APPENDIX 12 Study Protocol and Amendments

TWO-GENERATION REPRODUCTIVE STUDY IN MINK FED DIISOPROPYL METHYLPHOSPHONATE (DIMP)

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SIGNATURE PAGE

GLP: Yes, with provisions for mink husbandry.
Human Subject: Yes____ No XX
Controlled Sub.: Yes____ No XX

TITLE: Two-Generation Reproductive Study in Mink Fed Diisopropyl Methylphosphonate (DIMP)

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1.0 EXPERIMENT TITLE

Two-Generation Reproductive Study in Mink Fed Diisopropyl Methylphosphonate (DIMP)

1.1 FACILITY

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1.2 STUDY DIRECTOR

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2.0 OBJECTIVES

2.1. The primary objective of this study is 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. The animals will be evaluated by clinical observation, outcome of mating, analyses of blood and by necropsy and microscopic examination.

2.2. The logistics of GLP studies in mink are challenging and costly, and data on the effects of xenobiotics on mink reproduction are sparse. The primary objective requires that the challenge be met and the expenses be incurred, yet it measures a relatively narrow endpoint in the spectrum of possible reproductive effects.

The federal government has recently (July 1994) issued new, comprehensive draft guidelines to test reproductive effects (Health Effects Test Guidelines for Reproduction and Fertility Effects) from the US EPA's Office of Prevention, Pesticides and Toxic

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Substances (1994). These guidelines provide for all of the requirements for reproductive tests under EPA's FIFRA and TSCA rules, as well as for internationally harmonized requirements. The guidelines are the current "gold standard" test for reproductive effects.

In the course of pursuing the primary objective, we can follow these guidelines very closely, with suitable allowances for reproductive behavior of mink, and obtain most of those additional reproductive data in a highly cost-effective manner. The work proposed here will thus meet the highest standards recommended to test the reproductive toxicity comprehensively. The additional data to be obtained include effects on fecundity (number, sex and viability of offspring), presence of terata in offspring, effects on sperm and on the morphology of reproductive organs in the male. Videotechnology, as recommended in the guideline, will be used to evaluate potential effects on sperm.

There is no database in mink for genetic toxicology. We will have the opportunity to corroborate directly in treated mink, the previous (negative) in vitro mutagenicity studies of DIMP, as well as the in vivo mutagenicity studies performed with DIMP in mice and rats (Tice, 1993).

Thus a second objective is to take advantage of the study design to obtain additional reproductive data that will become available as Objective #1 is being pursued.

3.0 BACKGROUND

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

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

The specific studies that were considered on which to base the Lifetime Health Advisory included a 90-day study in dogs (Hart, 1980) and a 12-month study in mink (Aulerich,

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1979). The authors of the Advisory concluded that data from the mink study could not be used for this Advisory because of "high natural mortality in mink and uncertainties concerning the relevance of mink to human health assessment" (EPA, 1989).

The EPA also recommended a Longer-term (7-year) Health Advisory and set the value for adult humans at 30 mg/L(30,000 ppb) of DIMP. To arrive at this value, several animal studies, excluding the mink, were considered. Of the studies in mice, rats and dogs that were evaluated, the authors based their calculations on the data from the same 90-day dog study that they used for the Lifetime Health Advisory because it had the most conservative No Observable Adverse Effect Level (NOAEL) of these species, 75 mg/kg/day.

The Shell Oil 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 (1979) 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, Dr. Edward J. Calabrese, reviewed the EPA and Shell Oil Company recommendations at the request of the State of Colorado and proposed 0.36 µg/L (0.36 ppb) as the lifetime drinking water guideline (Calabrese, 1990). Calabrese used the data from the 12-month mink study as did Shell Oil 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 et al 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

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histopathologic examination, reproductive and teratogenic toxicity studies in other species, and additional in-vitro mutagenicity studies.

The Army awarded a contract to Pathology Associates, Inc. (PAI), Frederick, MD, 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 or 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. 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 also 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

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NOAEL 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 drinking 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, 2/24 at 50 ppm and 0/24 in untreated controls.

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. (It 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 was the same (10.8%) 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 controls 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 female mink in the Aulerich et al study was

"Nursing Disease", a stress-related fatal condition afflicting female mink that are unable to compensate for the energy demands of parturition and lactation. This condition has since been described in detail (Schneider and Hunter, 1993).

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 on the recent work by Bucci et al 1992 and 1994; Weiss et al 1994, that established the similarity of the response of mink to that of the dogs reported by Hart (the study used by EPA to set 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, with its controversial outcome.

The study proposed here is designed to repeat the administration of DIMP to mink during pregnancy and lactation, at the highest dose used by Dr. Aulerich's group. We will also extend those observations by using a higher dose, two generations of offspring and by examining the animals more closely in accord with EPA's current OPPTS test guidelines for health effects on reproduction and fertility. Regrettably, the facilities available to us preclude our using a substantially larger number of animals than was used by Aulerich et al.

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 incomparable experience in all the logistical aspects of preparing the dosed feed, caring for the animals and in analyzing DIMP for purity, and stability, and for concentration in feed. We will capitalize on this experience in this proposed study by using as many of the same personnel, suppliers, facilities, and techniques as can be accessed.

4.0 COMPLIANCE WITH EPA GUIDELINES

As stated earlier, this protocol will comply with EPA's TSCA Health Effects Guidelines, and specifically with the latest (1994 draft) Health Effects Test Guidelines for Reproductive and Fertility Tests, i.e., OPPTS 870.3800, which provide for appropriate modification to accommodate species other than the rat (para 2e). Modifications included in this protocol include:

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The F_0 animals cannot be exposed to the test article during the full period of gametogenesis, before the first mating. Because mink mate only in March, our proposal start date permits approximately 4 - 5 weeks of dosing before mating.

We will examine unequal numbers of males and females. Commercially-raised mink are bred in polygamous fashion, 4 - 6 females per male. Because of space limitations and because our primary objective is effects on females, we will adhere to this practice.

The mating procedure will adhere to that practiced in successful commercial mink operations. It includes two periods of copulation spaced 8 days apart, with post-coital vaginal aspiration for sperm, immediately after each mating. Vaginal smears to establish state of estrus are not used since ovulation is copulation-dependent in the mink. Mating pairs will be identified in the study data.

The litter size is expected to be 4 - 6 kits; standardization of litter size will not be practiced.

Pregnant dams will not be disturbed to determine weekly body weight. Food consumption will be measured one day per week.

There is no data base for mink regarding age at vaginal opening and preputial separation nor on anogenital distance; if feasible, we will attempt to record these data.

We propose gross necropsy and microscopic examination that are more extensive than the guidelines recommend, to capitalize on the availability of these treated animals. For economy, tissues from certain groups will be preserved and held, should retrospective examination be warranted; in other groups, only selected tissues (reproductive tract) will be examined, or only tissues from high dose and control animals will be examined.

The dose levels employed must include 450 ppm for one test group, to repeat the high-dose exposure employed by Aulerich et al (1979). This dose was innocuous in our previous 90-day study in non-pregnant mink (Bucci et al 1992 and 1994), and we are proposing 450 ppm here as the intermediate dose. From the metabolism and excretion pattern of DIMP in mink (Weiss et al 1994), we believe all lower doses should produce no evidence of toxicity. Selection of the high dose for the two-generation duration is less certain.

5.0 CHEMICAL COMPOSITION OF DIISOPROPYL METHYLPHOSPHONATE (Dacre and Rosenblatt, 1987)

CAS number: 1445-75-6
RTECS number: SZ9090000 (1983-1984 Supplement)
Edgewood Arsenal Number: EA 1250
Structure: ((CH3)2CHO)2(CH3)P=O
Molecular Formula: C7H,703P
Physical appearance: oily liquid at room temperature
Density at 25° C: 0.976 g/cc
Boiling Point: 174° C
Solubility in water at 25° C: listed as 1-2 g/L, however, our tests show at least 8 g/L
Hydrolysis rate at 1 OIC: (3.2xl 0-6M sec-')
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.

5.1 CHARACTERIZATION OF THE TEST ARTICLE

Purity of DIMP will be determined by the supplier, Lancaster Synthesis LTD, Windham, NH, and confirmed under conditions of Good Laboratory Practice by an independent laboratory. A specification sheet and a Material Safety Data Sheet will be provided with each lot number. (We anticipate use of only one lot.) This supplier provided the DIMP used in the previous studies by Bucci, et al (1992 and 1994) and Weiss, et al (1994).

Analytical methods for DIMP are well documented (Fasano et al 1982; Seiman, L. in Appendix of Bucci, et al 1992). When analyzed by an independent laboratory using ultraviolet/visible and infrared spectroscopy, nuclear magnetic resonance, and gas and thin-layer chromatography, the DIMP used by Bucci et al in 1991 was revealed to contain a single impurity (diisopropyl phosphonate), which comprised 5.3% - 5.4% of the sample. The water content was 0.2%. We will subject the DIMP used in the proposed study to the same purity analyses, by the same laboratory. The dosed food mixture will be analyzed for DIMP content by the same laboratory also, immediately after preparation.

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6.0 EXPERIMENT DESIGN

6.1 GENERAL

Mink (Mustela vison) were selected as the animal system by the sponsor. The husbandry of the mink is well established and was reviewed by Joergensen (1985).

Mink have a single annual breeding season of approximately one month in duration, which occurs between late February and late March in 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 concentrations of 50,000 to 500,000/mm^3. The males ejaculate approximately 80 million spermatozoa during a normal copulation lasting 30 to 60 minutes. In females each ovary contains a number of follicles having a diameter of 1 mm or more at the beginning of the breeding season. If the female is not mated, the mature follicles degenerate, and are followed by successive "waves" of replacement follicles. Ovulation is dependent on copulation and occurs 36 - 42 hours afterwards. Most of the 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 the rematings have higher survival rates.

The average gestation length in mink is 49 - 50 days. The dams are very excitable and subject to high rates of abortion if disturbed during pregnancy. In Northern U.S., parturition occurs predominately in late April to mid May. The average litter size is 5 kits. The lactation period lasts approximately 42 days. The kits consume mother's milk exclusively for 21 days. Kits will eat feed beginning around the fourth week.

The oral route of administration was selected for this study in accord with the EPA/FDA recommendations for toxicological studies. Administration of the DIMP in drinking water would be preferable since the issue concerns groundwater and drinking water, but the mink diet contains approximately 65% moisture and their daily requirement of 190-225 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. Dosed water would therefore contain high and possibly unpalatable concentrations of DIMP. Mink also play with their water sources and spill unquantified amounts, so determining the actual doses consumed

in water would require considerable approximation. Thus we are proposing the more cumbersome but less uncertain route of dosed feed.

All animals will be randomly assigned by weight rank (from heaviest to lightest) to treatment groups and cages. The identity of treatment groups and dosed feed will be color-coded, to minimize bias on the part of the animal technicians.

6.2 EXPERIMENT DETAIL

6.2.1 Animal Model

Mink, Mustela vison ("Ranch Wild" strain) First generation (F_0) born May 1994, approximately 7 months old at start on Feb 1995; F_1 born in May 1995. The F_0 animals will be supplied by the North Branch Fur Farm, North Branch, MN, the same source and strain used for the previous DIMP study (Bucci et al 1991). The mink will be vaccinated against Pseudomonas pneumonia, canine distemper, mink virus enteritis, and Type C botulism (United Vaccine Co., Middleton, WI). The ranch has been free of Aleutian Disease virus since 1977.

6.2.2 Protocol Duration

Six months for F_0; 12 months for F1 (18 consecutive months)

6.2.3 Route of Administration

Oral (mixed in food)

6.2.4 Frequency of Administration

Daily in Feed

6.2.5 Dose Levels

150 ppm, 450 ppm, 2500 ppm, in feed. Anticipated food consumption is approximately 200 gm/day for adult non-pregnant and non-lactating females having body weight of approximately one kilogram. These dose levels will thus provide approximately 25 mg/kg.

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75 mg/kg, and 415 mg/kg of DIMP daily, on the basis of body weight. Pregnant and lactating animals are expected to have increased food intake on a body-weight basis. (Duration of pregnancy is approximately seven weeks, lactation six weeks.)

6.2.6 Number of Animals Treated

30 females, 6 males per dose level per generation

6.2.7 Number Untreated Control Animals

60 females, 12 males per generation

6.2.8 Total Animals Required

30 males and 150 females per generation; 360 animals total, plus approximately 10% surplus in each group (220 animals will be purchased to assure sufficient mated females in the first generation).

6.2.9 Feed

Complete mink diet that fulfills the various nutritional needs throughout their life cycle. Mink rations are fabricated of locally available components based on cost. In the Minnesota area, mink ranchers use primarily by-products of meat, poultry and egg-packing industries. Fish by-products are also used, and cereal grains comprise about 20% of the rations by weight. Supplements and some water are added. The ingredients are ground finely and mixed, forming a moist paste. The diet is varied somewhat through the life cycle, to support growth, reproduction and pelt development. Nutritional requirements of mink have been well established and are defined in a National Research Council Handbook, (Nutritional Requirements of Mink and Fox, National Research Council 1982). Our ration will meet or exceed those requirements.

The feed used in this study will be purchased as the prepared paste, from the supplier of the animals (North Branch Fur Farm, North Branch, MN). It will be analyzed for macro and micronutrients, and for pesticides, fungicides, mycotoxins and other contaminants; each batch of dosed feed will be examined microbiologically for number of total bacteria and for E. coli and Salmonella species. (Bucci et al 1992).

The animal care subcontractor (U of MN) will thaw the paste, mix the DIMP into the feed using a commercial mixer, preparing the requisite doses in quantity sufficient for 45 days' feed. The dosed and control feed will be portioned with a commercial canning machine into single-meal metal cans, color-coded, and refrozen (-20°C) until used.

Each batch of dosed feed will be sampled and analyzed for DIMP content; the feed will be used if the actual concentration of DIMP is 90-110% of the target concentration. The sampling level will be one can per hundred prepared. For each dose level approximately 1400 cans will be prepared (each with approximately 250 g feed) every 45 days. The 250g/day should provide ad libitum feeding except during pregnancy and lactation, when the ration will be increased to continue to offer a slight excess.

In our previous study (Bucci et al 1992 and 1994), DIMP was stable in feed samples held at -20°C for 0, 7, 14, 28, 60, 90 and 120 days and in others held at room temperature for 24 hours.

6.2.10 Procedure for Individual Animal Identification

All animals will be identified by cage cards and by electronic transponder implanted subcutaneously in the interscapular region at the time the animals are anesthetized for blood sampling, during quarantine.

Dose Groups per Generation
Two-Generation Reproductive Study of DIMP in Mink

SEX	DOSE LEVEL (ppm in Feed)	NUMBER OF ANIMALS*
Male	2500	6
Male	450	6
Male	150	6
Male	0	12
Female	2500	30
Female	450	30
Female	150	30
Female	0	60

* number will be increased by 10% if space permits

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6.2.11 Housing Requirements

Material of cage - Galvanized wire mesh cage and pine wood nest box
Cage Measurement - Whelping cages 4000 cubic inches (65,548 cc)
Bedding Material - None - except in nest box at time of whelping (straw).
Animals/Cage - dams alone except with litter for @ 6 weeks
Temperature - 23°C ± 5°
Humidity - 30% - 70% relative humidity
Light/Dark Ratio- artificial light controlled by computer to equal duration of outdoor ambient light/dark cycle. The duration of daylight is critical to regulation of the mink breeding cycle. Light intensity will be 15 -25 lumens.

6.2.12 Waste Disposal

Animal waste will be disposed of by a licensed waste disposal firm.

7.0 CONDUCT OF THE STUDY

7.1 FIRST GENERATION (F_0)

The animals will be quarantined for at least two weeks prior to the study. The F_0 animals will be housed individually and will, after quarantine, receive the appropriate dose of DIMP in their food daily. Cageside clinical observations will be made twice daily, the mink will be weighed weekly, and food consumption will be measured once weekly for each animal.

During quarantine, each animal will be anesthetized with 30 mg ketamine and 1 mg xylazine/kg body weight, intramuscularly. An electronic transponder capsule will be implanted subcutaneously, in the intercapsular area to provide unique identification. A blood sample will be obtained from the jugular vein at this time, for analysis as described in the paragraphs below.

After mating, females will not be weighed until the day of whelping, just after delivery, at which time the kits will also be weighed individually.

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Males will be evaluated by manual palpation for proper testicular development during the month of December. Breeding will begin at approximately 7 a.m. and end at noon, March 6 - 10, 1995. The females will be placed in the males' cage for one-half to one hour, until a positive mating is observed. Positive matings will be confirmed by post-coital vaginal aspiration for sperm. A positive mating will be followed by a second mating of the same pair eight days later. Identity of mating pairs will be recorded.

During the whelping period, the nest boxes will be checked daily. Newborn kits will be weighed and sexed on the day of whelping, at four weeks and at sacrifice. Whelping females will be weighed on the day of whelping, four weeks later and at sacrifice. Length of gestation, litter size, sex ratio, kit mortality, increase in kit biomass at four weeks, and weight of lactating females will be recorded.

Animals that die or are removed in moribund condition during the course of the study (F_0 and F_1) will be subject to complete necropsy with microscopic examination of all protocol-specified tissues and gross lesions (see Paragraph 7.1.4.2). Clinical chemical and microbiological diagnostic procedures will be included where appropriate.

If feasible, kits will be monitored for date of vaginal opening, preputial separation and for determination of anogenital distance.

At weaning, 6 F_1 males and 30 F_1 females per treated group and 12 F_1 males and 60 F_1 females from the control group (at least one female per litter) will be selected randomly as parents for the second generation of their respective dose groups. Sibling matings will be avoided and the identity of mating pairs will be recorded.

Of the offspring not selected for mating, two kits of each sex per litter will be examined for gross anatomic defects (see Paragraph 7.1.4.2). All protocol-specified tissues will be preserved in 10% neutral-buffered formalin, for potential future microscopic examination. All gross lesions will be examined microscopically.

7.1.1 Clinical Pathology

The blood sample obtained during quarantine from F_0 males and females will be examined for a full panel of hematologic and chemical analytes as recommended by the National Toxicology Program (NTP, 1991), and will also include determinations of plasma and

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whole blood cholinesterase. Examination of blood specimens from all mink will be conducted by the Department of Pathobiology, College of Veterinary Medicine, U of MN. Dr. Douglas Weiss, Director of Hospital Laboratories, will be the responsible collaborator. Blood samples (approximately 8 ml) for hematological and chemical examination will be collected during quarantine, and at necropsy. Samples will be collected by jugular vein puncture under ketamine:xylazine anesthesia, prior to feeding.

7.1.1.1 Hematology

Complete blood cell count including cell morphology assessment, hemoglobin concentration, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, reticulocytes, and percentage of red cells with Heinz bodies will be determined.

7.1.1.2 Clinical Chemistry

Electrolytes (sodium, potassium, chloride, total carbon dioxide, glucose, calcium, and phosphorus), BUN, total protein, whole blood and plasma cholinesterase, creatinine, albumin and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) will also be measured.

7.1.2 Genetic Toxicology Data

These studies will include examination of bone marrow for induction of micronuclei (damage to chromosomes) and examination of hepatocytes and leukocytes for DNA strand breaks and alkali-labile sites, using single-cell gel electrophoresis (Tice, 1995). To establish the similarity of mink to other mammalian models, we recommend examination of phytohemagglutinin-stimulated lymphocytes from treated and control mink, for formation of micronuclei, as well as general karyotyping of metaphase nuclei. The latter can reveal the frequency of gross chromosomal aberrations (breaks and deletions, discontinuities, displacements, exchanges of genetic material in chromosomes). Assays to screen for restriction fragmentation length polymorphisms (DNA fingerprints) could reveal the extent of relative genetic similarity or diversity in this mink genotype, for comparison with other animal models of human toxicities.

7.1.3 Male Reproductive Evaluations (F_0)

After the second mating, a blood sample will be obtained from the F_0 males under xylazine/ketamine anesthesia and they will be killed by CO2 asphyxiation. A complete gross necropsy examination will be performed on each, but only testes, accessory sex glands and gross lesions will be collected. Semen will be obtained from one vas deferens, following PAI SOP for sperm analysis using the Hamilton-Thorn IVOS-10 Automated Semen Analyzer, the videotechnology referenced by EPA OPPTS (Slott and Perrault, 1993) and owned by PAI. Sperm from the epididymis will be evaluated for count and morphology. Testes and accessory sex organs will be weighed per EPA OPPTS, and preserved in 10% neutral-buffered formalin for future examination if warranted and requested. All gross lesions will be examined microscopically. Specimens of bone marrow, liver and peripheral blood will be obtained from untreated males for pilot studies to establish genetic toxicology baselines.

7.1.4 Pathology, F_0 Females

After weaning of the F_1 generation , a second blood sample will be obtained from the F_0 animals and analyzed as described in Paragraph 7.1.1, to reveal any effects of treatment. The animals will be subject to complete necropsy and microscopic examination following the specifications of the National Toxicology Program (NTP .1992, with organs weigned as specified in the EPA OPPTS. We propose to conduct a complete necropsy with harvest of the complete list of protocol-specified tissues (see 7.1.4.2) from all F_0 females. Organ weights will be determined for all animals in all groups. The complete list of tissues including gross lesions in the high dose group and both control groups will be processed into slides and examined microscopically. Only reproductive tissues and gross lesions will be initially processed and examined in the intermediate and low-dose groups. All other organs from the latter two groups will be examined at necropsy and stored in fixative for later microscopic examination if warranted. Microscopic alterations detected in the high dose animals and not in controls will be cause to examine the affected tissues in the lower dose groups.

Specimens of bone marrow, liver and peripheral blood for genetic toxicology assay will be obtained from 10 animals per treatment group, at random.

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7.1.4.1 Histopathology

Necropsy, tissue collection and processing, and histopathologic evaluations will be done following the procedures of the National Toxicology Program for 13-week studies (NTP 1991) and the Standard Operating Procedures of the Pathology Associates, Inc.

7.1.4.2 Tissues for Gross and Histopathologic Examination

Adrenal gland (2)
Brain (3 sections including frontal cortex, basal ganglia, parietal cortex and thalamus, and cerebellum and pons)
Esophagus
Eyes
Femur, including diaphysis with marrow cavity and epiphysis (femoral condyle with epiphyseal cartilage plate, articular cartilage and articular surface)
Gallbladder
nerve
Gross Lesions
Heart and aorta
Intestine, large (cecum, colon, rectum)
Intestine, small (duodenum, jejunum, ileum)
Kidneys (2)
Liver (2 sections including the left lateral and median lobe)
Lungs (2) and major bronchi median lobe
Lymph nodes(mandibular and mesenteric)
Mammary gland
Muscle, skeletal
Nose (3)
Ovaries (2), Oviducts
Pancreas
Parathyroid glands
Pituitary gland
Prostate
Salivary gland
Skin
Spinal cord and sciatic
Spleen
Stomach
Testis/Epididymis
Thymic area
Thyroid glands
Trachea
Urinary bladder
Uterus

7.1.5 Kits Not Selected for Breeding

All kits will be weighed individually at weaning. After selection of the F_1 animals to be parents of the second generation, two randomly-selected kits of each sex per litter will be necropsied. Special attention will be paid to those anatomic sites most often the site of

developmental malformations, i.e., limbs, digits, eyes, palate, brain, heart, and major blood vessels. Specimens of bone marrow, liver and peripheral blood for genetic toxicology assay will be obtained from 10 animals per treatment group, at random.

The full set of protocol-specified tissues (see Paragraph 7.1.4.2) will be preserved in 10% neutral-buffered formalin, for future examination if warranted. All gross lesions will be examined microscopically. Any remaining animals will be euthanized and discarded.

7.2 SECOND GENERATION (F_1)

The F_1 females will be selected at random, one from each litter to replace each dam. Six males will be selected randomly from each treatment group to serve as sires within their respective groups. Sibling matings will be avoided and identity of the mating pairs will be recorded. If space permits, the number of breeders will be increased approximately 10% to assure sufficient numbers of F_2 litters.

This phase of the study will repeat the F_0 procedures except these F_1 animals will have been exposed to the test article from conception. Dose levels in their feed will continue as it was for the first generation, unless results in the F_0 phase dictate reconsideration.

The animals will be identified uniquely with electronic transponders implanted at the time of their allocation to the F_1 parent generation (July, 1995) shortly after weaning. The identity of their litter of origin will be preserved. Blood samples will be obtained from each of these animals at this time and at 3- month intervals (approximately Oct. 1995 and Jan. 1996). They will be housed individually as were their parents; body weight and food consumption will be measured weekly.

They will be mated beginning mid-March, 1996, using the same procedures followed in the 1995 matings.

7.2.1 Male Reproductive Evaluation (F_1 Parents)

After completion of mating (March, 1996) a blood sample will be obtained from the F_1 breeder males, they will be killed with CO2 and necropsied. The blood samples and video -recorded semen samples will be evaluated as in the previous generation. Testes and accessory sex organs will be weighed and examined microscopically for all animals.

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All gross lesions will be examined microscopically. Bone marrow, liver and peripheral blood samples will be obtained for genotoxicity assay.

7.2.2 Clinical and Tissue Evaluation, F_1 Parental Females

After mating in March, 1996, food consumption by the F_1 parental females will be measured weekly, but these pregnant animals will not be weighed until the day of F_2 whelping. At whelping of the F_2 generation, the same parameters measuring kit numbers, sex, age and vitality will be recorded as were recorded for the F_1 kits. The characteristics of development of anogenital orifices will be recorded if possible. All kits and dams will be weighed on the day of whelping and four weeks later during lactation.

After weaning (early July 1996), a blood sample will be obtained from each F_1 parental female for hematologic, chemical and genetic toxicologic examination as was done for the F_0 females, and the animals will be examined by complete gross necropsy. The same organs will be weighed (per EPA OPPTS). All tissues will be preserved in 10% neutral-buffered formalin. Specimens for genetic toxicology assay will be obtained from 50% of the animals at random.

Tissues from all F_1 females from the high dose and intermediate dose groups and from all control animals will be examined microscopically. (The intermediate dose is the 450 ppm group comparable to the high-dose group in the study of Aulerich et al 1979.) Gross lesions from all groups will be examined microscopically. Microscopic changes detected in the high or intermediate dose groups and not in the controls will be cause for the affected tissue to be examined in the low dose group

Ovaries from ten high dose and ten control animals will be processed to evaluate ovarian toxicity as specified in the EPA OPPTS document.

7.3 F_2 KITS

All F_2 kits will be weighed at weaning, euthanized by C02. Two kits of each sex per litter will be selected randomly and examined by complete necropsy, with special attention to sites affected by common developmental defects, as was the F_1 generation. All protocol-specified tissues will be preserved, but no histopathology will be performed unless warranted by gross findings and requested by the sponsor. Specimens of bone marrow,

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liver and peripheral blood for genetic toxicology assay will be obtained from 12 animals of each sex per treatment group, at random.

8.0 RESULT EVALUATION CRITERIA

This study will be evaluated using the most appropriate analytical methods available at the conclusion of the study. The protocol will be amended to detail the statistical analysis. (e.g., see Allen, 1994).

9.0 DATA COLLECTION

Data collected (body weights, clinical observations, food consumption, mortality data and day (date) of sacrifice) will be entered on an automated data collection system, preparatory to review, tabulation, analysis and reporting. The system will be validated before use and annually during the study.

10.0 RECORDS TO MAINTAIN/REPORTS TO GENERATE

Data will be collected on an automated system.

An interim report of the pregnancy outcomes, and the survival and growth of the F_1 's to weaning, will be submitted to the Contracting Officer's Representative (COR) by 31 August 1995 (10 copies).

Ten copies of the draft report titled, "Two-Generation Reproductive Study of DIMP in Mink," will be submitted to the COR for review by 30 October 1996. Forty (40) copies of a draft final document shall be prepared, following receipt of Army comments, and submitted to the COR by 30 November 1996. The draft final report will be reviewed by the Environmental Protection Agency, State of Colorado, and the Agency for Toxic Substances and Disease Registry. Following receipt of all comments, the contractor shall submit forty (40) copies of the final report to the COR by 31 December 1996.

The "Two-Generation Reproductive Study of DIMP in Mink" reports shall be prepared according to the format from "Style Manual for USAMBRDL Technical Reports and Contractor-prepared Reports" USAMBRDL, Fort Detrick, Frederick, MD 21701,

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(Anonymous, 1980) and the Contractor Reporting Requirements for the Health Effects Research Division (Barkley 1984), or an alternate format approved by the COR.

11.0 QUALITY ASSURANCE

This study will be conducted in general agreement with Good Laboratory Practice Standards, Environmental Protection Agency, Toxic Substances Control Act (TSCA) 40 CFR Part 792 (Federal Register, Vol. 54, No.158, August 17, 1989, pp. 34034 - 34050), and EPA OPPTS 870.3800, EPA 712-C-94-208, July 1994. All aspects of the studies will be conducted in accordance with written Standard Operating Procedures (SOP) of the performing unit and all raw data and performance documents will be maintained in general agreement with GLP. All changes, deviations, and amendments will be documented and signed by the Study Director in accordance with Sections 792.190 and 792.195 of the Good Laboratory Practice Standards. An administratively separate quality assurance unit shall monitor the studies to assure adherence to good laboratory practices and the approved SOPs.

12.0 STORAGE OF RECORDS

All hard copies of data sheets for the present study will be stored in the PAI Archives under the control of the Quality Assurance Officer. Biological samples collected during the course of the study will be placed in secure storage in the PAI Archive. All records, data and reports will be maintained for a minimum of five years.

13.0 PERSONNEL

Principal Investigator: Dr. Thomas J. Bucci, PAI
Co-Investigator and Clinical Pathologist: Dr. Victor Perman, (U of MN)
Co-Investigator and Reproductive Toxicologist: Michael D. Mercieca, PAI
Co-Investigator and Genetic Toxicologist: Dr. Lawrence B. Schook, (U of MN)
Mink Specialist: Dr. William Wustenberg, PAI
Clinical Pathologist: Dr. Douglas J. Weiss, (U of MN)
Quality Assurance: Carol Hoffman, PAI
Pathologist: Dr. Robert M. Kovatch, PAI

14.0 CARE AND USE OF LABORATORY ANIMALS

This protocol will be conducted in accordance with Army Regulation 70-18, (1 June 1984) "The Use of Animals in DOD Programs," and with the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 85-23, Revised 1985). All caging, housing and husbandry will meet or exceed 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 Animal Care and Use Committee will review and approve the use of animals for this protocol.

15.0 TIME SEQUENCE (anticipated)

Draft Protocol	23 January 95
Final Protocol	10 February 95
Animal Allocation to Dose Groups	13-17 February 95
Start DIMP Feeding	20 February 95
Mate F_0 (Parents)	6-24 March 95
Necropsy F_0 Parental Males	27-31 March 95
Whelp F_1 (Approx.)	8-19 May 95
Wean F_1 (Approx.)	4-13 July 95
Necropsy F_0 Parental Females	26-30 June 95
Necropsy F_1 Kits Not Selected for Breeding	24-28 July 95
Mate F_1 (Parents)	7-25 March 96
Necropsy F_1 Parental Males	28-31 March 96
Whelp F_2 (Approx.)	9-20 May 96
Wean F_2 (Approx.)	5-14 July 96
Necropsy F_1 Parental Females	5-14 July 96
Necropsy F_2 Kits	19-23 July 96
Draft Final Report to OAS	30 November 96
Final Report to OAS	31 December 96

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STUDY NUMBER:	TP-001
AMENDMENT:	1
STUDY TITLE:	TWO-GENERATION REPRODUCTIVE STUDY IN MINK FED DIISOPROPYL METHYLPHOSPHONATE (DIMP)
DATE ISSUED:	May 1 1995
DATE EFFECTIVE:	April 1, 1995
	AMENDMENT 1

This amendment effects changes in the draft protocol of the same title, dated 23 Jan 1995 and signed by me on 16 Feb 1995, to officially begin the study. Written approval to progress with the study had been provided by the sponsor on 9 Dec 1994 (Letter contract DAAM02-95-C-0030), but the sponsor's review of the 23 Jan 1995 draft protocol had not been completed and a protocol signed by the sponsor was therefore not yet available on 16 Feb 1995, when treatment with the test article was scheduled to begin.

This amendment incorporates recommendations of reviewers as well as editorial corrections as follow:

1. Title page and all other pages: add Study Number TP-001. Change UMN to U of MN throughout the document.

2. Signature page (page i): delete all signature lines except Study Director and Sponsor. Reason: Others not necessary.

3. Page 1 Objectives, Section 2.1: expanded at reviewer's request, to explain the reason for the study. This section now reads:

   2.1 There is ongoing controversy concerning the cause of mortality in female mink fed DIMP in a previous reproductive study (Aulerich et al, 1979). The controversy involves whether or not the deaths were caused by exposure to DIMP, leading to disagreement among authorities regarding use of those data in 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 3, Background. The primary objective of this study is to repeat the essential features of the Aulerich study, 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. The animals will be evaluated by clinical observation, outcome of mating, analyses of blood and by necropsy and microscopic examination.

4. Page 2 Objectives, Section 2.2: In the third line on the page, the sentence, The guidelines are the current "gold standard" test for reproductive effects was deleted. Reason: Reviewer's recommendation, since the referenced guidelines are still in draft form.

5. Page 6, Compliance with EPA Guidelines, Section 4.0: the first three words, As stated earlier, were deleted. Reason: Editorial improvement.

6. Page 7, Section 4 (end of 2nd line on p 7): Change proposal to read "protocol". Reason: Editorial correction.

7. Page 7, Section 4: The fourth paragraph, second sentence, is amended to change the mating interval to read "8-10 days apart, with vaginal aspiration for sperm immediately after mating, once for each male", replacing the original [8 days apart.....after each mating.] Reason: The additional two days' time is needed to accomplish remating of all 175 females; aspiration for sperm cannot be accomplished after every mating because too much time is required.

   - The fifth paragraph beginning Pregnant dams will not be disturbed....was expanded to explain why pregnant and lactating dams will not be disturbed to obtain weekly body weight. Reason: Reviewer's request.

   - The sixth paragraph is deleted at the Study Director's decision. Reason: Determination of age (of kits) at vaginal opening and preputial separation, and measurement of ano-genital distance would require daily examination of all kits. This would be excessively upsetting to kits and dams and could result in dam's abandoning or cannibalizing the kits.

- The seventh paragraph: the 2nd word propose was changed to read "plan" (editorial correction) and the final phrase, or only tissues from high dose and control animals will be examined was deleted. Reason: Reviewer's request.

- The last paragraph was expanded to provide fuller explanation of the rationale for dose selection. Reason: Reviewer's request.

The amended portion of Section 4 now reads:

Because the dams are very excitable and subject to high rates of abortions when agitated, pregnant or lactating dams will not be disturbed to determine weekly body weight. Food consumption will be measured one day per week.

We plan gross necropsy and microscopic examinations that are more extensive than the guidelines recommend, to capitalize on the availability of these treated animals. For economy, tissues from certain groups will be preserved and held, should retrospective examination be warranted; in other groups, only selected tissues (reproductive tract) will be examined.

The dose levels employed must include 450 ppm for one test group, to repeat the high-dose exposure employed by Aulerich et al (1979). It was unexplained deaths at this dose that caused the controversy about the Aulerich study. The 450 ppm dose was innocuous in our previous 90-day study in non-pregnant mink (Bucci et al 1992 and 1994), and we are including 450 ppm here as the intermediate dose. From the metabolism and excretion pattern of DIMP in mink (Weiss et al 1994), we believe all lower doses should produce no evidence of toxicity. For the low dose in this study we will use 150 ppm, the intermediate dose used by Aulerich et al, with no adverse effect. Potential effect of the high dose for this two-generation duration is less predictable, but 2700 ppm was the LOAEL in our 90-day study. We therefore are using 2500 ppm as our target high dose in this study.

8. Page 8, Section 5, Solubility in water : I OIC was corrected to read 10°C. Reason: correct typographical error.

   5.1 The "independent laboratory" referred to in the original protocol was amended to read Midwest Research Laboratory (MRI), or simple MRI. Reason: improved specificity.

9. Page 9, Section 6.1 The first sentence was changed to include explanation of the sponsor's choice of test system by adding ".....to replicate the essential features of a previous study in mink (Aulerich et al, 1979)". Reason: Reviewer's request.

   - The 6th line of the second paragraph was corrected to "15 to 60 minutes" instead of the erroneous 30 to 60 minutes.

   - The third paragraph was expanded to begin with the sentence "Mink exhibit embryonic diapause (delayed implantation) with gestation varying between 39 and 72 days." Reason: Improved explanation.

10. Page 10, Section 6.2.1.: The first paragraph has been expanded to explain the genetic similarity of the test system to that of the Aulerich et al study being replicated. Reason: Improved explanation for selection of this test system. This section now reads:

    Mink, Mustela vison ("Ranch Wild" strain) First generation (F_0) born May 1994, approximately 7 months old at start on Feb 1995; F_1 born in May 1995. The F_0 animals will be supplied by the North Branch Fur Farm, North Branch, MN, the same source and strain used for the previous DIMP study (Bucci et al 1992). The mink will be vaccinated against Pseudomonas pneumonia, canine distemper, mink virus enteritis, and Type C botulism (United Vaccine Co., Middleton, WI).

    The ranch has been free of Aleutian Disease virus since 1977. The "Ranch Wild" strain is the strain currently available that is most closely related to the stock used by Aulerich et al in 1979-1978 (per telephone conversations with Dr. Richard Aulerich by Drs. Thomas Bucci and William Wustenberg, Dec 1990 and Feb, 1995).

11. Page 11 Sections 6.2.6, 6.2.7 and 6.2.8.: The number of treated animals used was increased from 30 females and 6 males per generation to 35 females and 7 males in F_0 and to 35 females and 12 males in F_1; the number of untreated controls was increased to 70 females and 14 males in F_0 to 70 females and 24 males in F_1. Reason: Study Director's intent, should space be available; also Reviewer's recommendation.

- A paragraph was added to the end of Section 6.2.7 to explain the rationale for the double control group. Reason: Reviewer's request.

These sections now read:

6.2.6 Number of Animals Treated

35 females, 7 males per dose level in F_0; 35 females and 12 males F_1.

6.2.7 Number Untreated Control Animals

70 females, 14 males in F_0; 70 females and 24 males F_1.

There is no theoretical statistical justification for this double-sized control group, if universal random biologic variation is assumed. However, experience with mink reveals a far 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, involving sudden death of dams, abortions and kit mortality. We have added the second control group of 35 females and associated males as a concurrent control replicate, to improve our prospects to observe representative variations among groups of only 35 female mink.

6.2.8 Total Animals Required

175 females and 35 males in F_o; 175 females and 60 males in F_1 (220 animals will be purchased for F_o to assure sufficient mated females in the first generation).

12. Page 11, Section 6.2.9: The second paragraph is amended to specify the laboratory that will analyze the feed for nutrients and contaminants (Lancaster Laboratories, Lancaster, PA.

- The first paragraph on p.12 is corrected to reflect that the mink feed (ground meat paste) is received thawed, and is frozen only after being canned.

- Second paragraph: A final sentence is added to explain that extra water will be added to the feed when the kits are adapting to solid feed before weaning. Reason: Explanation of a change in the feed mixing procedure.

- Third paragraph: this paragraph is expanded and a fourth paragraph is added to increase explanation of our confidence in the purity and stability of the diet.

The paragraphs on page 12 now read:

> The animal care subcontractor (U of MN) will mix the DIMP into the feed using a commercial mixer, preparing the requisite doses in quantity sufficient for 45 days' feed. The dosed and control feed will be portioned with a commercial canning machine into single-meal metal cans, color-coded, and frozen (-20°C) until used.
>
> Each batch of dosed feed will be sampled and analyzed for DIMP content; the feed will be used if the actual concentration of DIMP is 90-110% of the target concentration. The sampling level will be one can per hundred prepared. For each dose level approximately 1400 cans will be prepared (each with approximately 250 g feed) every 45 days. The 250 g/day should provide ad libitum feeding except during pregnancy and lactation, when the ration will be increased to continue to offer a slight excess. The consistency of the feed will be adjusted with added water when kits are adapting to the solid feed before weaning.
>
> In our previous study (Bucci et al 1992 and 1994), DIMP was stable in feed samples held at -20°C for 0, 7, 14, 28, 60, 90 and 120 days and in others held at room temperature for 24 hours. This test will not be repeated, but the actual DIMP concentrations achieved in each batch of dosed feed and the purity of the test article will be determined by analyses conducted by Midwest Research Institute, Kansas City, Mo.
>
> We will have the benefit that our untreated feed, supplied by North Branch Fur Farm at nearby North Branch, MN, will concurrently be fed to approximately 4000 breeding females at that farm until their young are weaned in June, 1995; thereafter, it will be fed to approximately 25,000 animals until pelting time in Dec, 1995. The same cycle will be repeated in 1996. Thus this large population will served as corroboration for any unanticipated variables potentially related to diet.

- The table on page 12 is corrected to reflect the increased number of animals used, as specified in Sections 6.2.6 and 6.2.7 (p 11). The footnote below the table is deleted as unnecessary now that the animal numbers have been increased.

The corrected table follows:

Dose Groups per Generation
Two-Generation Reproductive Study of DIMP in Mink

SEX	DOSE LEVEL (ppm in Feed)	NUMBER OF ANIMALS	
		F_0	F_1
Male	2500	7	12
Male	450	7	12
Male	150	7	12
Male	0	14	24
Female	2500	35	35
Female	450	35	35
Female	150	35	35
Female	0	70	70

13. Page 13, Section 6.2.11, Bedding Material : straw was replaced with "Aspen and Poplar wood chips" Reason: These wood chips are cleaner than straw.

- Humidity: 30% to 70% was changed to 15% to 70%. Reason: At very low outdoor MN temperatures, mink are accustomed to low humidity and 30% RH is difficult to maintain indoors during periods of heating when ambient outdoors is very cold.

- Section 6.2.12 Waste Disposal:

  Changed to read:

  Animal waste, excess treated feed and animal carcasses will be disposed of by a licensed waste disposal firm. Reason - Feed and carcasses will also be disposed of by the licensed waste disposal firm.

14. Page 13, Section 7.1, last paragraph on page: First sentence changed to reflect that kits will be weighed collectively per litter and not individually. Reason: they will not have individual identification, and the total biomass is the datum of interest.

    - The amendment adds a final sentence to the paragraph, stipulating that the dams and the kits (as litters) will be reweighed four weeks later. Reason: Editorial improvement; the four-week weight originally was described only later in the protocol.

15. Page 14, Section 7.1, First paragraph on page: Second sentence is changed to read "Breeding will begin March 6, 1995", deleting the time of day and the end of the breeding period. Reason: The original wording was too restrictive to accommodate the behavior of mink.

    - The fourth sentence of the paragraph is changed to specify that post-coital vaginal aspiration for sperm will be performed only until each male is confirmed to produce sperm, but not for every mating. Reason: Requires excessive technician time.

    - Second paragraph (p14), last sentence, is changed to read, "Length of gestation, still births, litter size (weight and number), sex ratio and kit mortality will be recorded". Reason: Editorial improvement.

    - The fourth paragraph is deleted. Reason: Date of vaginal opening, etc, will not be determined, as noted in # 7, above.

    - The fifth paragraph is amended to reflect the increased number of animals, as specified in #11 above (35 females and 12 males in the treated F_1 group; 70 females and 24 males in the F_1 control groups). The paragraph is also expanded to repeat the rationale for increased number of males.

- The sixth paragraph is amended to add clinical pathology determinations on ten kits per sex per treatment group at necropsy. Reason: Reviewer's recommendation.

Section 7.1, (page 13-14) is therefore changed to read:

7.1 FIRST GENERATION (F_0)

The animals will be quarantined for at least two weeks prior to the study. The F_0 animals will be housed individually and will, after quarantine, receive the appropriate dose of DIMP in their food daily. Cageside clinical observations will be made twice daily, the mink will be weighed weekly, and food consumption will be measured once weekly for each animal.

During quarantine, each animal will be anesthetized with 30 mg ketamine and 1 mg xylazine/kg body weight, intramuscularly. An electronic transponder capsule will be implanted subcutaneously, in the interscapular area to provide unique identification. A blood sample will be obtained from the jugular vein at this time, for analysis as described in the paragraphs below.

After mating, females will not be weighed until the day of whelping, just after delivery, at which time the kits will also be weighed collectively per litter (They will not be identified individually). The animals will be reweighed four weeks later.

Males will be evaluated by manual palpation for proper testicular development during the month of December. Breeding will begin March 6, 1995. The females will be placed in the males' cage for one-half to one hour, until a positive mating is observed. Positive matings will be confirmed by post-coital vaginal aspiration for sperm, once for each male. A positive mating will be followed by a second mating of the same pair 8-10 days later. Identity of mating pairs will be recorded.

During the whelping period, the nest boxes will be checked daily. Newborn kits will be weighed (per litter weight) and sexed on the day of whelping, at four weeks and at sacrifice. Whelping females will be weighed after whelping on the day of whelping, four weeks later and at sacrifice. Length of gestation, still births, litter size (weight and number), sex ratio and kit mortality will be recorded.

Animals that die or are removed in moribund condition during the course of the study (F_0 and F_1) will be subject to complete necropsy with microscopic examination of all protocol-specified tissues and gross lesions (see Paragraph 7.1.4.2). Clinical chemical and microbiological diagnostic procedures will be included where appropriate.

At weaning, 12 F_1 males and 35 F_1 females per treated group and 24 F_1 males and 70 F_1 females from the control group (at least one female per litter) will be selected randomly as parents for the second generation of their respective dose groups. Females do not accept every male for mating. Carrying the larger number (12) of males for the F_1 generation will assure at least 6-7 different males as sires within each treatment group and will also provide improved statistical representation of the males for sperm assays. Sibling matings will be avoided and the identity of mating pairs will be recorded.

Of the offspring not selected for mating, two kits of each sex per litter will be examined for gross anatomic defects (see Paragraph 7.1.4.2), and clinical pathology (Para 7.1.1.1 and 7.1.1.2) if two are available. All protocol-specified tissues will be preserved in 10% neutral-buffered formalin, for potential future microscopic examination. All gross lesions will be examined microscopically.

16. Page 14, Section 7.1.1: The first paragraph of this section (final paragraph on page 14) is amended to a) correct the NTP reference date to 1992 from ~~1991~~, and b) to include the F_1 kits among those sampled for clinical chemistry.

This paragraph now reads:

7.1.1 Clinical Pathology

The blood sample obtained during quarantine and again at necropsy from F_0 males and females will be examined for a full panel of hematologic and chemical analytes as recommended by the National Toxicology Program (NTP, 1992), and will also include determinations of plasma and whole blood cholinesterase. Blood from their offspring (F_1) scheduled for necropsy will be examined for the same analytes. Within each treatment group, 10 F_1 kits per sex (each pair from a different litter) will be sampled. Because insufficient volume may be available per animal for all assays, subsets of 10 litters each per treatment group will be defined, one subset each for hematology only, and one each for half the clinical chemistry profiles listed in para 7.1.1.2. The specific analytes allotted to each half will be selected before necropsy.

Examination of blood specimens from all mink will be conducted by the Department of Pathobiology, College of Veterinary Medicine, U of MN. Dr. Douglas Weiss, Director of Hospital Laboratories, will be the responsible collaborator. Blood samples (approximately 8 ml) for hematological and chemical examination will be collected during quarantine, and at necropsy. Samples will be collected by jugular vein puncture under ketamine/xylazine anesthesia, prior to feeding.

17. Page 16 - Section 7.1.3: This paragraph is amended to 1) delete the statement that the videotechnology instrument to be used was referenced in the EPA OPPTS document (Reason: requested by reviewer) and 2) define the semen analysis of these F_0 males as a pilot study rather than a definitive one. Reason: the animals will have been given the test article for only approximately 5 weeks before analysis, there will be only seven males per group and the procedures to be used are unprecedented in mink. The semen analysis in the F_1 males in March, 1996, to be performed on 12 animals per group after lifetime exposure (approximately 9 months) will be definitive. Change xylazine/ketamine to ketamine/xylazine.

Section 7.1.3 now reads:

7.1.3 <u>Male Reproductive Evaluations (F_0)</u>

After the second mating, a blood sample will be obtained from the F_0 males under ketamine/xylazine anesthesia and they will be killed by CO_2 asphyxiation. A complete gross necropsy examination will be performed on each, but only testes, epididymis, prostate and gross lesions will be collected. As a pilot study, semen will be obtained from the cauda epididymis, following PAI SOP for sperm analysis using the Hamilton-Thorn IVOS-10 Automated Semen Analyzer (Slott and Perrault, 1993). Sperm from the epididymis will be evaluated for count and morphology. Testes, epididymis and prostate gland (the only apparent accessory sex gland in mink) will be weighed per EPA OPPTS, and preserved in 10% neutral-buffered formalin for future examination if warranted and requested. All gross lesions will be examined microscopically. Specimens of bone marrow, liver and peripheral blood will be obtained from untreated males for pilot studies to establish genetic toxicology baselines from untreated males or the animals reserved for methods development. The sperm analysis procedures relating to the F_0 generation will be regarded as a pilot study, since there is no precedent for them in mink, since these males are few in number and since they will have been exposed to the DIMP for less than a full spermatogenic cycle. We consider the procedures to be definitive

and subject to GLP for the F_1 male parents, however.

18. Page 16 - Section 7.1.4: Pathology, F_0 Females - This section amended to add microscopic examination of all tissues from all F_0 females. The original protocol specified that only high dose and control females would have all tissues examined . A second amendment to this section adds analysis of brain tissue for cholinesterase activity, in ten F_0 parental females in each group. Reason: Reviewer's recommendation.

    Section 7.1.4 now reads:

    7.1.4 Pathology, F_0 Females

    After weaning of the F_1 generation, a second blood sample will be obtained from the F_0 animals immediately before necropsy and analyzed as described in Paragraph 7.1.1. The animals will be subject to complete necropsy and microscopic examination following the specifications of the National Toxicology Program (NTP, 1992), with organs weighed as specified in the EPA OPPTS. Organs will be weighed to the nearest 0.01 gm. We will conduct a complete necropsy, examine and collect the complete list of protocol-specified tissues (see 7.1.4.2) from all F_0 females. All tissues will be processed into slides and examined microscopically.

    A specimen of brain tissue to include basal ganglia, striatum and frontal cortex will be obtained from one hemisphere from 10 animals of each group selected at random (50 specimens) to assay for cholinesterase activity. Specimens of bone marrow, liver and peripheral blood for genetic toxicology assay will be obtained from the same 10 animals.

19. Page 17 - Section 7.1.4.2 - Changed Nose (3) to Nose (3) Sections

20. Page 17 - Section 7.1.5 - Kits Not Selected for Breeding. The first paragraph is amended to reflect that all kits will be weighed individually at weaning, and to add that a blood specimen for clinical pathology will be obtained from ten of each sex per treatment group, at necropsy (approximately 75 days of age). Reason: Reviewer's recommendation.

This paragraph now reads:

7.1.5 Kits Not Selected for Breeding

All kits will be weighed individually at weaning (approximately 50 days of age). After selection of the F_1 animals to be parents of the second generation, two randomly-selected kits of each sex per litter will be necropsied (approximately 75 days of age). A blood sample for analysis as described above will first be obtained from ten per sex per treatment group. At necropsy, special attention will be paid to those anatomic sites most often the site of developmental malformations, i.e., limbs, digits, eyes, palate, brain, heart, kidney and major blood vessels. Specimens of bone marrow, liver and peripheral blood for genetic toxicology assay will be obtained from 10 animals per treatment group, at random.

21. Page 18 Section 7.2: This section was amended to reflect the increased number of animals scheduled for each group as specified above.

- Section 7.2.1 This paragraph was amended to specify which male sex organs will be weighed (testes, epididymis, prostate). Reason: Editorial improvement.

This section now reads as follows:

7.2 SECOND GENERATION (F_1)

The F_1 females will be selected at random, one from each litter to replace each dam (thirty-five per treatment group). Twelve males will be selected randomly from each treatment group to serve as sires within their respective groups. Sibling matings will be avoided and identity of the mating pairs will be recorded. If 35 F_1 litters are not available (i.e., some barren F_0 females) a second female F_1 kit from sufficient litters within the same dose group will be retained as breeders, to assure 35 parental females for the F_2 generation.

This phase of the study will repeat the F_0 procedures except these F_1 animals will have been exposed to the test article from conception. Dose levels in their feed will continue as it was for the first generation, unless adverse results in the F_0 phase dictate reconsideration.

The animals will be identified uniquely with electronic transponders implanted at the time of their allocation to the F_1 parent generation (July, 1995) shortly

after weaning. The identity of their litter of origin will be preserved. Blood samples will be obtained from a cohort of these animals at this time and from the F_1 animals at 3 - month intervals (approximately Sept - Oct, 1995 and Dec - Jan, 1996). They will be housed individually as were their parents; body weight and food consumption will be measured weekly.

They will be mated beginning March, 1996, using the same procedures followed in the 1995 matings.

7.2.1 Male Reproductive Evaluation (F_1 Parents)

After completion of mating (March, 1996) a blood sample will be obtained from the F_1 breeder males, they will be killed with CO_2 and necropsied. The blood samples and video-recorded semen samples will be evaluated as in the previous generation. Testes, epididymis and prostate gland will be weighed and examined microscopically for all animals. All gross lesions will be examined microscopically. Bone marrow, liver and peripheral blood samples will be obtained for genotoxicity assay.

22. Page 19 Section 7.2.2.: This section was amended to reflect that analysis for brain cholinesterase activity was added, and that all tissues of all parental females will be examined microscopically.

- Section 7.3 This section was amended to repeat that blood sampling will be conducted on ten F_2 kits per sex per treatment, consistent with the evaluations performed on the F_1 kits at necropsy.

Sections 7.2.2 and 7.3 now read:

7.2.2 Clinical and Tissue Evaluation, F_1 Parental Females

After mating in March, 1996, food consumption by the F_1 parental females will be measured weekly, but these pregnant animals will not be weighed until the day of F_2 whelping. At whelping of the F_2 generation, the same parameters measuring kit numbers, sex, age and vitality will be recorded as were recorded for the F_1 kits. All litters and dams will be weighed on the day of whelping and four weeks later during lactation.

After weaning (early July, 1996), a blood sample will be obtained from each F_1 parental female for hematologic, chemical (including brain cholinesterase) and genetic toxicologic examination as was done for the F_0 females, and the

animals will be examined by a complete gross necropsy. The same organs will be weighed (per EPA OPPTS). All tissues will be preserved in 10% neutral-buffered formalin.

Tissues and gross lesions from all F_1 parental females will be examined microscopically.

- Ovaries from ten high dose and ten control animals will be processed to evaluate ovarian toxicity as specified in the EPA OPPTS document.

7.3 F_2 KITS

All F_2 kits will be weighed at weaning and euthanized by CO_2. At approximately 75 days of age (to match the age of examination of the F_1 kits) two kits of each sex per litter will be selected randomly and examined by complete necropsy, with special attention to sites affected by common developmental defects, as was the F_1 generation. A blood sample for clinical pathology as performed on F_0 parental females will be obtained from 10 kits per sex per litters. All protocol-specified tissues will be preserved, but no histopathology will be performed unless warranted by gross findings and requested by the sponsor. Specimens of bone marrow, liver and peripheral blood for genetic toxicology assay will be obtained from 12 animals of each sex per treatment group, at random.

23. Page 20, Section 8.0: A final phrase is added to the last sentence of this paragraph: ",..... if different from those specified in Section 9.0. " The reference (e.g. see Allen, 1994) has been changed to read (e.g. Allen, 1994) Reason: Editorial improvement.

- Section 9.0 This section is expanded to explain in additional detail the data to be collected and the statistical analyses to be employed. Reason: Reviewer's Recommendation

Sections 8.0 and 9.0 now reads:

8.0 RESULT EVALUATION CRITERIA

This study will be evaluated using the most appropriate analytical methods available at the conclusion of the study. The protocol will be amended to detail the statistical analysis if different from those specified in Section 9.0. (e.g. Allen, 1994).

9.0 DATA COLLECTION

Data collected (body weights, clinical observations, food consumption, mortality data and day (date) of sacrifice) will be entered on an automated data collection system (LabCat®, IPA Software Inc., Princeton, NJ.) preparatory to review, tabulation, analysis and reporting. The system will be validated before use and annually during the study.

The same system will be used to record, tabulate and report results of necropsy and histopathologic examinations.

The results of hematologic and clinical chemical analyses will be collated, tabulated and reported by standard methods of the U of MN Veterinary Hospital Laboratory.

Sperm Analysis data will be collected, tabulated and reported with procedures specified in PAI SOPs for data collected with the Hamilton-Thorn sperm analysis system. Genetic toxicology endpoints will be collected, tabulated and reported manually.

Statistically, our primary interest is in any difference that may be found between mean values for the control group and those of each treated group, i.e. pairwise comparisons of two groups. We may also treat the values from each control group as separate replicates, and also evaluate comparisons among all treated groups. Our approach to comparison of means among three or more groups will be a test for homogeneity of variance among the groups (e.g., Bartlett's) followed if negative by analysis of variance (ANOVA) appropriate for the data being analyzed. If differences are noted between means, the different mean(s) will be identified with Duncan's multiple range test if group sizes are not equal, or if more than two means are being compared. When groups are approximately equal, and comparisons are pairwise (e.g., mean body weights, mean blood analyte values) Dunnett's t-test will be used.

With pairwise comparison of group means, when Bartlett's test reveals significant heterogeneity of variance, normally-distributed data will be analyzed via F-test. If the variances are approximately equal and $N_1 = N_2$, the means will be compared by Student's T-test; if either or both are unequal, Cochran's t-test will be used.

If Bartlett's test reveals heterogeneity of variances in non-normally distributed data, non-parametric methods will be used. We anticipate at least 20 litters per treatment group, and would therefore use standard reproduction/teratology methods, e.g., Wilcoxon-Mann-Whitney U test. For N less than 10, non-parametric procedures such as Kruskal-Wallis ANOVA may be used. In particular, the small number of males being compared for sperm characteristics will probably require non-parametric analyses.

Evaluation of data relating to kits will be multivariate because several litters in each treatment group will have a common sire, requiring a nested or cross-over clustered design. An additional variable will be room number since animals of all groups are randomized among several rooms.

We anticipate continuing development of proprietary computerized statistical programs that address toxicologic data such as these, and will monitor such programs. Any deemed advantageous may be tried, with documentation of all procedures used.

24. Page 20, Section 10.0 - The first sentence in the third paragraph of Section 10.0 is amended to read ".... to the COR by 30 November 1996," replacing..... to the COR for review by 30 Oct 1996. The title of the report is deleted. The second sentence is deleted. Reason: Necropsy of 175 female mink in late June, and approximately 800 kits in mid-July, to include the requisite blood analyses and microscopic tissue examination will require more time than that available before 30 Oct 96. The title of the study in the third paragraph has been deleted.

Section 10 now reads:

10.0 RECORDS TO MAINTAIN/REPORTS TO GENERATE

Data will be collected on an automated system.

An interim report of the pregnancy outcomes, and the survival and growth of the F_1's to weaning, will be submitted to the Contracting Officer's Representative (COR) by 31 August 1995 (10 copies).

Ten copies of the draft final report will be submitted to the COR by 30 November 1996. The draft final report will be reviewed by the Environmental Protection Agency, State of Colorado, and the Agency for Toxic Substances and Disease Registry. Following receipt of all comments, the contractor shall submit forty (40) copies of the final report to the COR by 31 December 1996.

The final report shall be prepared according to the format from "Style Manual for USAMBRDL Technical Reports and Contractor-prepared Reports" USAMBRDL, Fort Detrick, Frederick, MD 21701, (Anonymous, 1980) and the Contractor Reporting Requirements for the Health Effects Research Division (Barkley 1984), or an alternate format approved by the COR.

25. Page 21, Section 11.0: Sections 792.190 change to 792.120 and Sections 792.195 change to 792.185.

26. Page 22, Section 15.0: The dates for weaning of the F_1 kits and necropsy of their F_0 female parents are amended to 26-30 June 95, from 4-13 July 95. Reason: Original protocol did not allow sufficient time between necropsy of F_0 females (4-13 July) and necropsy of kits (18-22 July), to prepare logistically for necropsy of kits.

Section 15 now reads:

15.0 TIME SEQUENCE (anticipated)

Draft Protocol	23 January 95
Final Protocol	10 April 95
Animal Allocation to Dose Groups	13-17 February 95
Start DIMP Feeding	20 February 95
Mate F_0 (Parents)	6-24 March 95
Necropsy F_0 Parental Males	26-31 March 95
Whelp F_1 (Approx.)	8-19 May 95
Wean F_1 (Approx.)	26-30 June 95
Necropsy F0 Parental Females	26-30 June 95
Necropsy F_1 Kits Not Selected for Breeding	18-22 July 95
Mate F_1 (Parents)	7-25 March 96
Necropsy F_1 Parental Males	28-31 March 96
Whelp F_2 (Approx.)	9-20 May 96
Wean F_2 (Approx.)	5-14 July 96
Necropsy F_1 Parental Females	5-14 July 96
Necropsy F_2 Kits	19-23 July 96
Draft Final Report to OAS	30 November 96
Final Report to OAS	31 December 96

APPROVED BY:

1 April 95 — 1 Apr 95

Sponsor Date — Thomas J. Bucci, VMD, PhD Date

STUDY NUMBER:	TP-001
AMENDMENT:	2
STUDY TITLE:	TWO-GENERATION REPRODUCTIVE STUDY IN MINK FED DIISOPROPYL METHYLPHOSPHONATE (DIMP)
DATE ISSUED:	May 1, 1995
DATE EFFECTIVE:	April 6, 1995

AMENDMENT 2

This amendment is necessary because of a decision announced by the Sponsor on 6 April 95 that all aspects of genetic toxicology should be removed from the protocol.

As a result, the name and title of Lawrence B. Schook, PhD, are removed from the title page from Section 1.3, page 1, and from Section 13, page 21.

All references to genetic toxicology are deleted from the following pages: 2, 15, 16, 18 and 19, and the two bibliographic citations of Tice, R (1993, 1995) are deleted from page 25.

Deletion of Section 7.1.2 Genetic Toxicology data from page 15 has resulted in the following renumbering of succeeding sections:

Original	Amended
7.1.2 Genetic Toxicology Data	Deleted
7.1.3 Male Reproductive Evaluation (F_0)	7.1.2 Male Reproductive Evaluation (F_0)
7.1.4 Pathology, F_0 Females	7.1.3 Pathology, F_0 Females
7.1.4.1 Histopathology	7.1.3.1 Histopathology
7.1.4.2 Tissues for Gross and Histologic Exam	7.1.3.2 Tissues for Gross and Histologic Exam
7.1.5 Kits Not Selected for Breeding	7.1.4 Kits Not Selected for Breeding

Add Douglas J. Weiss, DVM. PhD as Co-Investigator, to Title page and Section 1.3, page 1, replacing Dr. Schook.

APPROVED BY:

6 APRIL 95 — **Sponsor** **Date**

6 Apr 95 — **Thomas J. Bucci, VMD, PhD** **Date**

STUDY NUMBER:	TP-001
AMENDMENT:	3
STUDY TITLE:	TWO-GENERATION REPRODUCTIVE STUDY IN MINK FED DIISOPROPYL METHYLPHOSPHONATE (DIMP)
DATE ISSUED:	June 1, 1995
DATE EFFECTIVE:	June 1, 1995

AMENDMENT 3

1. Page 22, Section 15.0: The dates for the necropsy of the F_1 kits are amended to 10-22 July 95, from 18-22 July 95.

Reason: Original protocol did not allow sufficient time to do necropsies.

APPROVED BY:

6/20/95

Sponsor **Date**

1 June 95

Thomas J. Bucci, VMD, PhD **Date**

STUDY NUMBER:	TP-001
AMENDMENT:	4
STUDY TITLE:	TWO-GENERATION REPRODUCTIVE STUDY IN MINK FED DIISOPROPYL METHYLPHOSPHONATE (DIMP)
DATE ISSUED:	November 8,1995
DATE EFFECTIVE:	November 8,1995

AMENDMENT 4

1. Section 7.1.3.2 Tissues for Gross and Histopathologic Evaluation

Remove Cecum from list of tissues for histopathologic evaluation.

Reason: Mink do not have a cecum.

2. Section 15.0 Time Sequence

Change dates for F_1 and F_2 necropsies from July 5-23, 1996 to June 8-20, 1996.

Reason: To achieve significant economy without compromise of data, we have elected to euthanize the F_2 kits when they are six-seven weeks of age, rather than to maintain them beyond. We can perform post mortem and blood examinations on these kits at any age to fulfill the protocol requirements; holding them for six weeks will permit us to determine whether or not exposure to DIMP has any effect on the ability of the dams to sustain them. Holding them beyond six weeks entails considerable expense since they begin to consume large amounts of food over the next few weeks.

3. Section 6.2.9 Feed

Change 2nd paragraph to read:

feed will be analyzed by Lancaster Laboratories, Lancaster, PA to Corning Hazleton Laboratories of Madison, WI.

Reason: Lancaster Laboratories was unable to meet deadline.

APPROVED BY:

Sponsor	20 Nov 1995 Date	Thomas J. Bucci, VMD, PhD	8 Nov 95 Date

STUDY NUMBER:	TP-001
AMENDMENT:	5
STUDY TITLE:	TWO-GENERATION REPRODUCTIVE STUDY IN MINK FED DIISOPROPYL METHYLPHOSPHONATE (DIMP)
DATE ISSUED:	November 29,1995
DATE EFFECTIVE:	November 29, 1995

AMENDMENT 5

This amendment is necessary to document the changes required to the protocol required by the Critical Phase Inspection conducted April 19, 1995 including the "Working Copy" of the study protocol.

Page 8, last paragraph reads "with post-coital aspiration for sperm, immediately after each mating." to read "with post coital vaginal aspiration for sperm once for each male." (This was previously addressed in Amendment 1.)

Reason: Amended to make all occurrences of the statement to read the same in the text of the protocol.

The following were changed from the original protocol as the Revised Protocol (Working Copy) dated April 1995, however Amendment 1 did not include the changes. The changes are listed below:

1. Page 7, last paragraph, 4th sentence: The original reads "EPA's current OPPTS test guidelines"; in the amended version, the word "draft" has been inserted to read "current draft OPPTS test guidelines".

Reason: The OPPTS test guidelines as currently issued are "draft" and as such this document should consistently refer to these guidelines as "draft'.

2. Page 8, last paragraph, 5th sentence;: The original read "spaced 8 days apart"; the amended version reads "spaced 8-10 days apart".

Reason: The two periods of copulation are spaced from 8 to as many as 10 days apart for logistical reasons, this change is made to correctly describe the procedure used.

3. Page 10, last paragraph, first sentence: The original read "by an independent laboratory"; the amended version reads "by Midwest Research Institute (MRI) Kansas City, Mo".

Reason: The laboratory used for the determination of purity of the DIMP compound has been identified and referenced in the protocol.

4. Page 11, second sentence: Original reads "by an independent laboratory"; amended version reads "by MRI".

Reason: The laboratory used for the determination of purity of the DIMP compound has been identified and referenced in the protocol.

5. Page 12, second paragraph, 4th sentence: Original read, "parturition occurs predominantly in late April"; the word predominantly" has been deleted from the amended version. Fourth sentence: Original read, "litter size is 5 kits"; amended version says "4-5 kits".

Reason: Parturition can occur in April and sometimes into May. Therefore the word "predominantly" is inappropriately used. The litter size varies from 4-5 and is not always specifically 5. Average litter size of 4-5 more accurately describes the litter.

6. Page 15, third paragraph, second sentence: The original read: "It will be analyzed for macro and micro nutrients, and for pesticides, fungicides, mycotoxins and other contaminants; each batch of dosed feed will be examined microbiologically for number of total bacteria and for E. Coli and Salmonella species. Amended version reads: "It will be analyzed for macro and micro nutrients, and for pesticides, fungicides, mycotoxins and other contaminants by Lancaster Laboratories, Lancaster, NJ; each batch of dosed feed will be examined microbiologically by the U of MN."

Reason: Lancaster Laboratories of Lancaster, PA has been identified as the lab that will analyze the feed for contaminants. This amendment documents the lab selected.

7. Page 23, in the list of tissues for gross and histopathological evaluation, the original listed "Lungs (2) and major bronchi median lobe." This was corrected in the amended version to eliminate the words "median lobe." The original listed "Spinal cord and sciatic nerve"; these tissues are listed separately in the amended version.

Reason: Median lobe was incorrectly listed with Lungs (2) and major bronchi. Median lobe is eliminated from the tissue list. Spinal cord and sciatic nerve are listed separately to facilitate the accurate collection of these organs.

APPROVED BY:

20 DEC 95 — 15 Dec 95

Sponsor **Date** — **Thomas J. Bucci, VMD, Ph.D.** **Date**

STUDY NUMBER:	TP-001
AMENDMENT:	6
STUDY TITLE:	TWO-GENERATION REPRODUCTIVE STUDY IN MINK FED DIISOPROPYL METHYLPHOSPHONATE (DIMP)
DATE ISSUED:	November 29,1995
DATE EFFECTIVE:	November 29, 1995

AMENDMENT 6

The following changes are required in Amendment 1 of the Protocol:

1. Page 1, number 1: "Title page and all other pages: Change UMN to U of MN." "UMN" does not appear on the title page.

Reason: Corrected to read "Change all occurrences of UMN to U of MN.

2. Page 6, 2nd paragraph, 2nd sentence: The amendment read, "This test will not be repeated, but..". The amended protocol reads, "This test will not be repeated, and..."

Reason: Correct grammatical error.

3. Page 9, 5th paragraph, 3rd sentence: The ending of this sentence should read,"vaginal aspiration for sperm, once for each male".

Reason: Change required to make wording for this procedure consistent throughout protocol and its amendments.

4. Page 10, last paragraph, 1st sentence should read : "The blood sample obtained during quarantine and again at necropsy..." The words "and again at necropsy" have been deleted in the Amendment.

Reason: Blood samples will not be collected at necropsy in addition to the collection in quarantine. The wording change corrects the procedure change.

5. Page 11, paragraph 4, sentence 5: The word "and" has been inserted between "Testes" and "epididymis"; the amended protocol reads, "Testes, epididymis and prostate gland.."

Reason: The use of "and" is properly inserted to indicate a third organ to be collected in this series, making this sentence listing grammatically correct.

APPROVED BY:

20 DEC 95 — 13 Dec 95

Sponsor **Date** **Thomas J. Bucci, VMD, Ph.D.** **Date**

STUDY NUMBER:	TP-001
AMENDMENT:	7
STUDY TITLE:	TWO-GENERATION REPRODUCTIVE STUDY IN MINK FED DIISOPROPYL METHYLPHOSPHONATE (DIMP)
DATE ISSUED:	8/8/97
DATE EFFECTIVE:	8/8/97

AMENDMENT 7

1. **Change required:** This amendment amends the changes incorporated in Amendment 1 dated 5/1/95. Page 11 last paragraph numbered "7.1.1 Clinical Pathology", fourth sentence should read".... and will also include determinations of plasma, whole blood and RBC cholinesterase.

   Reason: RBC cholinesterase determinations were left out of the list of chemical analytes to be performed.

2. **Change required:** This amendment amends the changes incorporated in Amendment 1 dated 5/1/95. Page 12 last paragraph numbered "7.1.3 Male Reproductive Evaluations (F_0)", seventh sentence should read"Sperm from the epididymis will be evaluated for count, morphology and percent motility.

   Reason: Percent motility was not included in evaluations to be conducted on semen obtained from the cauda epididymis.

3. **Change required:** This amendment amends the changes incorporated in Amendment 1 dated 5/1/95. Page 19, first paragraph, last sentence, should read, "Following receipt of all comments, the contractor shall submit forty (40) copies of the final report to the COR by August 31.

Reason: Approval to extend the report deliverable date to August 31, 1997 obtained from the COR due to delayed response of reviewers comments for incorporation into the report.

APPROVED BY:

Sponsor 8/18/97 Date

Thomas J. Bucci, VMD, PhD 8 Aug 97 Date

APPENDIX 13 GLP and Protocol Deviations

GLP and Protocol Deviations

1. NUMBER OF KITS BLED FOR HEMATOLOGY AND CHEMISTRY .The Study Protocol specifies that 10 kits of each sex per treatment group will be bled for hematology and clinical chemistry. The data indicate that at the necropsy of the respective generations, the following number of kits was sampled:

Number of Kits Sampled for Hematology and Clinical Chemistry

	Control A	Control B	150 ppm	450 ppm	2500 ppm
July 95					
F_1 Female Kits	8	11	10	10	11
F_1 Male Kits	7	13	11	10	10
June 96					
F_2 Female Kits	10	11	8	10	11
F_2 Male Kits	9	12	8	12	11

Jugular venipuncture of these small kits was difficult, given the anatomy of the ventral cervical region of the mink, and the attempt was unsuccessful in some. Sufficient samples were obtained to represent the cohort. There were 19-30 litters in each group, from which two kits per sex per litter were selected for necropsy. From this subset, ten per sex per treatment group were selected for venipuncture preceding euthanasia.

IMPACT ON THE STUDY: There was no evident adverse impact on the study. The number of animals sampled per group was small but adequate statistically and the outcome data (e.g., Heinz body and reticulocyte counts) were internally consistent.

2. ERRONEOUS CLAUSE IN AMENDMENT 6. Protocol Amendment 6 deleted the requirement for blood collection at necropsy of F_0 animals yet blood was collected at necropsy. The deletion clause in Amendment 6 was a mistake that resulted from miscommunication by the Study Director; it was not intended and was not effected. The protocol, including Amendment 1, is correct as issued on this point.

IMPACT ON THE STUDY: None.

3. NUMBER OF SPECIMENS FOR BRAIN ACETYLCHOLINESTERASE. The number of brain specimens obtained at necropsy to analyze for brain acetylcholinesterase content did not match the protocol-specified 10 females per group, and the method of randomized selection of animals for this analysis was not documented.

The number of animals from which brain specimens were analyzed is tabulated below:

Number of Mink Sampled for Brain Acetylcholinesterase

	Control A	Control B	150 ppm	450 ppm	2500 ppm
F_0 Females	12	8	10	10	10
F_1 Females	9	10	8	10	10

The correct total number of specimens (50) was analyzed from the F_0 generation, but the distribution was not 10 per group. It is probable that two of the Control B specimens were mistaken for Control A, and misdesignated. In the F_1 generation, the correct number of animals was selected and analyzed from each group but values from three of them were deleted, among the several excess animals that were eliminated by lot at the end of the study (See Deviation # 18). If DIMP were an active anti-cholinesterase, the value would be expected to decrease, but there was no difference in brain acetylcholinesterase values among any of the groups i.e., no effect of DIMP exposure. In the F_0 generation, both control groups had lower values than did the treated groups; in the F_1 generation, the highest and lowest values were those of the control groups (Table 4.7.2.d).

IMPACT ON THE STUDY: None

4. SIGNATURES AND DATES ON RAW DATA. GLP regulations stipulate that raw data will be signed and dated by the person collecting such data. Many of the raw-data instrument printouts for hematology and clinical chemistry data as well as in-life data records were not signed and dated by the responsible technician. The printouts of clinical chemistry data did contain machine-printed date and initials of the operating technician.

IMPACT ON THE STUDY: Failure to sign and date the documents was clearly a GLP violation but the authors have no doubt that the designated technicians, all of whom were unequivocally qualified, performed these tests, or that the results are valid. The data are internally consistent, quality control samples were used appropriately and no other technicians were performing this work. We believe there was no compromise of data integrity.

5. RESERVE SAMPLES. GLP regulations stipulate that in long-term studies, reserve samples of the test, control and reference substances be retained throughout the duration of the study. In this study, the "neat" DIMP was available throughout, both at U of MN and MRI where samples were sent for characterization. However, samples of dosed feed from each mixing were not retained by U of MN. The first nine batches prepared were sampled for analysis by MRI; MRI retained aliquots of those. Each of the three dose levels was prepared, canned and frozen, in 363 kg batches at approximately 3-week intervals throughout the study, as were two batches of undosed control feed each time.

   IMPACT ON THE STUDY: None. There fortunately was no occasion to need a reserve sample.

6. ANALYSIS OF FEED FOR DIMP CONTENT. The Study Protocol stipulated that each batch of feed would be analyzed for DIMP content. After the first nine batches of feed had been analyzed for DIMP it was apparent (from the acceptably low relative standard deviation among samples of each dose) that the formulation SOP was dependable. It was determined at that time that feed and DIMP requirements had been underestimated in the pre-study calculation. To afford the extra feed and DIMP that would be required, the Study Director elected to forego continued analyses for confirmation of the mixing procedure, relying instead on the practiced methods used in the earlier 90-day study and in the first nine batches of this study. (Note that in the report of the Aulerich 1-year mink reproductive study {Aulerich, et al, l979}, which was pre-GLP, that there is no account of dose verification in the feed.)

   Every batch of feed was not examined microbiologically (per Protocol Amendment 5), but only Lots 3,4,6,9,10 and 12. The Study Protocol also specified analysis of the content of DIMP in the ration formulation immediately after preparation. During the period of approval of the draft protocol, the subcontract for DIMP characterization in feed was negotiated (with Midwest Research Institute, Kansas City, MO) and it was then clear that rapid turn-around

of analyses was not feasible. The protocol should have been amended at that time to describe the new timing, but it was not.

IMPACT ON THE STUDY: The analytic results, when they became available, indicated DIMP content to be 106%-111% of the target doses, with acceptable Relative Standard Error among samples, i.e., good homogeneity of mix. The delayed submission of sample (and slow return of result) thus caused no adverse impact on the study result. Had the mixtures been inappropriate, misdosing could have occurred.

7. MICROBIOLOGICAL ANALYSIS OF FEED. The proposal to conduct microbiological analysis of every batch of prepared feed was based on an understanding that the U of MN would be able to perform the analyses. When the time came, only Lots 3,4,6,9,10 and 12 were examined. The Study Director accepted these analyses for the record, rather than to purchase multiple additional analyses from a commercial laboratory where turn-around time would be too long to be useful. Mink feed contains raw slaughter-house products unfit for human consumption, including numerous potential pathogens. We knew from past experience and from the extensive concurrent experience at the mink farm that was the feed source that the feed was dependable, and more importantly, that the animals would have to tell us by their health whether or not the bacterial load was too high. The usual early manifestation of excessive bacterial growth in feed is loose stools in the animals. Commercial ranchers then add antibiotics to the feed therapeutically for a few days.

   IMPACT ON THE STUDY: None

8. AGE OF KITS AT NECROPSY. The Study Protocol stipulated that the F_1 kits not retained for breeding would be necropsied at about 50 days of age, at which date concurrent removal of the kits to be retained as breeders would define the weaning date for the F_1 generation. We failed to anticipate that insufficient space or cages would be available to house both the weaned kits and their dams separately. When we realized this, we decided to remove the dams for necropsy at the mid-June (50-day) period instead and leave the litters in their respective cages. Those retained for breeding were to be vaccinated at about 77 days of age, so we allocated them to individual cages at that time and sacrificed the remainder at the same time, and not at 50 days. They were weighed at this time and not at 50 days as originally specified.

In August of 1995 when it became obvious that a second lot of DIMP and more feed would be required than had originally been anticipated, we opted to sacrifice the F_2 kits at the earliest feasible date. This would minimize the amount of extra DIMP, feed and labor required. As a result, they were sacrificed in mid-June of 1996, along with their dams, when the kits were 4-6 weeks old, rather than around 77 days as the F_1 kits were.

IMPACT ON THE STUDY: These changes in the protocol caused the F_2 kits to be bled and necropsied at 4-6 weeks of age, in comparison with the F_1 kits (9-11 weeks) and thus precluded hematologic and biochemical comparisons as well as body weight comparisons between the generations. The F_1 kits were born to animals held under laboratory conditions for only four months; in contrast, the F_2 kits were born to parents that were born and raised in the laboratory, remaining there until after birth of the F_2 kits. The authors believe that these groups of kits were therefore quite different populations. Whereas failure to sacrifice the two generations of kits at the same age precluded direct comparison, this was outweighed by the financial advantage of removing the F_2's early, it permitted study of the response of kits of different ages to DIMP relative to age-matched controls, and also confirmed that younger mink, like most other mammals, have higher reticulocyte counts than adults. Thus the impact was more positive than negative.

9. DIMP CONTENT EXCEEDED 110%. The Study Protocol specified that feed containing DIMP within 10% of the target concentration would be acceptable. This range was specified by the Study Director based on its reasonableness and feasibility. The second batch of DIMP was 99% pure, compared with 97% purity of the original batch, yielding a proportionate increase in the DIMP concentration of the rations, causing the F_1 2500 ppm target dose group of mink to receive 111% of the target concentration from Oct 95 to the end of the treatment period. The Study Director elected not to change the formulation procedure and risk perturbation of a practiced procedure; the 111% was sufficiently close to the desired dose that it did not invalidate the rationale used in dose selection, and erred in the direction of making the test (of DIMP exposure) even more rigorous.

IMPACT ON THE STUDY: Given the small number and mild degree of adverse effects observed in the putative 2500 ppm group as compared with the 450 ppm group in which there were no adverse effects, accepting 111% of the target dose when up to 110% was specified is not expected to have any measurable effect on the study outcome.

10. STANDARDIZATION OF LITTER SIZES. The Study Protocol indicated that litter sizes would not be standardized, and Amendment 4 to the protocol changed the anticipated litter size from five to 4-5 kits. As the F_1 kits began arriving, litters varied from 1 -13 kits. Given the predilection of mink dams to experience "nursing disease" when under lactational stress, the Study Director sought to reduce the stress variability among dams from that source, by standardizing maximum litter size to six kits. All litters larger than six were reduced randomly to that number when the kits were 3-6 days old. The biomass (total litter weight) was determined for the full litter on the day of birth and the average kit weight was determined, to compare to the average kit weight taken at 28 days of age.

    IMPACT ON THE STUDY: Any impact should have been beneficial in that dams varied less in the lactational stress that they experienced, which is fatal in severe cases. No data were compromised since the number and weight of kits whelped were recorded.

11. WEEKLY DETERMINATION OF BODY WEIGHTS AND FOOD CONSUMPTION. The Study Protocol stipulated that the animals would be weighed weekly except during gestation and lactation. On several occassions weekly determination of body weights and food consumption were not recorded.

    IMPACT ON THE STUDY: Individual animal body weights were obtained on multiple occasions throughout the study, and were averaged over the full duration to determine average body weight. Weekly determination of 24-hour food consumption was performed in the same manner. Absence of an occasional weekly weight from that series does not have an adverse impact on the overall average weight or food consumption data.

12. EXTRA SPECIMEN OF FEED SUBMITTED FOR ANALYSIS. A sample of control feed was to have been sent to the Corning-Hazleton Laboratory, Madison, WI to analyze for a comprehensive set of nutrients and contaminants. The U of MN personnel responsible for shipping the sample sent both control and dosed feed. This was an oversight and was recognized by the recipient. After concurrence by the Study Director, only the control feed was analyzed. The Corning-Hazleton report is Appendix 1B.

    IMPACT ON THE STUDY: None.

13. CALIBRATION OF SCALES. Good Laboratory Practices require calibration of scales. Documentation of calibration of scales used to weigh feed and animals was not always present in the raw data.

IMPACT ON THE STUDY: Like individual animal body weights and food consumption, calibrations of scales used to weigh feed and animals were obtained repeatedly on this study. While failure to calibrate or record calibration results violates GLP and the Study Protocol, absence of an occasional weekly calibration from that series does not have an adverse impact on the overall average weight or food consumption data.

14. DETERMINATION OF SERUM OSMOLALITY AND ANION GAP. The biochemical analytes listed in the Study Protocol to be measured or derived from chemical analyses did not include Osmolarity, a calculated measure of the concentration of ions that reflect whether or not the patient is dehydrated, or Anion Gap, another calculated measure that indicates the degree of acidosis or alkalosis present. When the reports of blood analytes were first received from the U of MN laboratory where the analyses were performed, these values were included. Query by the Study Director revealed that the analytic instruments automatically calculated these values from the other requisite analytes already measured and they were included at no extra effort or cost, so they were accepted.

    IMPACT ON THE STUDY: The impact was beneficial. More data were available, and in particular, two of the early death animals were confirmed to have dehydration and acidosis through these values.

15. ANESTHETIC DOSE. The Study Protocol specified that the anesthesia to be used would be a mixture of 30 mg ketamine and 1 mg xylazine/kg BW, yet SOP 300.01 differed in specifying 40 mg ketamine in the mixture. The SOP was followed, but the protocol was not amended.

    IMPACT ON THE STUDY: None known. Recommended doses of ketamine for mink, rabbits and other laboratory and zoo animals range from 15-60 mg/kg BW, used alone or in combination with one or more other compounds. In this study, the anesthetic was used uneventfully for all bleedings except the mid-Dec95 and Feb96 period, when the animals were somewhat overweight. This resulted in larger anesthetic doses and since the adipose tissue does not contribute to metabolism of the drug, a relative overdose also resulted, causing longer (and probably deeper) sleep times. A number of animals went off feed in the days that followed. Five females and one male did not resume eating and developed the fatal stress syndrome after the Dec95 bleed, and one female after the Feb96 bleed. Whether or not this would have occurred with 30 mg ketamine in the mixture cannot be known--the other 213 animals survived this dose regimen.

16. BARBITURATE EUTHANASIA. The Study Protocol specified carbon dioxide as the euthanasia medium for the adult mink and kits, prior to necropsy. Experience with the F_1 kits revealed that the young animals were very resistant to the anoxia produced by the compound, requiring prolonged exposure before death occurred. To preclude a reoccurrence with the even younger F_2 kits, an injectible barbiturate was used for their euthanasia, with the concurrence of the Study Director. A protocol amendment should have been issued but was not.

    IMPACT ON THE STUDY: No adverse impact. The euthanasia was more humane, and less time was required for the necropsy procedure.

17. LABELS ON DIMP CONTAINERS. Good Laboratory Practices require that storage and shipping containers for test substances be labeled with CAS Number, Batch Number and Expiration Date. When the "neat" DIMP was shipped from U of MN to the Midwest Research Institute (MRI) for purity analysis, it was labeled only as "DIMP", a GLP violation. The 100-ml volume for the aliquot was requested by MRI based on their past experience with DIMP during the 90-day study in 1991. The Study Director had provided MRI with available literature concerning analytic methods, and with the list of characteristics listed in the Final Report on page 13, including the CAS Number. MRI had conducted stability tests with DIMP and was aware that it is stable (for "decades", according to the manufacturer). An expiration date had not been assigned.

    IMPACT ON THE STUDY: No adverse impact known.

18. DELETION OF EXCESS ANIMALS. A misunderstanding between the Study Director and Dr. Perman at U of MN resulted in an excess number of F_1 dams and males being maintained throughout the study (approximately 10%). When the F_1 kits were placed into individual cages at about 11 weeks of age, it was not known what their survival rate might be, and there was concern that a minimum of 20 dams per experiment group be available for breeding, gestation, whelping and lactation. Dr. Perman recommended that a slight excess (above the 35 specified in the protocol) be retained for at least a few weeks to determine how the kits would succeed. Dr. Perman also expressed his concern about lack of males competent for breeding, because the F_0 males did not perform as well after just six weeks in the laboratory as did their counterparts still on the mink farm. Dr. Perman's concern was that after 11 months in the laboratory, the F_1 males may be even more indolent, and he preferred the larger number if there were space for them. The Study Director concurred. Dr. Perman had space for 39 females and 13 males per treatment group, so this number was retained. This decision was made in July, 1995, during necropsy of the F_1 kits not retained as breeders. The Study Director's understanding was that the extra animals would

be carried no longer than the breeding cycle (March, 1996), at which time the excess females could be randomly eliminated.

At mating, the males did perform poorly, with only 3-4 good sires per treatment group, so there was cause for concern about the number of pregnant females. The F_1 breeding was less efficient than the F_0, with 83% of F_0 females delivering and only 66% in the F_1 group. The number of kits per litter and their weight were also slightly smaller (Tables 4.6.a & 4.6.b).

The subject of the extra animals was inexplicably not discussed again, and repeated spot checks of records by the Study Director failed to remind of their presence. When the complete set of records was analyzed and the extra females were still among them, the Study Director elected to delete them from the study rather than to incur the expense of histologic processing and microscopic examination. (The animals had been necropsied and the tissues are archived along with their full records).

The table below details the animals by group and individual number. The animals eliminated were drawn by lot from the total number in each treatment group, by the Study Director.

F_1 Dam Inventory
(Before reduction to 35/group)

DIMP (PPM)	Tmt Group	No. ♀ (Total)*	Excess No. In Group	Early Deaths	No. ♂ (Total)*	Early Deaths	Excess No. in Group*
0 (Control A)	1	39	4	1	13	0	1
0 (Control B)	2	39	4	1	13	0	1
150	3	39	4	2	13	0	1
450	4	38	3	3	13	1	1
2500	5	39	4	3	13	0	1
Totals		194	19	10	65	1	5

* Includes Early Deaths

Surplus Dams (F_1) and their Litters (F_2) that were Eliminated

DIMP (PPM)	Tmt Group	Animal Number eliminated
0	6	403, 418, 420, 428
0	7	440, 442, 444, 454
150	8	482, 487, 493, 506
450	9	519, 520, 538
2500	10	555, 570, 583, 593

In eliminating the extra animals randomly, identification numbers for all animals in the respective treatment groups were placed in the drawing, including those that had died early (their records and tissues were available). The animal numbers that were drawn are listed in the Surplus Dam table. All data from these animals and their kits were deleted from the study but are retained in the study archive. None of the males was deleted.

IMPACT ON THE STUDY: The only apparent impact on the study was the added cost and logistics of maintaining these animals. In retrospect, it was not an imprudent decision given the uncertainty of the outcome. But cost was a continual factor and the extra animals should have been deleted as early as possible. The overage was eliminated completely randomly, theoretically not biasing the outcome. With the data all in hand and compiled at this writing, it is difficult to see how inclusion of one or more of those animals to the exclusion of some now included, or not excluding any, could make a meaningful difference in outcome.

19. CLINICAL TREATMENT OF ANIMALS. Six females of the F_0 generation died of various causes but only after they had become anorexic, refused food for several days and developed electrolyte imbalance. This is a well-known syndrome in a number of species when they are under unusual metabolic stress (see Final Report, under "Early Deaths"). The Study Director and Dr. Victor Perman decided to attempt to salvage future mink that displayed this syndrome by supplementing their nutrition, offering them food that may be attractive, e.g., palatable cat foods, and by administration of electrolytes or electrolytes and glucose (lactated Ringer's solution) parenterally in more severe cases. A protocol amendment was not issued although an SOP (300.26) was. In practice, the SOP was too specific to allow for the latitude needed in clinical judgement,

and was not followed closely. The treatments administered involved only a few animals and a few days each, and are recorded in the study file.

IMPACT ON THE STUDY: A few animals were supported in this way and were not lost to the study when otherwise they might have died. The intervention did not always succeed in saving them, either; the six F_1 females and one F_1 male that died after becoming anorexic following anesthetization for blood collection were candidates for salvage but were lost. The animals so treated were from all experiment groups including controls not exposed to DIMP, whereas the vast majority of the animals in the highest dose group (2500 ppm) had no symptoms at all, thus we were not treating DIMP toxicity. The supportive therapy may have prevented a few early deaths from conditions that were unrelated to exposure to the test article.

20. ERRONEOUS IDENTITY OF SUBCONTRACTOR IN PROTOCOL AMENDMENT 5. When the original protocol was submitted to the sponsor for approval, a subcontractor to analyze the feed nutrients and contaminants had not been selected. The selected contractor, Lancaster laboratories, Lancaster, PA, was identified in Amendment 1 to the protocol. Lancaster then informed the Study Director that they would be unable to provide the analyses in the time stipulated, and they withdrew from the agreement. Corning-Hazleton Laboratories, Madison, WI was selected as the alternate and this was specified in Amendment 4. Amendment 5 erroneously includes a statement that Lancaster Laboratories was the contractor of choice for the analysis.

    IMPACT ON THE STUDY: None.

21. MISDOSINGS. Several instances (documented in the study/file data) of misdosing occurred during the study. They were random occurrences affecting different animals on a single occasion (fed the wrong can of food).

    IMPACT ON THE STUDY: None.

22. UNREQUIRED BLEED. The F_1 animals were bled 2/96. This bleed was not required by protocol. The data/values collected from this bleed will not reported.

    IMPACT ON THE STUDY: None.

23. ONCE DAILY CLINICAL OBSERVATIONS. According to the protocol, cageside clinical observations were to be made twice daily. This was unnecessary and was reduced to once daily by the Study Director.

IMPACT ON THE STUDY: None.

24. ROOM ILLUMINATION. The Study Protocol stipulated light intensity of 15-25 lumens. At 10.7639 lux/lumen, this requirement was 161.5-269 lux. The reported measurement was 325 lux. The rooms were therefore somewhat brighter during the day than prescribed.

    IMPACT ON THE STUDY. None known.

25. MISSING WEIGHT. Animal #93 (F_0 male) had no recorded testes weight and Animal # 84 (F_0 male) and #130 (F_0 female) did not have a body weight recorded at necropsy as required by the protocol.

    IMPACT ON THE STUDY: None.

26. MISSING CLINICAL OBSERVATIONS. On several occassions, protocol-required observations were not recorded. The personnel were in the animal rooms each day, and the animals were observed, however.

    IMPACT ON THE STUDY: None known.

27. MATING FEMALES TO MALES OF ANOTHER DOSE GROUP. In the F_1 generation some males were disinterested in mating and to assure an adequate number of pregnant females active breeders of a different dose group had to be used.

    IMPACT ON THE STUDY: None known. Equivalent outcome of reproduction among dose groups suggest that there was no effect. It should be noted that in the Aulerich study (1979) it was stated that females were mated within dose groups whenever possible.

28. CHEMICAL COMPOSITION OF DIMP. The protocol incorrectly listed the chemical structure and the molecular formula of DIMP. The molecular formula was listed as C7H7O3P instead of C7H17O3P. The structure was listed as ((CH3)2CHO)2(CH3)P=0 instead of [(CH3)2(CHO)]2(CH3)P=0. Both are listed correctly in the report.

IMPACT ON THE STUDY: None Known.

Thomas J. Bucci, VMD, PhD
Study Director

28 Aug 97
Date

APPENDIX 14 QA Statement

Two-Generation Reproductive Study in Mink Fed Diisopropyl Methylphosponate (DIMP)

Protocol Number TP-001

QUALITY ASSURANCE STATEMENT

This report has been inspected and audited by the PAI Quality Assurance Unit (QAU) as required by the Good Laboratory Practice (GLP) Standards promulgated by the U.S. Environment Protection Agency. The following table is a record of the inspections/audits performed and reported by the QAU.

Date of Inspection	Phase Inspected	Date Findings Reported to Management/ Study Director
	Protocol Review	
02/15/95	Protocol	02/22/95
	Phase Inspections	
02/16/95	Anesthetic Administration	02/22/95
02/16,17/95	Animal Identification	02/22/95
02/16,17/95	Blood Sample Collection and Analysis	02/22/95
02/17/95	Feed Mixing	02/22/95
03/27,28/95	Necropsy and Organ Weighing	05/03/95
03/27,28/95	Blood Sample Collection	05/03/95
03/27,28/95	Animal Feeding/Dosing	05/03/95
03/27,28/95	Animal Care	05/03/95
06/26-27/95	Necropsy and Organ Weighing	06/29/95
06/26,27/95	Blood Sample Collection	06/29/95
06/26/95	Anesthetic Administration	06/29/95
06/26,27/95	Animal Feeding/Dosing	06/29/95
06/26,27/95	Animal Husbandry	06/29/95
06/27/95	Body Weights	06/29/95
06/27/95	Feed Mixing	06/29/95
06/27/95	Feed Consumption Records	06/29/95
06/27/95	Clinical Observation Records	06/29/95
07/10,11/95	Necropsy and Organ Weighing, Teratology Examination	07/21/95
07/10,11/95	Animal Husbandry	07/21/95
09/21/95	Staining and Coverslipping	09/21/95
11/30/95	Feed Consumption	12/14/95
11/30;12/01/95	Animal Husbandry	12/14/95
11/30;12/01/95	Feed Mixing	12/14/95
12/01/95	Animal Feeding/Dosing	12/14/95
12/01/95	Training Records	12/14/95
12/01/95	Animal Observation, Body Weight, Food Consumption Records	12/14/96

03/05/96	Animal Feeding/Dosing	03/22/96
03/05,06/96	Animal Breeding Procedures	03/22/96
07/25/96	Tissue Trimming	08/05/96
09/06/96	Tissue Embedding	09/06/96
09/09/96	Tissue Microtomy	09/09/96
09/18/96	Sperm Count Sample Preparation	09/19/96
09/23/96	Sperm Motility	09/30/96
	<u>Report/Data Audits</u>	
06/10-14;07/28,29/96	In-life Data Inspection	08/05/96
09/30/96	Necropsy Forms, Data Entry, Interim Pathology Report	09/30/96
11/14,15,20/96	Sperm Analysis Report	11/20/96
11/18-30;12/10,11/96	Clinical Path. - Hematology	12/11/96
12/13/96	Brain Cholinesterase	12/15/96
12/16/96	Body/Organ Weights	12/16/96
12/16/96	Necropsy Forms	12/16/96
12/01-10,12/96	Clinical Path. - Clinical Chemistry	12/16/96
12/19-24,27,30/96	Body Weight, Scale Calibration	01/02/97
12/16-19,23,26/96	Food Consumption	02/02/97
01/06/97	Pathology Report	01/06/97
12/27-31/96; 01/01-05/97	Pharmacy, Feed, Anesthesia, Records	01/07/97
01/12,13/97	Miscellaneous In-life Data Review	01/13/97
01/06-10,14/97	Kit Data	01/16/97
01/09;02/06,26,27; 03/03-05/97	Temperature/Humidity Records	03/06/97
04/15/97	Pathology Report	04/15/97
03/12,13,19-25;04/01-04, 09-11,14/97	Clinical Observations	05/13/97
04/16-18,21-30; 05/01-05/97	Draft Report	05/05/97
06/18-21,24;08/26/97	Breeding Data	08/26/97
08/14,15,18-26/97	Final Report	08/26/97

Carol Hoffman

Carol Hoffman
Quality Assurance Director

8/26/97
Date

David T. Wilson

David T. Wilson
Quality Assurance Specialist

8/28/97
Date