (TSCA) guidelines. A protocol was developed by PAI and reviewed by the Army and the NAS for a two-generation reproduction study of DIMP in female mink that would replicate the study reported by Aulerich *et al* (1979) and would also include additional animals, additional outcome measurements and a second generation of offspring. Also developed and reviewed was a protocol for a two-year oral chronic toxicity study of DIMP in mink. The latter two studies were not funded at that time.

The mink in the Bucci *et al* 90-day study were fed DIMP at target doses of 0, 50, 450, 2700, 5400 or 8000 ppm in feed. Actual doses fed were 0, 57, 528, 2930, 6174 and 8990 ppm. There were no deaths or clinically apparent disease. The erythrocytes of animals in the two highest dose groups had a small but statistically significant increase in Heinz bodies, beginning at Week 3 in the highest dose group. The Heinz body count returned to normal within two weeks after cessation of treatment.

At the end of the 90-day treatment, plasma cholinesterase (but not red blood cell cholinesterase) was 30 - 60% of the value in the controls, in proportion to dose, in the 2700, 5400, and 8000 ppm groups. The cholinesterase values returned to normal by one week after cessation of treatment.

All major organs and tissues were examined microscopically at the end of the study. The only treatment-related change was increased hematopoiesis in the spleen of 7/8 animals of each sex in the 8000 ppm group, versus 6/10 and 4/10 among male and female controls, respectively. This change was not found in the two animals of each sex from the 8000 ppm group that were permitted a two-week recovery after cessation of DIMP treatment.

The authors of the 90-day study considered the lowered cholinesterase to be an adverse effect, observed in the 2700 ppm dose group but not in the 450 ppm dose group. The LOAEL corresponding to the 2700 ppm dose was 400 mg/kg body weight (using actual dose consumed); the NOAEL corresponding to the 450 ppm group was 73 mg/kg body wt/day. The NOAEL in the 90-day study in dogs by Hart (1976), upon which the EPA *Health Advisory* was based, was 75 mg/kg body wt/day.

In the metabolic study, DIMP was rapidly absorbed after oral administration to male and female mink. It was metabolized promptly by a saturable pathway to a single metabolite, isopropyl methylphosphonate (IMPA), which was excreted rapidly, primarily in urine (~90%). The rats in this study metabolized the DIMP in approximately the same manner. The authors concluded that mink metabolized DIMP in the same manner as reported by Hart (1980) in rats, mice and dogs.

During July 1993 - January 1994, the Colorado Water Quality Control Commission (CWQCC) held hearings for the purpose of setting a state ground water and surface water standard for DIMP (CWQCC, 1993). The State of Colorado Health Department, together with their consultant Dr. Edward Calabrese, recommended a standard of 8 ppb, based primarily upon the outcome of the study reported by Aulerich *et al* 1979. In that study, unexplained deaths occurred in female mink fed DIMP daily for one year, including the period from mating through lactation. The deaths occurred in 5/24 at 450 ppm, 3/24 at 150 ppm, 2/23 at 50 ppm and 0/24 in untreated controls. Two

of 24 males died also, one each of six in the control group and the low dose (50 ppm) group, both during the first 3 months of the study.

At the CWQCC hearings the US Army argued that the Aulerich study was not intended for standard-setting and the shortcomings in reporting that were associated with it precluded such use, as had been concluded earlier by the EPA and the NAS. (The Aulerich study was a screening test for reproductive effects and the animals that died were not examined.) Further, a second study, feeding dicyclopentadiene (DCPD) to mink, was being conducted simultaneously in cages that were intermingled with those of the DIMP study. The animals were of the same source and same lot for both studies, and the control animals were fed the same untreated control diet. In the DCPD study, the overall death rate (10.8%) was the same as that in the DIMP study (10.3%), except in the DCPD study the deaths were distributed approximately equally among all groups, including 4/24 untreated control females. There was no difference in reproductive performance between control and treated animals with either compound (Aulerich *et al* 1979).

An Army consultant (Dr. William Wustenberg, an expert on mink) expressed the opinion that the most probable cause of the deaths in the lactating female mink in the Aulerich *et al* study was "Nursing Disease", a stress-related fatal condition afflicting female mink that are unable to meet the energy demands of parturition and lactation. This condition has since been described in detail (Schneider and Hunter, 1993). The Aulerich report listed the deaths cumulatively at 3, 6 and 12 months of exposure, so it is not clear precisely when the animals died relative to mating and parturition. Seven of the 10 deaths in females occurred during the final 6 months; mating, whelping and lactation occupied the final four months.

At the CWQCC hearings, the Army asserted that the EPA recommendation of 600 ppb be upheld, on the strength of the EPA *Health Advisory* and work by Bucci *et al* 1992 and 1994, and Weiss *et al* 1994, that established the similarity of the response of mink to that of the dog reported by Hart (the study used by EPA to derive the *Health Advisory*). Throughout the hearing procedures, representatives of the Colorado Health Department and the CWQCC charged that the Army had not sponsored a reproductive study to repeat the Aulerich *et al* study.

The study reported here was designed to repeat the administration of DIMP to mink during pregnancy and lactation, at the highest dose used by Dr. Aulerich's group. Aulerich's study design was expanded by using a still higher dose, two control groups, two generations of offspring and by examining the animals more closely in accord with EPA's current Office of Prevention, Pesticides and Toxic Substances (OPPTS) test guidelines for health effects on reproduction and fertility.

The 90-day oral toxicity study and the metabolic/pharmacokinetic studies with DIMP in mink (Bucci *et al* 1992 and 1994; Weiss *et al* 1994) provided valuable experience in the logistical aspects of preparing the dosed feed, caring for the animals and in analyzing DIMP for purity and for concentration in feed. Many of the same facilities and experienced personnel and vendors used for the 90-day study were available again for this two-generation study.

2.0 KEY PERSONNEL AND FACILITIES

2.1 Pathology Associates International, Frederick, MD.

- Study Director - Thomas J. Bucci, VMD, PhD, Diplomate, American College of Veterinary Pathologists
- Co-Investigator - Michael D. Merceica, BS, Perinatal Biologist and Toxicologist
- Study Pathologist - Robert Kovatch, DVM, Diplomate, American College of Veterinary Pathologists

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

- Administrator - Victor Perman, DVM, PhD, Professor of Pathobiology, Diplomate, American College of Veterinary Pathologists
- Head of Hospital Laboratories - Douglas J. Weiss, DVM, PhD, Diplomate, American College of Veterinary Pathologists
- U of MN provided housing and animal care for the study, to include formulation of the dosed feed, and clinical chemistry and hematologic examination of all blood samples. Methods for and results of the U of MN analyses are described in the relevant sections of this report.

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 to document the homogeneity of mix.

2.4 Corning-Hazleton, Inc. Madison, WI

- Corning-Hazleton, Inc. analyzed the mink ration for macro and micronutrients and for contaminants.

3.0 GOOD LABORATORY PRACTICE STANDARD

The study was conducted in compliance with Good Laboratory Practice (GLP) Standards, Environmental Protection Agency, Toxic Substances Control Act (U.S. EPA 1989a) except as noted in the attached Compliance Statement. 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 study to assure adherence to GLPs and approved SOPs. Deviations from the protocol and GLP are noted in the raw data and are listed in Appendix 13.

The sponsor of the study and the Study Director were aware that established procedures (SOP's) did not exist for some of the tasks planned, since mink are not commonly used in toxicologic research. For example, US Environmental Protection Agency Office of Prevention, Pesticides and Toxic Substances Health Effects Test Guidelines, EPA (1994) recommends a count of ovarian follicles in sections of ovary, but this procedure has not been reported in mink, to our knowledge. PAI's approach was to adapt methods established for other species to the mink where none was described for mink and to use experienced personnel for all critical tasks.

There were instances where peculiarities of the species had to be accommodated, that were counter to common GLP experience. A few examples of those that were anticipated are noted here. Others are listed under Protocol or SOP deviations. One example was our adherence to the practice of modified harem-mating (several females per male) as is practiced successfully on commercial mink farms rather than to use pair-wise mating. Even so, female mink do not accept all males, and some males do not successfully mate with any females. Since the main purpose of this study was to study reproducing females, it was necessary to rely on the subjective skills and experience of a life-long mink husbandman (Mr. Patrick Molitor of the U of MN staff) to select mates and to judge whether or not successful mating had occurred.

The following are examples of concessions to the biology of the species that we made. In tests for possible reproductive developmental defects in rodents, near-pubertal F₁ offspring of treated dams are usually compared with those of control dams to note the age at which patency of their vaginal/preputial orifice occurs, and then to

compare the distance between the anal and genital openings in the F₂ generation if a treatment effect is noted in the age of patency in the F₁. We were unable to make these observations because they would have required extensive handling and examination of the kits, necessitating either firm restraint or anesthesia, an unnecessary risk to the animals. Another accommodation was the use of nest boxes (in which the kits were born and nursed) made of wood rather than a more impervious material. The USDA inspectors and institutional animal care committee authorized these as necessary for the species. Wood insulates better against abrupt change in noise and temperature, and may have some olfactory advantage ("den smell"), as well.

The basal mink diet as used successfully on mink farms is not as well defined or as uniform over time as ideal laboratory practice would dictate. There are no commercial "laboratory chow" rations for mink; those used for dogs or ferrets have not been successful in the past. The rations fed on commercial farms consist of freshly mixed highly perishable combinations of comminuted waste meat, fish, grain, eggs, and dairy products supplemented with vitamins. These rations are varied in content throughout the life cycle of the mink, to support reproduction, growth in offspring, development of cold tolerance for winter (and production of dense fur). These rations typically are ground, mixed and fed in a matter of days, and at times medications are added empirically or by prescription. For example, in summer, larvacides may be added. These pass undigested through the gut of the animals and prevent breeding of flies in the feces. If the animals experience gastrointestinal upsets (loose stools), the rancher may add antibiotics for a few days. Mink are prone to develop a poorly understood urinary condition known as "wet belly" which can be fatal and is sometimes associated with urinary calculi. Increasing the acidity of the diet is palliative, so phosphoric acid may be added until symptoms subside. The protocol of this study included use of the ration prepared and used on the same farm that produced the mink, where 30-40,000 similar animals were being raised during the course of this study. The mink in this study thus received the same ration as fed on the farm. The study file includes proximate analyses of the ration provided, as well as notation of any additives. The authors are unable to recommend any feasible alternative to such a food source and believe that no adverse impact on the study was experienced because of these variables. Further, one of the objectives of the study was to compare the results with those of the study reported by Auerlich *et al* (1979), and the ration used by that group was similar (Kavanaugh, 1993). They also used harem mating.

This report is designed to provide sufficient description of all procedures to document that the data are complete and reliable.

4.0 CARE AND USE OF LABORATORY ANIMALS

The study was 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, 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 U of MN Institutional Animal Care and Use Committee reviewed and approved the protocol employed for this study.

B. STUDY DESIGN

1.0 PRIMARY OBJECTIVE

There is ongoing controversy concerning the cause of mortality in female mink fed DIMP in a previous one-year, one-generation reproductive study (Aulerich *et al* 1979). The controversy involves whether or not deaths in that study were caused by DIMP, leading to disagreement among authorities regarding use of those data for human risk assessment. The disagreement is central to past and present litigation between the US Army and the State of Colorado, summarized in part in Section 1, Background.

The primary objective of this study was to repeat the essential features of the Aulerich study, in particular to determine the toxic effects, if any, of DIMP on female mink that undergo mating, gestation, parturition and lactation while ingesting DIMP in their feed.

1.1 Secondary Objectives

The logistics of GLP studies in mink are challenging and data on the effects of xenobiotics on mink reproduction are sparse. To achieve the primary objective of this study was resource-demanding, yet the end-point measured (mortality of dams) was relatively limited. The federal government had recently (July, 1994) issued new, comprehensive draft guidelines for testing reproductive effects (Health Effects Test Guidelines for Reproduction and Fertility Effects) from the US EPA's Office of Prevention, Pesticides and Toxic Substances (OPPTS). These guidelines encompass all of the requirements for reproductive tests under EPA's FIFRA and TSCA rules.

In pursuit of the primary objective, these guidelines were followed as closely as the reproductive behavior of mink would allow. As a result, we were able to obtain in a highly cost-effective manner, additional data useful for human risk assessment that were not sought by Aulerich *et al.*

1.2 Overview of the Study

1.2.1 Mink Reproduction

Mink (*Mustela vison*) were selected by the sponsor as the animal system to replicate the essential features of a previous study in mink (Aulerich *et al* 1979). The rationale for the strain selected is described in Section 2.6. The husbandry of mink is well established (Joergensen, 1985).

Mink have a single annual breeding season of approximately one month in duration, occurring between late February and late March in the Northern U.S. In males there is an annual recrudescence of testicular activity followed by regression. The spermatogenic cycle is at least 40 days. Mature sperm are reported to have an average age of 65 days at mating, with sperm concentration between 50,000 and 500,000/cubic millimeter. The males ejaculate approximately 80 million spermatozoa during a normal copulation lasting 15 to 60 minutes. If the female is not mated, the mature follicles degenerate and are followed by successive "waves" of replacement follicles. Ovulation is induced by copulation and occurs 36-42 hours afterwards. Most ova fertilized by the first mating do not develop. However, females will ovulate several times during the mating season when matings are spaced several days apart. Ova from rematings have higher survival rates. Mink exhibit delayed implantation of fertilized ova (embryonic diapause), with gestation varying between 39 and 72 days. The average gestation period is 49-50 days. The dams are very excitable and will abort if disturbed during pregnancy. In the Northern U.S., parturition occurs in late April to mid May. The average litter size is about 6, but ranges from 1 - 15. The lactation period lasts approximately 42 days. The kits consume mother's milk exclusively for 21 days and will begin to eat adult food beginning around the fourth week.

1.2.2 Plan of the Study

To determine the potential reproductive toxicity of DIMP to mink during a standard two-generation reproductive study, 35 female and 7

male animals were allocated randomly by weight to each of 5 groups for the first (F_0) generation. Two groups served as replicate untreated control groups while the others were fed DIMP-containing diets *ad libitum* at concentrations of 150, 450 or 2500 ppm, as the low, intermediate and high-dose groups, respectively (Table 1.2.2). All received water *ad libitum*. The F_0 generation was placed on dosed feed on 24Feb95, when they were 9 months of age. They were mated (within dose group) during late March; their offspring, (the F_1 kits) were born during late April-early May and were weaned in June, 1995. The F_0 sires were killed after mating was completed in March, and the F_0 dams were killed in June, 1995, after the kits were weaned. These F_1 kits were weaned onto the same diets as that consumed by their respective parents (same treatment group). In July, 1995, 35 female F_1 kits per group were selected as parents for the second generation, and the number of parental F_1 males was increased to 13 per treatment group (Table 1.2.2). All remained in the same dose group as their parents. The F_1 kits not selected as parents were killed in July, 1995, when they were 10-12 weeks old.

Like the F_0 animals, the F_1 's were mated in March (1996), they whelped in late April-mid May, and their young (the F_2 generation) were weaned in June, 1996. The F_1 males were killed and examined at the end of breeding in March, and all F_1 dams and their kits were killed at the time of weaning in June, 1996, for their respective examinations.

Table 1.2.2 Allocation of Mink to Treatment Groups

Dose (ppm)	F_0		F_1	
	Female	Male	Female	Male
0 (Control A)	35	7	35	13
0 (Control B)	35	7	35	13
150	35	7	35	13
450	35	7	35	13
2500	35	7	35	13

1.2.3 Rationale for Dose Selection

In Aulerich's one-year (one-generation) reproductive study (Aulerich *et al* 1979), groups of 30 mink (6 males, 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 that study, the offspring were born at the end of the ninth and beginning of the tenth month. In the females of the 450 ppm (high dose) group there were no deaths before 3 months, two deaths by six months and three more by 12 months. There were 5/24 (21%) deaths in the high dose females (450 ppm), 3/24 at 150 ppm and 2/23 at 50 ppm. No deaths occurred in the 24 untreated controls. The overall mortality (including two males that died during the first three months, one control and one at 50 ppm) was therefore 12/119 (10%).

The dosages employed in the current study necessarily included 450 ppm for one test group, to repeat the high-dose exposure employed by Aulerich *et al* 1979. It was the unexplained deaths at this dose that caused the controversy about the Aulerich study. The 450 ppm dose was innocuous in a 90-day study in nonpregnant mink (Bucci *et al* 1992, 1994) yet suspect in the Aulerich one-generation study, so this dose was included as the intermediate dose in the present study. The pattern of metabolism and excretion of DIMP in mink (Weiss *et al* 1994) suggested that all doses lower than 450 ppm should produce no toxicity. For the low dose in this study, 150 ppm was used; this had been Aulerich's intermediate dose. The potential effects of doses higher than 450 ppm over the one-year duration of each generation in this study were less predictable, but 2700 ppm was the lowest dose observed to cause a minor adverse effect in the 90-day study by Bucci *et al* 1992, 1994. The target high dose for the present study was therefore set at 2500 ppm.

Replicate control groups were used in this study. While there is no theoretical statistical justification for a double-sized control group when universal random biologic variation is assumed, this assumption may not be met with groups of mink of this size. Experience with mink reveals a larger range of outcomes than is seen with similar-sized groups of more conventional laboratory, farm and pet animals. Experience with mink on ranches and in the laboratory is replete with documented as well as anecdotal reports of unexplained adverse events, including abortions, kit mortality, and sudden death of dams. The second control group of 35 females and associated males was included to improve the likelihood of

observing representative variations.

1.2.4 Assessment of Toxicity

The animals were evaluated during the study by periodic measurement of food consumption, body weight, hematological and clinical chemical characteristics, clinical examination and reproductive performance. In both generations all parental animals and two kits per sex per litter were subjected to gross necropsy examination and eight to 13 kits per sex per litter were bled for clinical pathology evaluation. Numerous tissues in the parental females, the reproductive organs of the parental males and gross lesions from kits were examined microscopically. Additionally, semen analyses were performed on the F₁ males, and ovarian follicles were counted in 20 high dose and ten control F₁ females. Because DIMP is a potential inhibitor of acetylcholinesterase, cholinesterase was measured in plasma and whole blood and calculated in red blood cells of all parents and approximately ten kits per sex per treatment group. Acetylcholinesterase was also measured directly in brain tissue (basal ganglia, frontal cortex and striatum) in 8-12 female parental animals of each dose group of both generations.

1.2.5 Study Dates

The F₀ generation was placed on dosed feed beginning 24 February, 1995, and the in-life phase of the study ended with necropsy of the last F₁ dams and F₂ kits on 21 June, 1996.

2.0 MATERIALS AND METHODS

2.1 Chemical Composition of Diisopropyl Methylphosphonate (Dacre and Rosenblatt, 1987; Midwest Research Institute, 1991)

- CAS number: 1445-75-6
- RTECS number SZ9090000 (1983-1984 Supplement)
- Structure: $[(CH_3)_2(CHO)]_2(CH_3)P=O$
- Molecular Formula: C₇H₁₇O₃P
- Molecular Weight: 180.18
- Physical Appearance: clear, slightly viscous liquid
- Density at 23.3°C and 708.7 mm Hg: 0.9754±0.0008 g/ml
- Boiling Point: 174°C

- 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: $3.2 \times 10^{-6} \text{M sec}^{-1}$
- 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 Inc., LTD, Windham, NH, to be approximately 97% pure. Two batches were used in the study, Lancaster Synthesis batch # 10005098 and # 10009217. Both were analyzed by Midwest Research Institute (MRI), Kansas City, MO. The characterization consisted of determination of physical properties, spectroscopy, water analysis and chromatography. The first lot is described in the 16Oct95 report from MRI, Appendix 1A, detailing the methods and instrumentation, the spectra obtained by ultraviolet (UV), 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. Results of similar analyses of the second batch (#10009217) were reported by MRI in their 18Mar96 report (Appendix 1A).

In brief, infrared and ultraviolet/visible spectroscopy and nuclear magnetic resonance and physical characteristics were all consistent with the structure of DIMP.

In the first batch, chromatographic analyses indicated two impurities of 1.3% and 0.6% relative to the major component (DIMP), and water content of 0.99%. The test substance was identified as DIMP, with a purity of 97.1%. The density was 0.979 grams/milliliter.

In the second batch, a slight increase in absorbance was observed between 320 and 200 nanometers with UV, and a trace impurity detected with NMR was attributed to the solvent. Chromatographic analyses confirmed the major component to be DIMP, with minor impurities (approximately 0.1 and 0.7%). Water content was determined to be 0.17%. No other impurities were

observed up to 20 minutes after the major peak was eluded. The test substance was identified as DIMP, with a purity in this batch of approximately 99%. The density was 0.976 grams/milliliter.

The first batch (#10005098) was fed to the F₀ generation and to the F₁ generation through October, 1995. Thereafter, the second batch (#10009217) was used.

2.3 Animal Feed

The basal ration was a commercial product formulated at the North Branch Fur Farm, the source of the mink used, to meet or exceed the minimum nutrient requirements for mink as outlined in the National Research Council (1982). Specific components of the diet varied as a function of availability and cost. The following is a typical example of the composition: 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 was 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 since the issue concerned groundwater and drinking water, but the mink's diet contains approximately 65% moisture, and their daily requirement of approximately 200 gm of water is met principally through feed. (Full-grown animals consume 225-285 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 route of dosed feed. The DIMP used in the study reported by Aulerich *et al* 1979 was administered in feed, as well.

For each dose level of DIMP, dosed feed was prepared as described in Section 2.5, below, and dispensed into individual-portion cans, sealed with "pop-tops", color coded by dose group, frozen and stored at -20°C until feeding.

From the batch of control feed prepared on 1Aug95 one can of control feed was selected at random for nutrient and contaminant testing and was shipped frozen to Corning-Hazleton Inc., Madison, WI, for the following analyses: moisture, ash, crude protein, crude fat, crude fiber, vitamin E, aflatoxin (B1, B2, G1, G2), free fatty acids, peroxide value, aerobic plate count, arsenic, cadmium, lead, mercury and a general pesticide screen. The screen included 124 organochlorinated compounds, 130 organophosphate compounds, 33 organonitrogen compounds and 20 N-methylated carbamate compounds. The compounds are listed in Appendix 1B together with the analytical methods and results, in the Corning-Hazleton report dated 10Dec96. No results were available from the organonitrogen part of the screen because of chromatographic interference from the sample. All other values were within acceptable ranges; in particular, organophosphate insecticides were below the screening limit of 50 parts per billion.

2.4 Water Analysis

The water provided to the University of Minnesota was analyzed in February, 1996 (St. Paul Water Utility, St. Paul, MN) for heavy metals and other chemical characteristics, and water samples from the rooms in which the mink were housed were analyzed commercially (SERCO Laboratories, St. Paul, MN.) for coliform bacteria and heavy metals in June, 1996. None were present.

The list of compounds measured and the results are included as Appendix 1C.1 (St. Paul Water Utility), and 1C.2 (SERCO Laboratory).

2.5 Formulation and Verification of Dosed Feed

2.5.1 Formulation

The basal mink ration was obtained from North Branch Fur Farm, North Branch, MN, the supplier of the mink. It was delivered to U of MN by truck in steel drums containing about 200 kg each, and held at approximately 4°C in a U of MN refrigerator for one to three days, until U of MN personnel added the DIMP, dispensed the feed into single-serving cans and stored the prepared formulation at -20°C until used.

The amount of freezer space available for storage limited the dosed feed that could be prepared at one time to about 3 weeks' supply. Thus, dosed feed was prepared at approximately three-week intervals. Limits of available space and personnel precluded preparation of the

entire three-week supply of dosed feed in a single day; four consecutive days were required, with two dose levels prepared on one of the four consecutive days. The dose level prepared first was rotated with each consecutive batch, so the pre-mix storage time at freezer temperature would be randomized across treatment groups.

The dosed feed was prepared according to the formula tabulated below (Table 2.5.1), based on 97% purity of the DIMP.

Table 2.5.1 Quantity of DIMP Incorporated in Feed

Target Dose (ppm:mg/kg)	Milliliters of DIMP per 363 kg feed	Milliliters DIMP/363 kg (0.98 gm/ml; 97% pure)
0	0	0
150	57.6	60
450	172	180
2500	959.6	980

This formula was not adjusted after the purity was demonstrated to be 99% in the second batch. Actual concentrations of 106.7-108.9% of the target doses were achieved with the 97% product. At 99% purity, the amount of DIMP offered to the F₁ generation was approximately 2% greater (Table 2.5.2, below).

The DIMP for each dose was diluted to 15 liters with tap water and mixed for 5 minutes by agitation. This DIMP premix was added to 181 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 then 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.

The food-handling equipment was thoroughly emptied between batches, washed daily with hot water and sanitized 15 minutes with 5% sodium hypochlorite solution.

2.5.2 Verification of Target Doses and Homogeneity of Mix

For the first nine batches of feed prepared (spanning the period February, 1995 through July, 1995), one can per 100 of each dose group was selected randomly and shipped to MRI to analyze for DIMP content and lot homogeneity. Results per batch and specific instrumentation and analytical methods used for these analyses are detailed in the MRI report dated February, 1997 (Appendix 1D).

To determine actual DIMP concentrations in dosed feed samples, MRI weighed approximately 40 g of feed into 200 ml centrifuge bottles that contained ten 5/16" stainless steel ball bearings. A 100 ml aliquot of methanol was pipetted into the bottle, and the bottle was sealed. The DIMP was extracted into the methanol with 15 minutes 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 duplicate.

The target versus average actual concentrations of DIMP in nine lots of the dosed feed (prepared with DIMP of batch #10005098, 97% pure and density of .98 g/ml) are tabulated below, Table 2.5.2. percent relative standard deviation (%RSD) is a measure of variance among samples from the same dose levels (homogeneity of mix), with %RSD of 5% or less being desirable. MRI found the %RSD to be 6.7%, 2.3%, and 3.7%, respectively in the 150, 450, and 2500 ppm formulations. These were considered acceptable.

Table 2.5.2 DIMP in Dosed Feed-Target Concentration vs. Actual
(ppm; mg/kg)

Target Dose (mg/kg) ppm	Batch #10005098 (97%) Fed to F ₀ and F ₁		Batch #10009217 (99%) Fed to F ₁	
	Actual Dose (mg/kg) ppm*	Percent of Target Dose*	Actual Dose (mg/kg) ppm **	Percent of Target Dose **
150	162	108.3	165	110.0
450	480	106.7	490	108.7
2500	2722	108.9	2774	111.0

* Results of analysis of nine lots of feed prepared over a period of five months, using DIMP of Batch # 10005098 (97% pure). This product was fed to all F₀ mink and to the F₁ generation from weaning through October, 1995 (approximately 4 months).

** Result of calculation, using the same formula for incorporating DIMP as for the 97% pure batch (Table 2.5.1), and using the percent of target dose achieved by that formulation procedure. Assuming constant preparatory methods, this calculation establishes the increased exposure to DIMP resulting from the greater purity (99%) of the second batch. This product was fed to the F₁ generation from November, 1995, to completion of the study in June, 1996.

These values were very close to the 10% variance from the target dose that was (arbitrarily) specified in the protocol. The rationale for selecting the target doses is given in Section B.1.2.3 of this report. The actual doses achieved were judged to be as acceptable as the original target doses, since they effectively represented the same 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 (prorated, in the F₁ generation, between the 97% and 99% purity).

A stability study conducted by MRI previously (MRI, 1991) had demonstrated DIMP at 50 ppm in mink feed to be stable for 24 hours at room temperature (to simulate feed in the animal cage) and also frozen at -20°C for 7, 14, 28, 61, 90, and 120 days. In an accelerated stability study, DIMP was also 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. This stability analysis was not repeated for the present study.

2.6 Animals and Animal Husbandry

"Ranch Wild" brown mink were used in this study because the "dark" variety genotype used by Aulerich (Aulerich et al, 1979) is no longer produced. Fur market forces determine which stocks are favored and maintained. Dr. William Wustenberg, our mink consultant, Mr. Gene Rabel, breeder of the mink used in this study, and Dr. Aulerich himself all assured the Study Director that the "Ranch Wild" strain is the current stock that is genetically most closely related to the strain used by Dr. Aulerich.

For the first generation, 220 nine-month-old brown "Ranch Wild" type mink (185 females and 36 males), obtained from North Branch Fur Farm, North Branch, MN, were randomized by body weight into five experimental groups as outlined in Section B. Table 1.2.2. The mink used had been vaccinated at eleven 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.

The animals were transported by truck approximately 40 miles between the source ranch and the U of MN, on February 8 and 9, 1995. They were housed in 14 rooms on two floors of the Veterinary Science Building, College of Veterinary Medicine, U of MN and were quarantined for approximately 2 weeks. Animals of all dose groups were represented randomly in all rooms. Males were in separate rooms from females.

Female mink 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 "penthouse", approximately 22x22x45 cm atop the rear of the cage, to which the mink had unrestricted access. Males were housed individually in cages that were 75x53x38 cm and had no penthouse; the larger dimension and plain

floor plan facilitated their use as breeder cages. The females were transferred to individual, large whelping cages a few days before being placed with males for breeding, and were returned to them after mating. Acclimation to the new larger cage before mating avoided the stress of this change immediately after breeding, which could have affected conception. These "nesting" cages were also 75x53x38 cm, with a 32x18x26 cm wooden nest box affixed to the rear. The USDA, NIH, and U of MN Animal Care and Use Committee approved wood for these boxes as an "environmental necessity" for mink. Wood provides warmth as well as insulation from noise and vibration. Aspen shavings were used for bedding.

Daily photoperiod determines the breeding season for mink, therefore control of the artificial lighting was critical. Lighting in the animal rooms was adjusted approximately weekly to match the outdoor sunrise/sunset time, although no dawn or dusk "fade" was provided. Light intensity was 325 lux measured one meter from the floor. Animal rooms were held at $23\pm 5^{\circ}\text{C}$ and 15 - 70% relative humidity. The ventilation system replaced the room air with 100% fresh air, 13 - 17 times per hour.

After one week in quarantine, the animals were weighed, anesthetized with 40 mg ketamine and 1 mg xylazine/kg BW administered intramuscularly, bled via the jugular vein for hematologic and clinical chemical baseline pretreatment values, and an electronic transponder (INFOPET, St. Paul, MN) was emplaced hypodermically in the interscapular space.

The transponder provided positive unique identification of each animal in the form of a seven-character alphanumeric. This same "number" was available in barcode form on each cage card and blood tube, together with other cage card data that provided room number and cage number. This alphanumeric was cumbersome to use manually, so the F_1 and F_0 generation members were assigned a sequence number (which appears as their individual identification number in the tables of this report). A tabulation of the transponder alphanumeric, the corresponding sequence number and the animal's cage number is included in Appendix 2.

The mink cages, color-coded for dose, were on metal supports, with plastic-backed absorbent-lined paper on the floor beneath. Feces that did not fall through the mesh floor of the cage were removed 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 U of MN.

The cages were equipped with aluminum drinking cups that were cleaned and refilled twice daily. Residual water was siphoned off manually and was replaced with fresh water. Individual mink that habitually played in their water and caused spillage were attended to more frequently.

Clinical observations were recorded once daily. Food consumption over a 24-hour period was measured once weekly. Animal body weights were obtained once weekly, but to avoid upsetting the pregnant or lactating dams, mink were not weighed after mating until the day they whelped, then were left undisturbed during lactation, except weights were taken on the 28th day after whelping, and again at weaning, approximately 6 weeks later. The kits of both generations were weighed as litters (including any dead kits) on the day of birth and surviving kits were weighed again at 28 days and individually at necropsy.

The F₀ females were weighed at allocation (16Feb95), 6Mar95, 15Mar95, the day of whelping, again on 22Jun95 and at necropsy (26-28Jun95). In the Individual and Group Mean Body Weight tables (Appendix 3B), the whelping weight column for F₀ females is dated 5-4-95. This date was used for convenience; it was the midpoint of the whelping period of 20Apr-12May95. The 28-day weights were obtained on the correct day for each litter, but the column in the 28-day tables is similarly dated 1Jun95, the approximate mid-point, for convenience.

The F₀ males were weighed at allocation (16Feb95), on 23Mar95, and again at necropsy, 27-28Mar95.

The F₁ kits retained as breeders for the second generation were selected randomly with each litter represented by one or more kits, vaccinated as described above, and weighed weekly until mating, when a schedule similar to that used for F₀ animals was followed. Those F₁ kits not retained were sacrificed at approximately 11 weeks of age.

The F₁ generation was tabulated beginning 18Jul95, when they were on average 10 weeks old. They had no interruption in exposure to their respective DIMP diets since weaning, however. Weekly body weights were obtained through 26Mar96 for the males, but ended 13Mar96 for the females. The whelping weight column is dated 5-3-96 (midpoint) for the F₁ dams, as noted for the F₀'s. They were weighed again 28 days after whelping (tabulated as 6-1-96) and at necropsy 10-21 June 96. The F₂ kits were weighed at birth, at 28 days and at necropsy 10-21 June 96, when they were approximately 6 weeks old.

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, one day per week 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, weighed as accurately as possible, and recorded as uneaten food.

Animals requiring veterinary attention were examined and treated by Dr. Victor Perman, in consultation with our mink consultant, Dr. William Wustenberg. The owners and managers of the North Branch Fur Farm, the source of the animals and feed, also provided experienced counsel. The sporadic medical conditions encountered were typical of commercial mink operations, unrelated to DIMP exposure. They included bone fragments (from feed) lodged in the mouth, "wet belly", (a urine scalding condition of unknown cause), central nervous system seizures, excess body fat in males, and nursing disease which is related to the stress of lactation (associated with inappetance, electrolyte loss and, sometimes, death). These conditions were treated symptomatically using methods standard to current veterinary practice.

Food weights and individual animal body weights were collected with Mettler brand balances and recorded manually during the period February, 1995 through August, 1995, at which time a proprietary software system (LABCAT, Innovative Programming Associates, Princeton, NJ) was installed. Thereafter, weights of animals and cans were entered directly into a computer system, using the bar coded cage card to identify the animals. The earlier, manually collected data were entered into this system retrospectively.

The 400-gm daily food ration was sufficiently excessive to provide *ad libitum* feeding except during lactation, when additional feed was required by some animals. Additional feed was provided as a second partial or full can.

2.7 Mink Breeding

Beginning in mid-March for each generation, the females were placed individually with males of the same treatment group in the male's cage and observed for copulatory behavior. Within minutes of copulation, seminal fluid was aspirated from the vagina of the female, to confirm the presence of spermatozoa. This procedure was conducted once for each male, to confirm his ability to inseminate. The males differed in their apparent interest in mating, independent of treatment; some were consistently efficient and others were indifferent. This behavior on the part of F_0 males prompted our protocol amendment to increase the number of available males in the F_1 generation. Several days were required to mate all females. Each female was mated a second time, nine days after the first mating, to the same male or within the same dose group whenever possible. The identity of the mates at each mating was recorded. Sibling matings were avoided in the F_1 generation since they were born in the laboratory and their genealogy was recorded. We did not know the familial relationship of the F_0 animals but because they were drawn from a pool of 20,000 animals on the source ranch, we believe it unlikely that siblings were mated in our small sample of that population.

During the whelping period of each generation the nest boxes were checked daily. The sex of each kit was determined and the newborn kits were weighed as a litter (not individual kit weight), on the day of birth. The kits were weighed again when 28 days old and at sacrifice. Litter data that were recorded included length of gestation, number of still births, sex ratio, litter size (weight and number of kits) and kit mortality.

Litter size ranged from 1 to 14 kits. In multigeneration studies in rodents it is customary to standardize litters to a maximum size, to reduce variation in the competition among offspring and the amount of stress on the dams. We expected less variation in litter size; our original protocol specified that litter size would not be standardized. In view of the large range in number of kits born per litter, litters were reduced to a maximum of six kits when the litter exceeded six. Excess kits were removed by seven days of age; the number of kits of each sex that was removed was recorded and is retained in the study file.

2.8 Hematology and Clinical Chemistry

Chemical and hematological examination of blood specimens from all mink was conducted by the Hospital Laboratories, College of Veterinary Medicine, U of MN. Dr. Victor Perman, a veterinary clinical pathologist and co-

investigator on this study, performed the venipunctures. Samples (approximately 8 ml) 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 three tubes, one plain to allow blood to clot (approximately 4 ml for general biochemistry) and two 2-ml tubes containing potassium EDTA anticoagulant, one for hematology and one for whole blood and plasma cholinesterase analysis. The samples were submitted to the laboratory for processing within one hour.

Dr. Douglas Weiss of U of MN, Chief of Hospital Laboratories at the College of Veterinary Medicine, supervised analysis of the specimens. The Study Director, Dr. Bucci, evaluated the results.

Each F₀ animal was sampled at allocation in February, 1995, and again at necropsy (end of March, 1995 for males, and June, 1995 for females). The F₁ kits not retained for breeding were sampled when necropsied in July, 1995. They were represented by 7-13 males and 8-11 females per treatment group.

Blood samples were taken from the F₁ parents of the second (F₂) generation in the same manner, first as kits (8-11 weeks old) in July, 1995, and then in September 1995, December 1995, and at necropsy in March, 1996 for the males and June, 1996, for the females. The F₂ generation were also sampled from each treatment group, for the same blood analytes, at their necropsy in June, 1996. They were represented by 8-12 males and 8-11 females per treatment group.

2.8.1 Hematology

For leukocyte differential cell counts and red cell morphology, two wedge blood films were made and stained with a Hema-Tek Model 4480 stainer (Miles Scientific, Naperville, IL) with a Hema-Tek Stain Pak 4481 (Miles Scientific, Naperville, IL), a Wright's stain. For reticulocyte counts, 2 drops of anticoagulated 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 (500 from each film) 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 manually as to cell type, i.e., polysegmented neutrophil, immature granulocyte, eosinophil, basophil,

monocyte, lymphocyte, blast cell or other(s). Leukocyte data were reported as an 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 are listed below.

Established criteria (Bessis, 1973; Jain, 1986; Viskochil *et al* 1978; Perman and Weiss, 1991) were used to classify the morphologic changes, and they were quantified subjectively as "normal" or "rare" and, when in greater number, graded as 1+ to 4+.

- 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 caused by dehydration or membrane alteration.

Quantification of erythrocyte indices, platelets and leukocytes was done on a S Coulter Model+ 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.

2.8.2 Clinical Chemistry

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, Brea, 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 CX3/CX3 DELTA Analyzer system (Beckman Instruments, Brea, CA) using the manufacturer's recommended methodology. The analyses for serum cholinesterase, whole blood cholinesterase, brain acetylcholinesterase, albumin, total protein, plasma cholinesterase, alanine aminotransferase and aspartate aminotransferase were conducted with Beckman CX4 and CX7 analyzers (Beckman Instruments, Brea, CA). The methods in the manufacturer's manual (Beckman Instruction 015-2477712-G) were followed except for albumin, which was measured by the method of Poumas and Bigg (1972), using Boehringer Mannheim Diagnostic-PN 5107242H reagent, and the cholinesterases, for which the Boehringer Mannheim kit No. 124117 was used (Ellman method).

Red Blood Cell Cholinesterase was calculated with the formula:

$$\text{RBC ACHE} = \frac{\text{whole blood CHE} - [\text{Plasma CHE} \times (1 - \text{Hct})]}{\text{HCT}}$$

The instruments were coupled to a computer system which created a database and printed data in chart form. All data were verified and entered into the LABCAT system for statistical analysis.

2.9 Necropsy and Histopathology

At the scheduled termination of the study for each generation, all animals were anesthetized for collection of the final blood sample and were then euthanized by carbon dioxide asphyxiation while anesthetized. For euthanasia of F₂ kits in June, 1996, sodium phenobarbital was used instead of carbon dioxide, because young kits are resistant to high concentrations of carbon dioxide.

Detailed gross necropsy examination was performed on all parents of both generations and on approximately two kits of each sex per litter from both generations.

Necropsy, organ weight determination, tissue collection and processing, and histopathologic evaluations followed the procedures of the National Toxicology Program (NTP, 1992) and the Standard Operating Procedures of Pathology Associates International. At necropsy of the kits, special emphasis was placed on examination of common sites of developmental malformation (birth defects). These included limbs, digits, eyes, palate, brain, heart, kidney and major blood vessels. In addition, a specimen of brain including the striatum, basal ganglia and frontal cortex was obtained from 8 -12 parental females from each treatment group of both generations.

The complete Clinical Pathology Report is included as Appendices 5, 6 and 7 and the Pathology Report is Appendix 9A.

3.0 STATISTICAL ANALYSIS

Body weight, organ weights, food consumption, ovarian follicle counts, blood chemistry and continuous hematology data were analyzed using a two-tailed analysis of variance followed by Dunnett's T test, with significance set at Type I error of 5% or less. This is an appropriate test for pair-wise comparison of means (each treated group compared separately to the control group). Aulerich *et al* (1979) also used this analysis. In the present study with two untreated control groups, one of them, Control Group A, was used as the basis for comparison. Control Group B was compared to Group A in each analysis, as was each treated group. Control Group B was expected to be similar to Group A. For clarity, the option to combine the two control groups was not elected.

For most parameters evaluated, the individual animal data were entered into a proprietary computer system (LABCAT, Innovative Programming Associates, Princeton, NJ) that calculated mean values per treatment group, together with the standard deviation, two-tailed analysis of variance and Dunnett's T test. Each computational

function of the system was validated by a parallel calculation using an alternate system.

Sperm motility, total count and morphology data were calculated and compared across groups using the Kruskal-Wallis nonparametric ANOVA test. If a significant effect occurred ($p < 0.05$), the Wilcoxon (Mann-Whitney U) test would be used for pair-wise comparisons of each treated group to the control group. Statistical analyses were performed using an IBM™ compatible computer with SAS computer programs (SAS/STAT User's Guide, 1989).

Derived values that were not collected as raw data, for example, red blood cell cholinesterase and the overall average body weight, average food consumption and amount of DIMP consumed per kg BW/day were calculated with Microsoft Excel spreadsheets or with an electronic calculator. Group mean values derived in this manner were also entered into the LABCAT system for statistical comparison.

The males and females were analyzed separately.

4.0 RESULTS AND DISCUSSION

Within each endpoint evaluated, the study outcome will be described first for F_0 and F_1 females. The outcome in the F_0 and F_1 males will then be described, followed by the respective kit (F_1 and F_2) data.

The authors elected to include "Discussion" together with "Results" because of the length of the Results section. Including Discussion precludes having to refer continually between sections.

For brevity, the data presented in this section have been extracted from reports that are more comprehensive. The findings tabulated in this section are those judged by the authors to be most relevant. For all data except Clinical Observations, the referenced Appendix contains Individual Animal Reports and for most endpoints, Summaries of Mean Values per Treatment Group. Included in the Summary Tables are group means and standard deviations, the number of animals in the group, and statistical analysis (comparison of variance and equality of means by two-tailed analysis of variance followed by Dunnett's T-test). For Clinical Observations the Appendix includes a comprehensive summary; individual observations are included in the Study Files. In most tables that follow, the group means from the respective Summary Tables have been presented without the standard deviations that accompany them in the referenced Appendix. Statistical differences are shown, however.

4.1 Early Deaths

During the study period of the F₀ generation (February through June, 1995) six of the 175 females (3.4%) died before the scheduled sacrifice. In the F₁ generation (July, 1995 through June, 1996) there were eight early deaths among 175 parental females (4.6%). The dates of the deaths and the distribution of the mortality among dose groups are tabulated in Tables 4.1.a and b, followed by a short summary of the clinical course of each animal.

Most of these animals were noted to be ill before death, and were examined carefully. In several instances medical treatment was attempted. Blood was obtained from two to assist in diagnosis, and complete necropsy and microscopic examination were performed on all. As a general rule, mortality caused by a toxic agent is expected to occur earliest in those exposed to higher doses, and the overall number affected per treatment group is expected to be proportional to dose. There was no such pattern of dose or duration in the occurrence of mortality in this study, to incriminate exposure to a toxic agent. A probable cause of death was identified in all but one F₀ female (450 ppm) that died after 90 days on study, and 3 F₂ kits from an F₁ control mother. There was no evidence that exposure to DIMP was the cause of the early deaths.

The most frequent constellation of clinical signs included partial to complete lack of appetite (anorexia), markedly reduced activity (lethargy), and occasionally, dark or light diarrheal feces. Examined at necropsy, these animals had yellow (fatty) livers, yellow kidneys and reduced or absent thymic tissue. Some also had hemorrhage in the stomach or small intestine. Microscopically, such animals had vacuolated (fatty) livers, degeneration of kidney tubules and thymic atrophy. These are non-specific changes that follow severe metabolic stress associated with sharp reduction in nutrient intake in animals having a very high metabolic demand. The constellation of changes described above was described comprehensively in mink having "nursing disease", in which dams unable to meet the heavy metabolic requirement of milk production late in the lactation period succumb to the negative nutritional balance (Schneider and Hunter, 1993). A comparable syndrome occurs in high-producing dairy cattle where the condition is known as "ketosis", in diabetics with acute insulin/glucose imbalance, and in more chronic form, starvation from all causes. In dairy cattle and in humans, where the condition has been well characterized, lack of sufficient glucose from dietary sources causes the body to metabolize its fat stores and its muscle protein. With deficiency of dietary glucose, this process is incomplete, producing metabolic byproducts (e.g., ketones) that further suppress appetite (Bruss, 1989). The fulminant clinical course in mink appears to be

related to their inherently high metabolic rate. In them, any event that causes reduced food intake for only a few days precipitates the negative balance. In Table 4.1.a and b below, animals in which we diagnosed this condition are identified as having "stress syndrome" as the cause of death.

Table 4.1.a Early Deaths, F₀ Female

Treatment Group (ppm)	No. Deaths (Animal No.)	Date of Death	Cause of Death
0 (Control A)	0/35		
0 (Control B)	1/35 (#220)	7May95	Stress Syndrome associated with fetal death in uterus
150	2/35 (#27) (#103)	14May95 11May95	Stress Syndrome Stress Syndrome - Possible Nursing Disease
450	2/35 (#136) (#202)	23May95 26Mar95	Undetermined Stress Syndrome
2500	1/35 (#201)	12Mar95	Stress Syndrome assoc/with abscess behind one eye

Table 4.1.b Early Deaths, F₁ Female

Dose Group (ppm)	No. Deaths (Animal #)	Date of Death	Cause of Death
0 (Control A)	1/35 (#414)	26Dec95	Stress Syndrome associated with anesthesia
0 (Control B)	1/35 (#475)	27Feb96	Stress Syndrome associated with anesthesia
150	2/35 (#502) (#495)	15Aug95 2Jan96	Seizure disorder Stress Syndrome associated with anesthesia
450	1/35 (#535)	1Jan96	Stress Syndrome associated with anesthesia
2500	3/35 (#573) (#574) (#582)	25Dec95 1Jan96 24May96	Stress Syndrome associated with anesthesia Stress Syndrome associated with anesthesia Stress Syndrome associated with ruptured uterus/peritonitis

Animal # 201 (2500 ppm, F₀), the first unscheduled death, is an example in which the precipitating cause was apparent: this animal had an infected eye and went "off feed" as a result. If she had returned to normal appetite, she might have survived. Her demise was characterized by a less acute course of the consistent but nonspecific changes described above. Animal #201 was noted to have an inflamed eye as early as 24Feb95, the first day of her exposure to dosed feed. She had weighed 1077 grams at allocation on 16Feb95. She was noted to have eaten 10.2 gm of feed on 23Feb95 and 7.5 gm on 3Mar95, when her cohort was averaging about 130 gm/day. At her death on 12Mar95 she weighed 565 gm. At necropsy she had a periorbital abscess, a tan, mottled liver and she was markedly thin. This animal received dosed feed for only a few days and ate little of it; she consumed less DIMP than all other dosed animals in the study. The sudden and almost complete loss of appetite in this animal and her rapid loss of condition prompted the decision to attempt to restore appetite in future cases.

Animal #202 (450 ppm) died on 26Mar95. This animal weighed 1483 gm on 16Feb95, ate normally on 23Feb95 (184.7 gm), but only 13.8 gm on 3Mar95.

Animal #136 (450 ppm) had a record of sporadic inappetance, whelped normally on 3May95 and was found dead on 23May95, having not eaten on 21 and 22May. She had weighed 1152 gm on 16Feb95, 959 gm on 4May95, and 679 gm on 23May. She was very thin, but her organs appeared normal at necropsy and microscopically. Her four kits were euthanized. She appears to be a "nursing disease" victim, but without fully-developed liver lesions.

Animal #103 (150 ppm) was sacrificed when moribund on 11May95. She had whelped four normal kits on 29Apr95 but her food consumption decreased abruptly from normal (~150 gm/day) through mid-April to 50-80 gm after about April 20. Her body weight had been 1259 gm on April 29 immediately after whelping and was 882 gm at necropsy, where she was found to have the now expected swollen fatty liver. Her clinical illness began before her kits were born, so she is not a typical case of nursing disease.

Animal # 220 (control), died on 7May95. Her food consumption had been erratic but she was anorexic terminally (14 gm on 4May95, nothing on 5May95). She had a history of mucoid and bloody discharge associated with soft feces, beginning on 1May95. At necropsy the major finding was 10 macerated fetuses of expected (near-term) size, that apparently had died in utero in the past few days. The earlier discharges most probably signaled impending fetal death, followed by fetal death, then death of the dam.

Animal #27 (150 ppm) died on 14May95, having not eaten since 7May95. She weighed 1419 gm at allocation on 16Feb95 and 890 gm at death. She had bloody diarrhea, fatty liver, degeneration of kidneys and her uterus contained five normal late-term fetuses.

It should be noted that these F₀ animals all died within 90 days of beginning ingestion of DIMP (except one was an untreated control). In 1991 we fed groups of 10 females 2930, 6174 or 8990 ppm for 90 days and none died. They were not pregnant but pregnancy *per se* did not appear to be a susceptibility factor for DIMP in this study. Of the 14 deaths in both generations of females, only six were in pregnant or lactating animals. Of those, one control had intrauterine fetal death, a 2500 ppm animal had a ruptured uterus and a 450 ppm animal died within the first week of breeding and may not have been pregnant.

Several of the F₁ females succumbed to this "final common path" after experiencing a definitive stressful event that caused reduced food intake, even before the animals were pregnant. Animals #414, 495, 535, 573 and 574, representing all treatment groups including the untreated Control A group, were anesthetized for the blood collection of mid-December, 1995. The record reveals that a number of animals had difficulty coming out of the anesthesia and several including those above went "off feed". Because the consequences of that symptom were ominous, subcutaneous injection of lactated Ringer's solution and tempting the animals with cat food were tried, to provide nutrient energy. Some animals recovered their appetite over several days, but the foregoing animals did not, and died over a short span of time in late Dec 95 and early Jan 96. Animal #475, another untreated control, died on 27Feb96 after several days of not eating following anesthesia, similar to the five animals that had died in late December 95 - early Jan 96. Necropsy and microscopic examination again revealed fatty liver, degeneration of kidneys and atrophy of the thymus. In evaluating this situation, we concluded that the mink were overweight, i.e., their body fat percentage was high, their anesthetic dose was administered on a per kilogram basis. The heavier weight caused them to receive relatively high doses of anesthetic (the extra fat does not help to metabolize the anesthetic). The overweight condition was difficult to avoid. Mink naturally put on fat as the winter progresses, except these laboratory-bound animals had no cold stress to overcome. We were reluctant to restrict food consumption, to avoid restricting access to the test article. The result was mink that were about 25% heavier than their counterparts in unheated housing on the source ranch.

Animal # 582 (2500 ppm) was euthanized when moribund on 24May96. She had been lethargic and not eating for ten days, and had dramatic weight loss. At necropsy, she had five decomposing full-term fetuses free in the abdominal cavity, the result of a ruptured uterus. There was generalized peritonitis. Animal #502 (150 ppm) was one of several animals that had repeated brief seizures, especially when excited by handling, cage cleaning, etc. This is an affliction of mink that apparently has a genetic basis. This animal's unexplained death at the age of 3 months followed several days of lethargy. (The seizure disorder is not usually fatal.) Necropsy and histology were non-contributory.

4.1.1 Early Deaths, F₀ and F₁ Male

One parental male died, an F₁ from the 450 ppm dose group, on 2Jan96. This animal was one of the group which included five females that died following anesthesia for the mid-December blood collection.

4.1.2 Early Deaths, Kits

Three F₁ kits died before the scheduled sacrifice at 11 weeks. Each came from a different litter. One control-group male died on 28Jun95 with a dilated esophagus and food impaction. The dilated esophagus may be a genetic condition. On 2Jun95 a female from the 150 ppm group and on 8Jun95 a male from the 2500 ppm group died, each having had its tail tip bitten off by a litter mate. The mother then continually licked the stump, preventing the blood from clotting and the kits died from blood loss.

Three F₂ kits died, all males from the litter of F₁ control animal #430; one died on 27 May and two on May 31. The cause of death could not be determined by necropsy and microscopic examination.

4.1.3 Early Deaths, Interpretation

Our conclusion is that each of these deaths was a consequence of mink husbandry and the inherent nature of the species. The deaths had no systematic relation to dose and are considered unrelated to exposure to the test compound. Note also that in the study reported by Aulerich *et al* (1979), 10 of 95 females in the 12-month study were early deaths (10.5%), with 450 ppm as the highest dose. In the F₁ phase of the present study, the 175 female mink experienced only 4.6% early mortality, despite consumption of 2774 ppm by the highest dose group and 490 ppm by the middle dose group. If the deaths in the Aulerich study had been caused by DIMP with 450 ppm being the highest dose used, we should have seen comparable lethality at that dose, and greater mortality in our 2500 ppm group, but we did not.

The incidence of nursing disease typically peaks around 42 days after parturition, when the kits are at weaning age (large) and the dams have been under stress for the entire lactation period to balance energy intake with depletion. Of the 14 females that died during this study, only one was nursing (F₀, #103). She had become anorexic before her kits were born and she died when they were only 12 days old, so she was not a typical nursing disease. The number of kits being nursed is a major factor in precipitating the disease (Schneider and Hunter, 1992a) as is warmer ambient temperature (*Ibid*, 1992b). Our removal of kits in excess of six per litter, plus using a controlled-temperature environment, may have had a protective effect. Kits were not reported to be removed in the Aulerich study (Aulerich, 1979). Schneider and Hunter (1992b) also

reported a difference in susceptibility to nursing disease among different color phases of mink on the same ranch, but the differences were not consistent between ranches or in succeeding years.

Variations such as those noted above may well have contributed to the differences in mortality that occurred between the Aulerich study and the one reported here.

4.2 Food Consumption

Individual daily food consumption was recorded and tabulated, based on approximately one measurement per week. The mean daily food consumption per treatment group was calculated and compared statistically (Appendix 3A). There was no difference in average daily food consumption among the groups of F_0 males, approximately 200-240 gm/day. Among the F_0 female groups the mean daily food consumption ranged from approximately 182 to 212 gm/day. The 182 gm average for the 150 ppm dose group was significantly less statistically than the 212 gm average for the Control A group. Because there was no dose-related pattern of decreased food consumption, this difference was considered not significant biologically.

Mean daily food consumption per group of female mink is plotted (at monthly intervals) in Fig. 4.2.a (F_0) and Fig. 4.2.b (F_1).

Tables 4.2.a (F_0) and 4.2.b (F_1) contain a column showing the overall mean food consumption per day by the respective treatment groups.

In the F_1 generation, within sex, the treatment groups all consumed approximately the same amount of feed overall, 217 - 235 gm/day by males and 140 - 144 by females. The similarity in amount of food consumed by the males of both generations was notable. The food consumed per 100 gm of body weight was consistent across all groups, particularly in the F_1 generation. (See gm food consumed/100 gm BW in Table 4.2.b).

Whereas the food consumption listed in Table 4.2.b for the F_1 generation reflects the overall daily intake averaged over the 11-month period when the animals were between 3 and 13 months of age (July 95 - May 96), that for the F_0 's reflects only the 4-month interval from Feb 95 - June 95, when that generation was 9-12 months old. During the corresponding age period for the F_1 generation, the F_1 's food consumption was not significantly different from their overall lower average, so the age difference does not explain the higher food

usage by the F₀ generation (see Fig. 4.2.a&b).

In the 90-day study of DIMP in mink, (Bucci *et al* 1992, 1994), there was no effect of DIMP on food consumption up to 6174 ppm DIMP in the diet and the overall food consumption by the males (10 per dose) was 240 gm/day, very close to the 217 - 235 gm/day seen in the present study. The females in the 90-day study consumed an amount of food about midway between that eaten by F₀ and F₁ females in the present study. The 90-day study was conducted with mink and feed from the same source as the present study and was conducted in the same facility.

Table 4.2.a F₀ Group Mean Food Consumption, Body Weight, DIMP Ingested (DIMP at 97% Purity)

DIMP Dose Group (ppm)	DIMP Actual (ppm in diet)	Feed Consumed (gm/mink/day)		DIMP Consumed (mg/mink/day)		Mean Body Wt. (kg)		DIMP Consumed (mg/kg/day)		gm/food consumed / 100 gm Body Wt.	
		male	female	male	female	male	female	male	female	male	female
	(μ g/gm)										
0	0	223.5	212.4	0	0	2.274	1.142	0	0	9.8	18.6
0	0	239.1	205.3	0	0	2.296	1.200	0	0	10.4	17.1
150	162	202.8	182.7*	32.85	29.60	2.199	1.156	14.94	25.61	9.2	15.8
450	480	221.4	200.9	106.27	96.43	2.244	1.137	47.36	84.81	9.9	17.7
2500	2722	233.0	191.6	634.23	521.54	2.227	1.132	284.79	460.72	10.5	16.9

* Significantly different statistically from Group A Control at p < 0.05

Figure 4.2.a - Daily Food Consumption, F0 Females

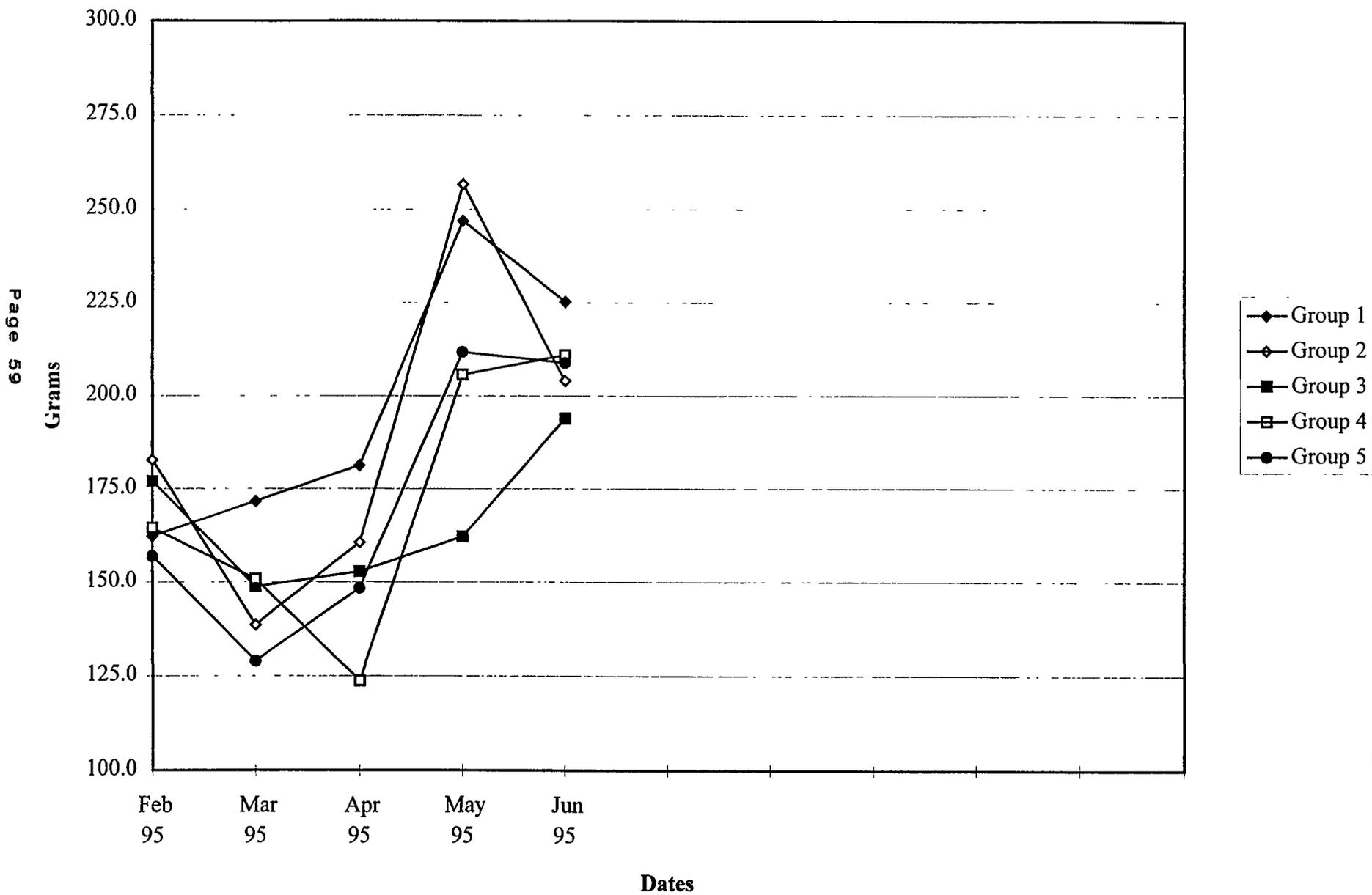


Figure 4.2.b - Daily Food Consumption, F1 Females

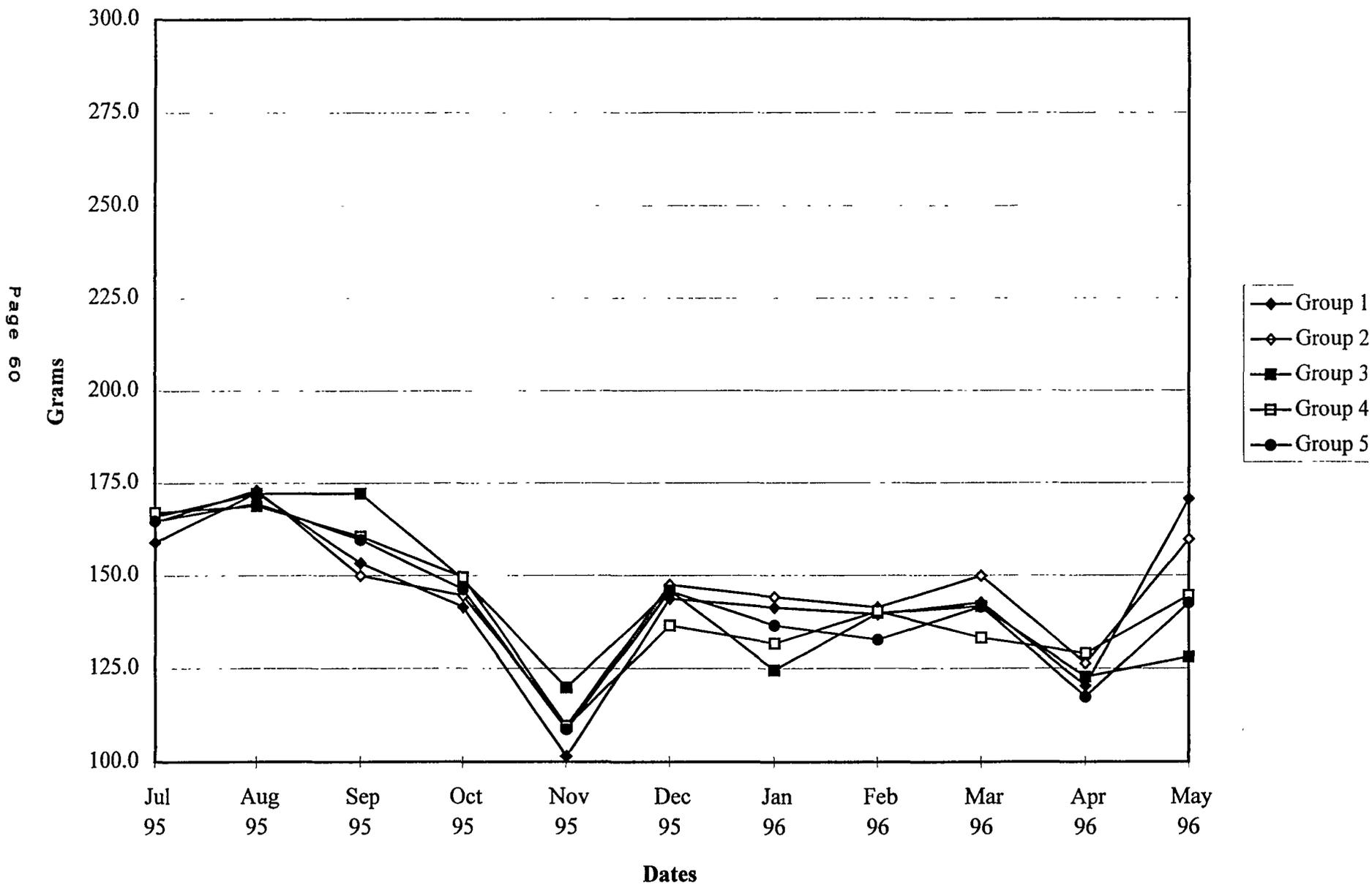


Table 4.2.b F₁ Group Mean Food Consumption, Body Weight, DIMP Ingested (DIMP weighted for 97% and 99% purity)

DIMP Dose Group (ppm)	DIMP Actual (ppm in diet)	Feed Consumed (gm/mink/day)		DIMP Consumed (mg/mink/day)		Mean Body Wt. (kg)		DIMP Consumed (mg/kg/day)		gm food consumed/ 100 gm Body Wt.	
		male	female	male	female	male	female	male	female	male	female
0	0	225.6	139.5	0	0	2.400	1.208	0	0	9.4	11.5
0	0	235.0	144.0	0	0	2.521	1.292	0	0	9.3	11.1
150	168	218.6	142.4	36.72	23.92	2.343	1.212	15.67	19.74	9.3	11.7
450	490	222.6	139.5	109.07	68.36	2.425	1.210	45.00	56.50	9.2	11.5
2500	2774	217.2	141.1	602.51	391.41	2.302	1.188	261.73	329.47	9.4	11.9

Figure 4.3.a - Daily Body Weights, F0 Female

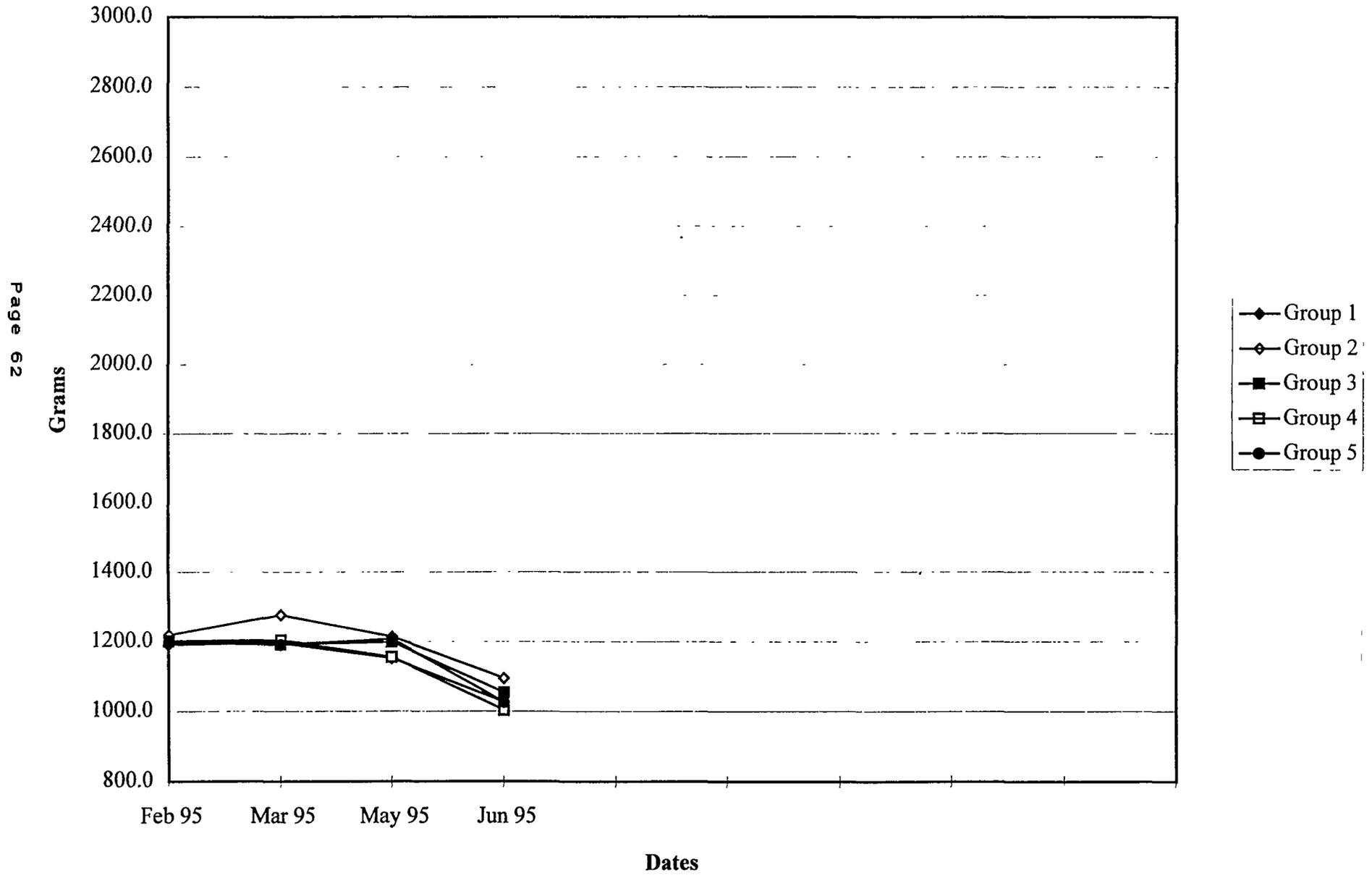
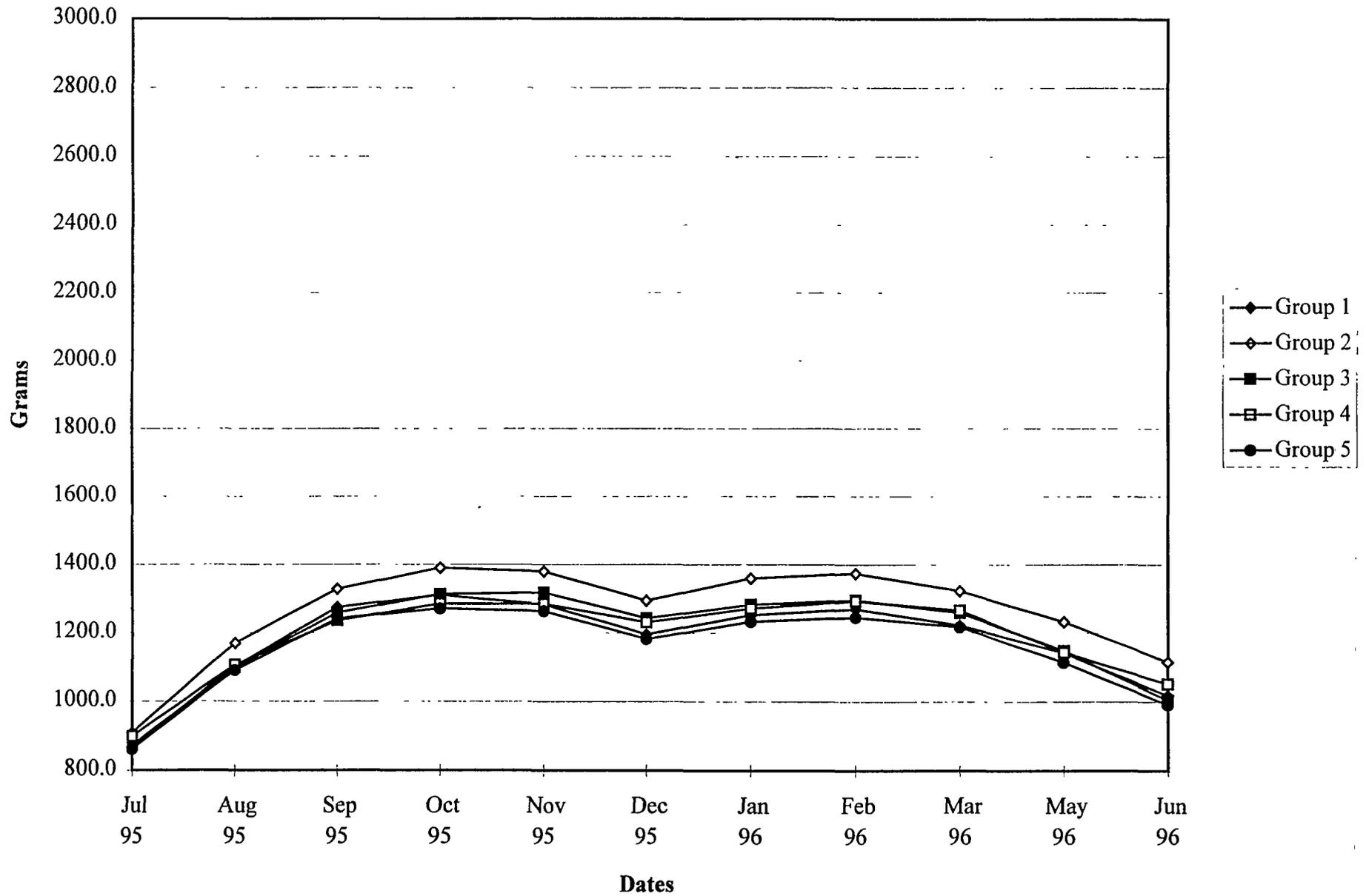


Figure 4.3.b - Daily Body Weight, F1 Females



4.3 Body Weight

Individual animal body weight within treatment group, and statistical comparison of overall mean body weight per treatment group are tabulated in Appendix 3B. There were no statistically significant differences between the mean weight of treated groups and controls. Group mean body weights at monthly intervals for females are plotted in Fig. 4.3.a (F_0) and in Fig. 4.3.b (F_1).

Tables 4.2.a (F_0) and 4.2.b (F_1) contain a column showing the overall mean weight for each treatment group. The F_0 females were about 6% smaller than the F_1 females, 1.15 kg versus 1.22 kg, respectively, despite having used nearly 50% more feed.

There was relatively little difference in overall group mean body weight among male groups in this study.

4.4 Consumption of DIMP

The quantity of DIMP incorporated into the feed is depicted in Table 2.5.1. The actual concentrations were 6 - 8% above target levels in the F_0 generation using a DIMP lot of 97% purity. This lot was depleted 4 months into the F_1 generation and the replacement lot was 99% pure, adding some 2% to the DIMP content of the ration. The formulation recipe was not changed to accommodate the increased purity. Thus the latter two thirds of the F_1 generation (8 months) was fed DIMP at 108-111% of target dose (Table 2.5.2).

To derive the actual amount of DIMP consumed per kg/BW by the F_1 generation, their mean food consumption was calculated for the first four months, then separately for the next eight months to account for the higher DIMP content during the latter months, with the weighted average calculated for the entire study.

The F_0 females were marginally smaller than the F_1 's yet had accounted for approximately 50% greater food consumption (17.2 gm/100 gm BW, averaged across all groups, versus 11.5 gm in the F_1 females). As a result, the average daily DIMP consumption/kg BW was also higher in the F_0 generation.

At least two explanations are possible for the apparent lower food consumption by the F_1 female animals. The food consumed could have been measured more accurately during the F_1 generation, with some of that attributed

to consumption by the F_0 generation actually having been wasted but accounted for as eaten, i.e., the F_0 food consumption value is inflated and thus also is the DIMP consumption value. Another possibility is that the F_0 's were nine months old when they arrived in the laboratory and spent only five months there, thus retaining more of their ranch leanness, whereas the F_1 's were housed under laboratory conditions for their entire 13 months and could have become heavier on less food.

In the opinion of the authors, this difference is not material to the outcome of the study, since differences in dose as small as that between F_0 and F_1 appeared to have little effect over the dose range tested. It is the recommendation of the authors that the more conservative values for DIMP exposure be used, i.e., the lower ones (F_1), should these data be employed to estimate exposure standards for humans.

4.5 Clinical Observations

Tabulated Clinical Observations are listed as a summary in Appendix 4. A variety of clinical manifestations was observed ranging from birth to death. Included are many that are common in caged mink such as occasional soft feces, bone fragment (from food) wedged across the roof of the mouth, etc. Among the several adults that died, anorexia and lethargy were common.

There were no clinical observations specifically related temporally or in number or severity to exposure to the test substance. All were sporadic in time and affected all dose groups, and thus there were no clinical manifestations attributable to DIMP exposure.

4.6 Fecundity

Several parameters of reproductive outcome are tabulated in Tables 4.6.a (F_0) and 4.6.b (F_1), and additional data are included in Appendix 11, to include sex ratios, kit mortality on Day 3 and weights on Day 28. Except for the smaller number of pregnancies in the 150 ppm group of F_0 females, there were no significant differences in breeding outcome as a function of exposure to DIMP. The isolated effect in the 150 ppm F_0 group is not interpreted to be a treatment effect.

Table 4.6.a F₀ Breeding Outcome

Treatment Group (ppm)	Number of Dams	Number of Litters	Total Kits	Mean Kits per Litter	Mean Live Kits per Litter	Litter Biomass (gm)	Mean Individual Kit Wt (gm)
0 (A)	35	32	226	7.06	6.50	75.35	10.97
0 (B)	35	31	229	7.35	6.32	74.42	10.69
150	35	19	134	7.05	6.53	71.84	10.13
450	35	32	232	7.25	6.63	74.81	10.59
2500	35	29	217	7.48	6.38	73.26	10.46

Table 4.6.b F₁ Breeding Outcome

Treatment Group (ppm)	Number of Dams	Number of Litters	Total Kits	Mean Kits per Litter	Mean Live Kits per Litter	Litter Biomass (gm)	Mean Individual Kit Wt (gm)
0 (A)	35	28	188	6.71	5.75	67.27	10.37
0 (B)	35	19	128	6.74	5.58	68.44	10.58
150	35	22	147	6.68	4.95	63.54	9.54
450	35	23	144	6.26	4.83	61.37	9.54
2500	35	23	144	6.26	5.70	65.40	10.43

The F₁ generation were slightly less efficient as breeders, attributable to their suboptimum conditioning in the laboratory environment. They were heavy and significantly less active than their commercial ranch cohorts in "outside" housing. The F₀ generation had been newly acquired in the laboratory and was in better condition for their breeding season. There was no effect of DIMP exposure on the fecundity endpoints tabulated, in either generation.

4.7 Clinical Pathology

4.7.1 Hematology

The values of all analytes measured for each animal are tabulated in Individual Animal reports in Appendix 5. Mean values for each treatment group, including statistical comparison of treated groups with the control value, are tabulated in Appendix 5.

4.7.1.1 F₀ Female

The F₀ females were examined hematologically at allocation (pretreatment) on 16, 17 and 20Feb95 and again at necropsy, 26 - 29Jun95. None of the group mean values for the parameters evaluated differed among groups in the pretreatment samples. In June, after pregnancy, lactation and 3½ months ingestion of DIMP, the 2500 ppm group's mean RBC count was decreased and their mean red cell volume was increased. Both changes were statistically significant at $p < 0.05$, but were of minimal biologic severity. Consistent with these changes, the reticulocyte and Heinz body counts were also increased in the 2500 ppm group, to a degree that was of biologic significance (Tables 4.7.1.a-d). The reticulocyte count was 4.1 versus 1.7 - 1.9% in all other groups, and 2.8% of RBC's contained Heinz bodies, with zero in the other groups. Both increases were also significant statistically at the 1% level.

4.7.1.2 F₁ Female

The F₁ females were evaluated hematologically on three occasions, Sept 95, Dec 95, and June 96. (The RBC parameters are summarized in Tables 4.7.1. (a - d).

Among all parameters compared in all dose groups and at all three time points, only the mean Heinz body count differed as an effect of DIMP exposure in this generation, and the effect (increase) was mild (Table 4.7.1.d).

In the December 95 measurement, the Heinz bodies were present in 0.2% of RBC's in the 2500 ppm group, with none seen in the control and 0.1% in the 150 and 450 ppm groups. Statistically the 2500 ppm group's count was increased significantly at the $p < 0.05$ level.

In the June 96 measurement, the Heinz body count in the 2500 ppm group was 1.3% of RBC's affected, whereas in all other groups it was 0.1% or 0.2% (statistically significant increase at the $p < 0.01$ level).

The presence of increased Heinz body count was the principal adverse effect noted in the earlier 90-day study in mink (Bucci *et al* 1992). In that study, conducted with mink of similar age to the F_0 generation in the present study, females given 528 and 2930 ppm DIMP in their diet (82 and 455 mg DIMP/kg BW/day) had 0.17 and 1.7% Heinz body counts, respectively at 90 days exposure. The Group 4 and 5 F_0 females in the present study, by comparison, consumed approximately 85 and 460 mg DIMP/kg BW/day, respectively, for approximately 110 days (Table 4.2.a). They had 0 and 2.8% of RBC's containing Heinz bodies. This result of the two studies (1991 90-day study and the present F_0 generation in 1995) is notably similar.

The present F_1 females consumed slightly less DIMP, 57 and 329 mg/kg BW/day for the 450 and 2500 ppm groups, respectively than did the F_0 group, (Table 4.2.b), but for a longer period, approximately 13 months versus 3 ½. Despite this increased lifetime exposure to DIMP, the 2500 ppm F_1 group had fewer Heinz bodies than did the F_0 group.

The pathophysiology of DIMP-induced oxidative injury to hemoglobin (i.e., production of Heinz bodies in RBC's) is characterized by shortened survival of affected RBC's in the circulation. Affected red cells are removed prematurely by the

spleen. The accelerated removal rate can result in a reduced RBC count and provide stimulus to the spleen and bone marrow to produce additional RBC's. These replacement red cells may be released to the circulation a few days before they are completely mature, some while they still contain nuclear remnants. Such cells are recognized in the hemogram as having increased volume (MCV), and in some, reticular remnants of the nucleus (reticulocytes). In instances where the production of replacement cells does not fully compensate for accelerated removal, RBC count and total hemoglobin would also be reduced (Fan *et al* 1978).

In the present study, evidence of slightly accelerated removal of RBC's was evident to a biologically important degree only in animals consuming the 2500 ppm dose, and the F₁ animals were less affected than the F₀ despite their nearly 3-fold longer exposure. The authors suggest that this decreased effect on the F₁ generation is a result of adaptation to exposure to the DIMP over the course of the lifetime of these F₁ animals.

Table 4.7.1.a Mean Red Blood Cell Count (X 10⁶/μL) - Female

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	7.59	7.72	7.80	7.63	7.72
June 95	7.79	7.86	8.00	7.80	7.38*
F ₁ Group	1	2	3	4	5
Sept 95	7.97	8.17	7.99	8.17	7.94
Dec 95	8.45	8.55	8.61	8.53	8.36
June 96	8.29	8.21	8.38	8.39	8.20

* Significantly different statistically from Group A Control at p < 0.05

Table 4.7.1.b Mean Reticulocyte Count (% of RBC's) - Female

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	3.1	3.6	3.0	3.9	3.3
June 95	1.8	1.7	1.7	1.9	4.1**
F ₁ Group	1	2	3	4	5
Sept 95	3.1	3.3	3.0	2.9	3.1
Dec 95	3.4	3.2	4.0	3.5	3.9
June 96	1.7	2.4	2.1	2.1	2.0

** Significantly different statistically from Group A Control at p < 0.01

Table 4.7.1.c Mean Cell Volume (um³)- Female

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	62	61	61	61	61
June 95	58	58	57	58	60*
F ₁ Group	1	2	3	4	5
Sept 95	60	60	60	59	60
Dec 95	60	60	61	60	61
June 96	61	60	61	60	61

* Significantly different statistically from Group A Control at p < 0.05

Table 4.7.1.d Mean Heinz Body Count (% of RBC's) - Female

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	0.0	0.0	0.0	0.0	0.0
June 95	0.0	0.0	0.0	0.0	2.8**
F ₁ Group	1	2	3	4	5
Sept 95	0.0	0.0	0.0	0.0	0.0
Dec 95	0.0	0.0	0.1	0.1	0.2*
June 96	0.1	0.1	0.2	0.2	1.3**

* Significantly different statistically from Group A Control at $p < 0.05$

**Significantly different statistically from Group A Control at $p < 0.01$

4.7.1.3 F₀ Male

The F₀ males (7 per group) were evaluated in Feb 95 and the end of the mating season in late March 95. None of the hematology parameters in the DIMP-exposed males differed from the control values. The relevant RBC parameters are summarized in Tables 4.7.1. e-h.

4.7.1.4 F₁ Male

The F₁ (12-13 per group) males were evaluated in Sept 95 when they were about 18-20 weeks old, again in December and after breeding in late March, 1996. There was no adverse effect of DIMP exposure on any of the hematologic parameters, although both the 450 ppm and 2500 ppm groups had 0.1% Heinz body counts in September, versus zero in the other groups. In December, one control group (B) had 0.1%, as did the 2500 ppm group again. The others were zero. In March, the 2500 ppm group still had 0.1% whereas all others were zero. The extent of the Heinz body effect was trivial biologically, but it does serve as a clue that some biological effect was occurring (Tables 4.7.1. e-h).

Table 4.7.1.e Mean Red Blood Cell Count ($X10^6/\mu L$)- Male

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	8.34	8.75	8.32	8.26	8.06
Mar 95	8.79	8.66	8.35	8.99	8.43
F ₁ Group	1	2	3	4	5
Sept 95	8.01	8.28	8.35	8.12	8.06
Dec 95	8.49	8.66	8.88	8.49	8.43
Mar 96	8.84	9.11	8.83	9.01	8.85

Table 4.7.1.f Mean Reticulocyte Count (% of RBC's) - Male

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	2.6	2.6	2.2	2.1	3.4
Mar 95	0.8	0.8	0.8	1.0	1.0
F ₁ Group	1	2	3	4	5
Sept 95	2.7	3.2	3.3	3.3	4.2
Dec 95	3.3	3.1	3.3	3.6	2.9
Mar 96	2.2	1.5	1.8	2.1	2.1

Table 4.7.1.g Mean Cell Volume (um³) - Male

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	59	60	59	60	60
Mar 95	59	58	58	60	59
F ₁ Group	1	2	3	4	5
Sept 95	60	60	62	61	61
Dec 95	61	60	62	61	60
Mar 96	61	60	62	61	60

Table 4.7.1.h Mean Heinz Body Count (% of RBC's) - Male

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	0.0	0.0	0.0	0.0	0.0
Mar 95	0.0	0.0	0.0	0.0	0.0
F ₁ Group	1	2	3	4	5
Sept 95	0.0	0.0	0.0	0.1	0.1*
Dec 95	0.0	0.1	0.0	0.0	0.1
Mar 96	0.0	0.0	0.0	0.0	0.1

* Significantly different statistically from Group A Control at p < 0.05

4.7.1.5 Kits

The F₁ kits not retained for breeding were sampled when necropsied in July 1995, when they averaged about 11 weeks of age. They were represented by 7-13 males and 8-11 females per treatment group. There were no hematologic changes in these kits that were attributable to exposure to DIMP. Relevant RBC parameters are extracted and summarized in Tables 4.7.1. i-l.

The F₂ kits were sampled at necropsy in June, 1996, at 4-6 weeks of age. They were represented by 8-12 males and 8-11 females per treatment group. Their values are also included in Tables 4.7.1. i-l. The Heinz body counts were unaffected by DIMP exposure in these young kits. The RBC and reticulocyte counts had nonsignificant and inconsistent trends toward lower RBC and higher reticulocytes counts in the treated animals (the F₂ male 2500 ppm group did have an RBC count that was lower than the control values to a statistically significant degree). The mean cell volume (MVC) was 70 μm^3 in the 150 ppm females, higher than the controls (66 and 68) and the two higher dose groups (68 μm^3). In males, both control groups and the 450 ppm group had an MCV of 69 μm^3 , with 71 in the 2500 ppm group and 72 in the 150 ppm group. These findings are difficult to interpret since the hemogram is maturing rapidly in animals of this age as they develop adult values. If the MCV increase and the decreased RBC count are DIMP-related functions in these young kits, they are both mild and transient, since they were absent in all but the 2500 ppm group at later times.

Unrelated to DIMP exposure, the younger F₂ kits had more reticulocytes and larger MCV than did the older F₁ kits.

Table 4.7.1.i Mean Red Blood Cell Count ($\times 10^6/\mu\text{L}$)

Female Kits					
Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₁ Group	1	2	3	4	5
July 95	6.36	6.74	6.49	6.77	6.59
F ₂ Group	1	2	3	4	5
June 96	4.68	4.63	4.51	4.62	4.39
Male Kits					
F ₁ Group	1	2	3	4	5
July 95	6.04	6.16	6.16	6.15	6.21
F ₂ Group	1	2	3	4	5
June 96	4.51	4.34	4.16	4.41	4.06**

** Significantly different statistically from Group A Control at $p < 0.01$

Table 4.7.1.j Mean Reticulocyte Count (% of RBC's)

Female Kits					
Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₁ Group	1	2	3	4	5
July 95	4.5	5.0	3.4	5.4	4.9
F ₂ Group	1	2	3	4	5
June 96	9.2	10.8	11.6	10.5	11.0
Male Kits					
F ₁ Group	1	2	3	4	5
July 95	4.8	6.2	4.5	5.1	6.1
F ₂ Group	1	2	3	4	5
June 96	9.6	10.7	9.9	9.7	11.2

Table 4.7.1.k Mean Cell Volume (μm^3)

Female Kits					
Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₁ Group	1	2	3	4	5
July 95	64	63	65	64	63
F ₂ Group	1	2	3	4	5
June 96	66	68	70**	68	68
Male Kits					
F ₁ Group	1	2	3	4	5
July 95	65	65	67	65	65
F ₂ Group	1	2	3	4	5
June 96	69	69	72**	69	71

** Significantly different statistically from Group A Control at $p < 0.01$

Table 4.7.1.1 Mean Heinz Body Count (% of RBC's)

Female Kits					
Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₁ Group	1	2	3	4	5
July 95	0.0	0.0	0.0	0.0	0.0
F ₂ Group	1	2	3	4	5
June 96	0.2	0.1	0.1	0.1	0.3
Male Kits					
F ₁ Group	1	2	3	4	5
July 95	0.0	0.0	0.0	0.0	0.1
F ₂ Group	1	2	3	4	5
June 96	0.2	0.1	0.3	0.1	0.3

4.7.2 Clinical Chemistry

The values of all analytes measured for each animal are tabulated in Individual Animal Reports in Appendix 6 and 7. Mean values for each treatment group, including statistical comparison of treated groups with the control value, are tabulated in Appendix 6 and 7. The tables in this section (Tables 4.7.2. a-j) summarize the cholinesterase assay results.

4.7.2.1 F₀ Female

The F₀ females were evaluated in February 95 and again at necropsy in June 95, after pregnancy and lactation. There were no meaningful differences among the group mean values for any analyte, in the February 95 (pretreatment) blood samples.

In June, there were significant decreases in mean values for whole blood and RBC cholinesterase (450 and 2500 ppm) and plasma cholinesterase (all three treated groups), but not in brain acetylcholinesterase (ACHE) (Tables 4.7.2. a, b, c and d).

The reduction in plasma cholinesterase (CHE) was presumably also the principal source of the reduction in whole blood CHE. Although the plasma and blood CHE were reduced sufficiently to reach the 1% level of significance statistically, there were no adverse clinical effects noted. Reduction in plasma cholinesterase is evidence of exposure, but is not an adverse effect until the reduction exceeds 20% (EPA 1989b, 1995). It is rapidly reversible and, of itself, of no demonstrable biologic consequence. In the F₀ females only the 2500 ppm group had reduction in plasma cholinesterase that exceeded 20%.

In contrast, conventional wisdom holds that reductions in RBC and brain tissue ACHE correlate with symptomology. While this may be true in some instances, it is not universal. In this set of 33-35 F₀ females per dose group, there were no changes in brain acetylcholinesterase, and no symptoms of excess acetylcholine activity despite (small) reductions in RBC cholinesterase.

4.7.2.2 F₁ Female

The 34 - 35 F₁ females per group were evaluated in September 95, December 95 and at necropsy in June 96. In September, plasma cholinesterase was decreased about 25% in the 2500 ppm group, a marginal degree. The whole blood CHE was also decreased about 7%, an inconsequential amount biologically even though statistically both decreases reached the 1% level of significance. The RBC cholinesterase was reduced 4% in this group as well (5% level of significance statistically), but this was also considered marginal biologically. There was no effect on brain acetylcholinesterase (Tables 4.7.2. a, b, c, and d). Serum phosphorus was increased in the 2500 ppm group, probably reflecting a minimal degree of hemolysis in the blood samples.

In December, the plasma and whole blood cholinesterases remained decreased to about the same level as in September, and RBC ACHE was normal. Albumin, AST, phosphorus, total protein, and creatinine were all elevated to a marginal degree in the 2500 ppm group. These changes suggest a degree of hemoconcentration but this group had normal osmolarity (and hematocrit) to mitigate against that.

The cholinesterases remained decreased to the same degree in June 96, but again only in the high dose group, and RBC ACHE remained unaffected; brain ACHE was normal in all groups, as well (Table 4.7.2.d). Serum phosphorus was slightly elevated in the 2500 ppm group, but no other treatment-related changes were evident. The values for osmolarity were low across all dose groups, for unexplained reasons.

4.7.2.3 F₀ Male

The seven F₀ males per group had similar group mean values for all analytes across all treatment groups, at the Feb95 sampling and also at the end of March. There was no effect of DIMP during this period, at any of the doses (Tables 4.7.2. e-g).

4.7.2.4 F₁ Male

In September 95 the mean plasma CHE for the 2500 ppm group of F₁ males (13/group) was decreased about 15% compared with the control value, and the 450 ppm group was also somewhat decreased, but not significantly (10%) (Table 4.7.2.e). The US EPA considers that $\pm 20\%$ in plasma cholinesterase is not important biologically....within the "noise" range of variability. The 150 ppm group had whole blood CHE elevated about 6% above control values, for example, while the higher dose groups had values equivalent to the control groups. RBC ACHE was unchanged in all dose groups (Tables 4.7.2. f and g). Urea nitrogen, sodium and creatinine were marginally elevated in the 2500 ppm group and osmolarity was 314 mOSM/L in the 2500 group while the controls were 307 and 306, probably reflecting the higher creatinine and sodium.

In the December 95 analysis, whole blood ACHE, but not plasma or RBC levels, was decreased (less than 10%), while it was elevated still in the 150 ppm group (about 6 - 7%). Albumin, total protein, calcium, sodium, and osmolarity were all elevated slightly in both 150 and 2500 ppm groups. The 450 dose had slightly elevated sodium and osmolarity.

In March 96, the mean value for all analytes did not differ among the groups. In particular, no cholinesterase values were affected (Tables 4.7.2. e, f, and g).

4.7.2.5 F₁ and F₂ Kits - Female

The 8 - 11 female F₁ kits that represented those not chosen for breeding were sampled in July 95. Mean plasma CHE was decreased (16%) in the 2500 ppm group. There was no change in the RBC or whole blood CHE. (Tables 4.7.2. h, i and j). Total protein and albumin were mildly increased in Control B and in the 450 and 2500 groups. There were no differences in results among treated and untreated F₂ female kits.

4.7.2.6 F₁ and F₂ Kits - Male

There were no treatment-related differences in mean values for any analyte, among the groups of F₁ male kits. All were within the normal range.

Among the F₂ male kits, the 450 ppm group had a 6% decrease in whole blood cholinesterase relative to controls, but the other cholinesterase parameters, and those for the 2500 ppm group, were not biologically or statistically different from control values (Tables 4.7.2. h, i and j). All other analytes were unchanged.

Table 4.7.2.a Mean Plasma Cholinesterase (U/L) - Female

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	1109	1089	1041	1033	1040
June 95	1426	1384	1250**	1215**	860**
F ₁ Group	1	2	3	4	5
Sept 95	1279	1293	1215	1272	999**
Dec 95	1133	1134	1180	1090	894**
June 96	1310	1288	1254	1271	905**

** Significantly different statistically from Group A Control at p < 0.01

Table 4.7.2.b Mean Whole Blood Cholinesterase (U/L) - Female

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	4378	4279	4324	4298	4355
June 95	3725	3730	3557	3347**	3115**
F ₁ Group	1	2	3	4	5
Sept 95	4589	4638	4587	4532	4285**
Dec 95	4793	4811	4919	4803	4572**
June 96	4335	4337	4278	4318	3966*

* Significantly different statistically from Group A Control at p < 0.05

** Significantly different statistically from Group A Control at p < 0.01

Table 4.7.2.c Mean Red Blood Cell Acetylcholinesterase (U/L)- Female

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	8118	7854	8006	8010	8099
June 95	6479	6532	6255	5943**	6001**
F ₁ Group	1	2	3	4	5
Sept 95	8207	8159	8247	7993	7859*
Dec 95	8301	8304	8356	8427	8174
June 96	7332	7457	7428	7349	7103

* Significantly different statistically from Group A Control at p < 0.05

** Significantly different statistically from Group A Control at p < 0.01

Table 4.7.2.d Mean Brain Cholinesterase (μ mol/gm/min) - Female

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
June 95	6.53	6.78	6.98	6.91	7.13
F ₁ Group	1	2	3	4	5
June 96	7.24	6.33	7.07	7.05	6.81

Table 4.7.2.e Mean Plasma Cholinesterase (U/L) - Male

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	1085	1205	1102	1181	1222
Mar 95	1312	1286	1253	1177	1002
F ₁ Group	1	2	3	4	5
Sept 95	1412	1529	1393	1256	1214*
Dec 95	1103	1196	1358	1065	972
Mar 96	1289	1350	1249	1159	1056

* Significantly different statistically from Group A Control at $p < 0.05$

Table 4.7.2.f Mean Whole Blood Cholinesterase(U/L) - Male

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	4511	4719	4543	4280	4524
Mar 95	4407	4229	4056	4281	3937*
F ₁ Group	1	2	3	4	5
Sept 95	4539	4627	4805*	4417	4402
Dec 95	4835	4832	5096*	4693	4544**
Mar 96	4487	4332	4509	4276	4185

* Significantly different statistically from Group A Control at $p < 0.05$

** Significantly different statistically from Group A Control at $p < 0.01$

Table 4.7.2.g Mean Red Blood Cell Acetylcholinesterase (U/L) - Male

Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₀ Group	1	2	3	4	5
Feb 95	8004	7930	8052	7405	8013
Mar 95	7317	7118	6989	6930	6898
F ₁ Group	1	2	3	4	5
Sept 95	7860	7819	8049	7682	7722
Dec 95	8338	8219	8205	8093	7997
Mar 96	7270	6823	7181	6740	6960

Table 4.7.2.h Mean Plasma Cholinesterase (U/L)

Female Kits					
Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₁ Group	1	2	3	4	5
July 95	1807	1884	1732	1675	1524*
F ₂ Group	1	2	3	4	5
June 96	2315	2308	1997	1865**	1983
Male Kits					
F ₁ Group	1	2	3	4	5
July 95	2244	2171	2075	1975	2044
F ₂ Group	1	2	3	4	5
June 96	2391	2610	2242	2349	2517

* Significantly different statistically from Group A Control at p < 0.05

Table 4.7.2.i Mean Whole Blood Cholinesterase (U/L)

Female Kits					
Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₁ Group	1	2	3	4	5
July 95	4070	4106	3991	3958	3856
F ₂ Group	1	2	3	4	5
June 96	4035	4119	4040	3929	3832
Male Kits					
F ₁ Group	1	2	3	4	5
July 95	4087	3745	3970	3792	3754
F ₂ Group	1	2	3	4	5
June 96	4193	4162	4098	3933*	3947

* Significantly different statistically from Group A Control at $p < 0.05$

Table 4.7.2.j Mean Red Blood Cell Acetylcholinesterase (U/L)

Female Kits					
Dose (ppm)	0 Control A	0 Control B	150	450	2500
F ₁ Group	1	2	3	4	5
July 95	7345	7280	7067	7153	7408
F ₂ Group	1	2	3	4	5
June 96	7766	8183	8261	8470	8346
Male Kits					
F ₁ Group	1	2	3	4	5
July 95	6973	6558	7311	6918	6366
F ₂ Group	1	2	3	4	5
June 96	8072	7714	8223	7522	7612

4.7.3 Summary of Biologically Relevant Adverse Hematological and Biochemical Effects of DIMP Exposure

The only biologically significant adverse manifestation of exposure to DIMP occurred in animals exposed to the highest dose, 2722 ppm in F₀ females and 2774 ppm in the F₁ females. The effects were mild in degree and were limited to decreases in cholinesterase and to increases in the percentage of red blood cells that contained Heinz bodies.

Cholinesterase was decreased in plasma and whole blood, and rarely in RBC, but brain acetylcholinesterase was unaffected. Reduction in plasma cholinesterase is not considered biologically significant until the value is more than 20% below the control value. In the erythron, related to Heinz body formation (oxidative change to hemoglobin in RBC's), there were shortened RBC survival and correlated increases in reticulocyte count and occasional increases in mean cell volume. RBC total count was decreased.

These changes are summarized in the following tables:

Table 4.7.3.a DIMP-Related Hematologic Changes
(2500 ppm Female/ Control A Female)

	RBC (x 10 ⁶ /μL)	Reticulocytes (% of RBC's)	MCV (um ³)	Heinz Bodies (% of RBC)
F ₀				
Feb 95	7.72/7.59	3.3/3.1	61/62	0.0/0.0
June 95	7.38*/7.79	4.1**/1.8	60*/58	2.8**/0.0
F ₁				
Sept 95	7.94/7.97	3.1/3.1	60/60	0.0/0.0
Dec 95	8.36/8.45	3.9/3.4	61/60	0.2*/0.0
June 96	8.20/8.29	2.0/1.7	61/61	1.3**/0.1

* Statistically different from Control A value with p < 0.05

** Statistically different from Control A value with p < 0.01

Table 4.7.3.b Cholinesterases (2500 ppm Female/ Control A Female)

	Plasma (U/L)	% ↓	Whole Blood (U/L)	% ↓	RBC (U/L)	% ↓	Brain (Micromol/gm/min)
F₀							
Feb 95	1040/ 1109	---	4355/ 4378	---	8099/8118	--	---
June 95	860**/1426 ^a	-40	3115**/3725 ^b	-16	6001*/6479 ^c	-8	7.13/6.53
F₁							
Sept 95	999**/1279	-22	4285**/4589	-7	7859*/8207	-4	---
Dec 95	894**/1133	-21	4572**/4793	-7	8174/8301	--	---
June 96	905**/1310	-31	3966*/4335	-8.5	7103/7332	--	6.81 / 7.24

* Statistically different from Control A value with $p < 0.05$

**Statistically different from Control A value with $p < 0.01$

Not shown: Reductions at lower doses that were significant statistically but less than 20%:

^a at 450 ppm (-15%) and 150 ppm (-12%)

^b at 450 ppm (-10%)

^c at 450 ppm (-13%)

4.8 Necropsy and Histopathology

4.8.1 Sperm Analysis

Automated sperm analysis was conducted on the F₀ and F₁ males at their respective necropsies in March 95 and March 96. The Hamilton-Thorne automated sperm analyzer was used to evaluate mean percent motility and total count of sperm removed from the epididymis (Slott and Perrault, 1993). Morphology was evaluated manually on stained smear preparations.

No treatment-related effects were observed for the sperm motility data. Mean values were 99.8 and 100.0 for the two controls, and 100, 99.6 and 99.9 percent for the 150, 450 and 2500 ppm groups, respectively.

The number of sperm/gram of cauda epididymis was comparable among the study groups. Mean values were 1244.9/1398.5, 1417.2, 1434.3, and 1561.5 million sperm/gram for the control, 150, 450 and 2500 ppm groups, respectively. No treatment-related differences were observed.

A low incidence of head/tail abnormalities was observed for animals in each treatment group. No treatment related differences were observed.

In the F₁ males (n= 13), there were no treatment-related differences among the groups. The detailed report of sperm analysis is included as Appendix 8.

4.8.2 Gross Necropsy

A variety of incidental lesions was recognized at necropsy in all groups of animals, but they were distributed among all dose groups and were not related to dose or duration of exposure to DIMP. In particular, there were no DIMP-related developmental defects recognized in the kits at necropsy. Refer to Appendix 9A for the complete pathology report.

4.8.3 Organ Weights

In F₀ and F₁ females, the adrenal glands, brain, kidney, liver, ovaries, spleen, thymic area and uterus were weighed. Testes, right epididymis and prostate were weighed in males. Group mean weights were compared to the value for Control Group A, as absolute organ weight, and as percentage of brain weight and of body weight. The individual values are reported in Appendix 9A, Pathology report and the group mean summaries with statistical analysis are presented in Appendix 10, Organ Weight Summary.

The group mean body weight was decreased in all groups administered DIMP, but the values were not different statistically from the control group value in the F₀ females. The F₀ female mink given the 2500 ppm diet had a statistically significant increase in the mean absolute weight of spleen ($p < 0.01$), and as a ratio of spleen to both body weight and brain weight, when compared to the control values (Table 4.8.3). This increase was considered biologically significant, related to the

spleen's role in removing Heinz body-containing red blood cells and supporting production of new erythrocytes.

In the female F₀ generation, the mean uterine weight was decreased to a statistically significant degree in both the 2500 ppm and 150 ppm groups, when compared to control values (Table 4.8.3).

Table 4.8.3 Organ Weights (Group Mean values, gm)

F ₀ Females					
Organ	0(Control A)	0(Control B)	150 ppm	450 ppm	2500 ppm
Spleen	3.17	3.32	3.21	3.32	4.50**
Spleen/BW%	0.294	0.298	0.306	0.322	0.441**
Spleen/Brain %	38.1	39.9	38.7	40.4	54.1**
Uterus	0.24	0.24	0.20**	0.22	0.21*
Uterus/BW%	0.023	0.022	0.020	0.022	0.020
Uterus/Brain%	2.94	2.89	2.46**	2.68	2.50**
F ₁ Males					
Testes	4.54	5.10	5.36*	4.85	4.54
Prostate	0.32	0.35	0.46**	0.36	0.37
Prostate/BW%	0.014	0.014	0.020*	0.016	0.018
(Brain not weighed)					

* Statistically different from Control A value with p < 0.05

**Statistically different from Control A value with p < 0.01

These variations in uterine weight were not considered treatment-related because they were inconsistent among dose groups and were measured during the period of post-partum uterine involution (see below).

In the 150 ppm group of F₁ males there was a significant increase in the absolute prostate weight (p < 0.01), absolute testes weight

($p < 0.05$) and a significant increase in the prostate as a percentage of body weight ($p < 0.05$) compared to the untreated control group value (Table 4.8.3). The testicular and prostatic weight increase observed in this group was considered to be unrelated to DIMP exposure as it was not observed in mink administered higher doses.

In the F_1 females, none of the organ weights differed between treated and untreated animals. The individual organ weight values are included in Appendix 9A and the statistical evaluation of organ and body weights are included as a summary in Appendix 10.

The decreased uterine weight observed in F_0 female groups administered 150 or 2500 ppm was considered unrelated to the administration of DIMP, as there was no dose-related trend and there were no group-related microscopic findings. The weight differences likely reflect a physiological variation in postpartum uterine involution as similar uterine weight decrease did not occur in F_1 female mink. This interpretation is reinforced by the observation that the weights of the uteri in F_1 groups, which were about 6 weeks post-partum, averaged around 0.5 gm, whereas the F_0 uteri, some 11 weeks post-partum, had involuted to about 0.22 gm (with range of 0.14 to 0.61). Normal involution was occurring in the reproductive tracts and population variance is to be expected. The testes and prostate glands in the F_1 males were also beginning involution at the time of their necropsy, and the slightly increased mean weight observed only in the low dose group is interpreted as normal variation unrelated to dose.

4.8.4 Histopathology

The increased weight of the spleen in the F_0 females fed the 2500 ppm diet was associated with increased incidence and severity of hematopoietic cell proliferation. These were the only morphologic findings considered related to the administration of DIMP. In a previous study (Bucci *et al* 1992, 1994) the administration of DIMP at 2930 ppm and above for 90 days resulted in oxidative injury to hemoglobin in erythrocytes. The life span of erythrocytes was reduced and a similar compensatory hematopoiesis was elicited.

The absence in F_1 female parents of microscopic changes in the spleen similar to those in the F_0 generation suggests beneficial adaptation

in the metabolism and excretion of DIMP with their longer exposure.

The Pathology Report is included in Appendix 9A.

4.8.5 Ovarian Follicle Count

Histologically, all ovaries examined were normal (Appendix 9B). To examine the ovaries for evidence of specific ovarian toxicity, the procedure described in the EPA (1994) US Environmental Protection Agency Office of Prevention, Pesticides and Toxic Substances Health Effects Test Guidelines was followed.

One formalin-fixed paraffin-embedded ovary from 10 F₁ animals of each control group and the 2500 ppm group was sectioned longitudinally. Five step-sections, 100 micrometers apart, were prepared from the central third of each ovary. The number of small ovarian follicles (as classified by Pederson and Peters, 1968) was counted in each ovary section and the number of follicles in each ovary and mean number of follicles in each treatment group were tabulated (Table 4.8.5).

Table 4.8.5 Mean Ovarian Follicle Counts

Dose Group N=10/Gp	Mean count of small follicles/group	ST DEV
0 (Control A)	329	153
0 (Control B)	460	147.8
2500 ppm	645**	157.1

** Significantly different from control at $p < 0.01$

The number of follicles present in the ovaries of the mink given 2500 ppm DIMP was significantly increased over the control values, $p < 0.01$.

In most reports of ovarian toxicity, the number of ovarian follicles is decreased with associated decrease in fecundity (Heindel *et al* 1989; Smith *et al* 1991). However, the published work was conducted with rodents; they experience regular estrous cycles on a 4 - 6 day schedule. Mink are copulation-dependent ovulators that are seasonally polyestrous.

To our knowledge this measure of ovarian toxicity has not previously been applied to mink and we are uncertain of the meaning of this elevated count.

This increased count in treated animals could represent disrupted follicular maturation with retention of ova, caused by the exposure to DIMP. However, in actual breeding experience, the treated dams of both F_0 and F_1 generations produced as many offspring as did the controls. Also, the ovaries from these animals were normal histologically (Appendix 9B). If ova were actually being retained by the 2500 ppm group, then a large surplus must mature to also permit fertilization of the normal number.

We believe this one test outcome should be regarded as inconclusive and that the actual breeding outcome should be the present test of reproductive effect. Additional investigative work is needed to validate this oocyte count in mink.

5.0 SUMMARY AND CONCLUSIONS

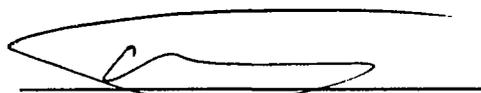
The female F_0 generation in this study was exposed to DIMP for approximately four months (February to June, 1995) whereas the F_1 females were exposed from birth through weaning of their offspring, approximately 13 months from May, 1995 to June, 1996. They were also exposed *in utero* for six weeks between their conception and birth, assuming that DIMP crosses the placenta. The conclusions that follow are therefore based on data derived from the female F_1 animals unless otherwise specified.

Biologically significant adverse effects of exposure to DIMP in this study were mild and were limited to animals given the highest dose level (2500 ppm target dose). The actual concentration received by the F_1 females in this group was 2774 ppm, averaged over the duration of the study. The adverse effects measured chemically were reduction in plasma and whole blood cholinesterase (21-40% less than control values; 4-8% reduction in RBC cholinesterase). Hematologically the 2500 ppm groups had significantly increased Heinz body counts in their red blood cells, which correlated with shortened red blood cell survival that was manifest also by lower RBC count, increased reticulocyte count and, inconsistently, increased cell volume. The hematologic change was evident only in the 2500 ppm group of the F_0 females at necropsy and microscopic examinations, manifest as increased weight of spleen grossly and as proliferation of replacement red blood cells microscopically. There were no clinical signs of illness attributable to exposure to DIMP, and no other gross,

no clinical signs of illness attributable to exposure to DIMP, and no other gross, microscopic or biochemical abnormalities associated with exposure. There were no treatment-related effects on reproductive efficiency, kit development or semen quality in males.

The study revealed that female mink during their reproductive cycle are not uniquely susceptible to DIMP toxicity, and no deaths resulted from exposure to DIMP. The two generations experienced 3.4% and 4.6% mortality in groups of 175 dams, respectively, distributed approximately equally among experiment groups including untreated controls and with an identified cause of death in all but one.

No adverse effects occurred at the 450 ppm dose level and below. The actual amount of DIMP at that target level was 490 ppm. **The No Observed Adverse Effect Level (NOAEL) in the study, based on the F₁ females, was 490 ppm.** This concentration was used to calculate the average quantity of DIMP consumed per day, which was 56.50 mg/kg body wt/day for the F₁ females (84.81 for the F₀ females).



Thomas J. Bucci, VMD, PhD
Study Director

28 Oct 97

Date

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7.0 LOCATION OF SPECIMENS AND RAW DATA

All study records and computer printouts to include animal husbandry, clinical observations, breeding, randomization, litter data, temperature/humidity records, anesthesia records, dosing, clinical chemistry, hematology, sperm analysis and pathology records, as well as pathology slides, blocks and wet tissues are stored in the Pathology Associates International Archive at Frederick, Maryland, until specified otherwise by US Army representatives. The point of contact for archival status is:

Tim Kilgannon
Project Officer
Remedial Planning and Monitoring Branch
Rocky Mountain Arsenal
Commerce City, CO 80022-1748

8.0 PERSONNEL

The following is a list of personnel employed on this project:

Pathology Associates International

Fred Argilan
Dr. Thomas J. Bucci
Mike Mercieca
Dr. Robert Kovatch
Dr. Mark Evans
Dr. Willam Wustenberg

University of Minnesota

Evelyn Townsend
Dr. Victor Perman
Pat Molitor
Dr. Doug Weiss
Les Westendorf

9.0 ACKNOWLEDGMENTS

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 Jefferson AR 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. Budget considerations were paramount throughout.

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

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The last of the mink in this study were sacrificed in June of 1996. Patrick Molitor

died on 1 August 1996, while vacationing among the lakes and forests of British Columbia.

The authors wish to express here their appreciation and gratitude for his many contributions to this work. Patrick S. Molitor worked with mink for most of his life. We were extremely fortunate to have the benefit of his detailed knowledge of the species and his skill in their husbandry, throughout the study. His work ethic was exemplary; his attitude was positive, his resourcefulness seemingly endless. He became the indispensable pillar, daily solving the multiple problems that occur and threaten to disrupt complex undertakings such as this study. Pat was a strong advocate for the welfare of the mink and, in his taciturn manner, strove constantly to preserve the serenity of their environment and excellence of their care. The animals certainly benefitted from his dedication.

All who associated with Pat also benefitted from his quiet intelligence, his humor, his encyclopedic knowledge of local plants and animals, his curiosity about the natural world and his deep devotion to conservation of all of nature. We had to admire his dedication to acquiring this knowledge; he unwittingly forced us to reevaluate our own standards.

10.0 DOCUMENT DISTRIBUTION LIST

- 40 Tim Kilgannon
Project Officer
Remedial Planning and Monitoring Branch
Rocky Mountain Arsenal
Commerce City, CO 80022-1748

- 2 Dr. Victor Perman
University of Minnesota
Department of Veterinary Pathobiology
College of Veterinary Medicine
205 Veterinary Service
1971 Commonwealth Avenue
St. Paul, Minnesota 55108

- 1 Dr. Douglas Weiss
University of Minnesota
Department of Veterinary Pathobiology
College of Veterinary Medicine
205 Veterinary Service
1971 Commonwealth Avenue
St. Paul, Minnesota 55108

- 1 Colonel Eugene Bishop,
Program Manager for Rocky Mountain Arsenal
Bldg 111
Rocky Mountain Arsenal
Commerce City, CO 80022

- 1 Major Thomas Cook
Rocky Mountain Arsenal
Bldg 111
Rocky Mountain Arsenal
Commerce City, CO 80022

- 1 Ray Fatz
Deputy Assistant Secretary Army
Environment, Safety, Occupational Health
RM 2E577
Pentagon
Washington, DC 20310-0110

- 1 Dr. Nancy Munro
Oak Ridge National Laboratory
P O Box 2008 - Bldg 2001 MS-6051
Oak Ridge, TN 37831-6051