DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Agency for Toxic Substances
and Disease Registry
Atlanta GA 30333

September 19, 1995

Hasmukh C. Shah, Ph.D.
Manager, Vinyl Chloride Panel
Chemical Manufacturers Association
2501 M Street, NW
Washington, DC 20037

Dear Dr. Shah:

This letter is to inform you that the Agency for Toxic Substances and Disease Registry's (ATSDR) peer review of the study protocol, "Vinyl chloride: Combined inhalation two-generation reproduction and developmental toxicity study in CD rats", has been completed. The protocol was submitted by the Chemical Manufacturers Association (CMA) as a candidate to address ATSDR's data needs for vinyl chloride via voluntary research. Enclosed are the comments of the three peer reviewers.

As stated in the 1992 Federal Register notices on procedures for voluntary research, the ATSDR approved study plan and the peer reviewers' comments, among others, will be available for public inspection at the Agency (57 FR 4758 and 57 FR 54160). Therefore, the Agency requests that CMA respond to the peer reviewers' comments and forward the response to ATSDR within three weeks of the date of this letter. Upon approval of your written response, ATSDR and CMA may then choose to move forward with the signing of a Memorandum of Understanding.

The Agency looks forward to continuing dialogue and collaborative research with CMA on vinyl chloride. Please contact me at 404-639-6306 if you have any questions.

Sincerely yours,

William Cibulas, Jr.

William Cibulas, Ph.D.
Chief, Research Implementation Branch
Division of Toxicology

Enclosures

cc:
Dr. Christopher DeRosa
Mr. Caffey Norman

CMA 115505

"VINYL CHLORIDE: COMBINED INHALATION TWO-GENERATION REPRODUCTION AND DEVELOPMENTAL TOXICITY STUDY IN CD RATS"

PEER REVIEWER COMMENT FORM

September 1995

Name: Mildred S. Christian, Ph.D., ATS

(Please note that the form sent references this information; this original form has comments on it recommending the protocol with required changes; my signature with the review date is provided on the form.)

1. **Are the study objectives clearly stated and appropriate?**

   Yes

Because most of the comments below apply to both questions 2 and 3, these two questions were addressed as one.

2. **Is the study design appropriate for the study objectives?**
3. **Are the methods selected appropriate for the study objectives?**

Essentially. Justification should be provided for some areas and some clarification is needed (see below).

Route/Method of Exposure

If there is concern regarding ingestion of the material from grooming, should a nose-only system, rather than whole-body exposure, be used? Use of a nose-only system would allow better control of exposure and prevent dermal absorption of the test material and exposure of the litters as the result of contact with the dams. Such considerations are important in these types of studies because individual exposure and actual routes of exposure are difficult to quantify. It is recognized that there are practical limitations in these types of studies, but as methods improve, they can be considered. Use of the nose-only approach would allow better monitoring of exposures and limit dermal exposure, as well as exposure of the offspring. However, it is also more expensive and more time consuming than whole body exposure.

The type of exposure method used should be justified, including reasons for use of a 5-day/week exposure except during pregnancy and lactation (without toxicokinetics information regarding metabolism and attained blood levels and elimination, there is no logical reason to have rats mimic a human worker exposure during one period but not

during other periods). If the exposure period differences are justified on the basis of practicality for the parental generations versus differences in time-dependent sensitivities for developmental toxicity, such should be identified.

Areas in the protocol identifying information regarding exposure, housing and duration of exposure:

Page 3, paragraph 3 - identifies that rats will be housed singly in wire mesh stainless steel cages in racks, with the exception that females will be housed in plastic nesting boxes with nesting materials during late gestation and lactation.

Page 7, line 9 - identifies that all rats will be housed continuously in exposure chambers following the initial exposure to the test material, except during late gestation and throughout lactation periods, when female rats will be housed outside the exposure chambers.

Page 7, beginning line 12 - Maternal rats will not be exposed to the test material after day 20 through the fourth day postpartum, in order to allow for parturition and initiation of lactation.

Exposure Levels Attained

Oral mg/kg/day dosages were referenced on the basis of monitored chamber exposures and ventilation rates. It is suggested that an actual evaluation of attained blood levels be identified, so that comparisons of blood levels resulting in toxicity could be compared with those attained by humans in the workplace (it would also identify whether the animal model was appropriate for use in human risk assessment). Such information is very helpful in risk assessment.

The design includes exposure on 5 days/week, 6 hours/day before mating and for 7 days/week during mating, gestation and lactation (page 6, reproduction - experimental design). It is unclear why all intervals do not require 7 days/week exposure.

Some clarification is needed regarding why the cages are housed in the chambers during the period before mating, but not housed in the chambers during gestation and lactation (the presumed reason is that the pups will not be potentially exposed to test material on the cages or in the bedding, BUT, they will be exposed to test material on the dams).

Although it helps in the practical conduct of the study, it is unclear why the dams are not exposed during the last few days of gestation and the first four days postpartum. There is no scientific reason why such should not occur. Are there historical data available demonstrating that pup mortality occurs as the result of separation of the dam and litter during only the first four days postpartum?

Selected P2 animals are exposed beginning at weaning (3 weeks of age). However, the litters will be produced over as much as a two-week time period, based on two weeks of cohabitation (page 7, breeding procedure). Body weight and feed consumption data are collected weekly for the P1 rats (page 8); a similar schedule is to be followed for the P2 generation (page 9, lines 9-10). The information regarding the P2 generation should be clarified. Differences in ages when first exposed can skew body weight data (growth curves). The P2 animals should be weighed when they are the same postpartum ages, so that body weight gain patterns are useful in identifying toxicity (i.e., all selected rats should be at the same age, not at ages that may vary as much as 2 weeks on any one calendar date).

Day Weaning Occurs (page 8, culling and weaning)

Day 0 postpartum is elsewhere defined as the day birth occurs, while day 1 postpartum is the day after birth. It appears that weaning on 21 days after birth indicates weaning on day 21 postpartum. The definitions regarding days 0 and 1 postpartum need to restated in this section.

Culling (page 8, culling and weaning)

The protocol states that culling occurs to reduce the variation in the growth of the pups. However, if one reduces the variation in the growth of the pups, the possibility also exists that evaluation of comparative measures of growth are confounded (e.g., increased mortality in high dosage group litters pre- or postbirth may have affected growth, increasing growth in smaller litters, hiding an effect of the agent, when compared with control litters, etc. There is considerable controversy regarding culling of litters. The current trend is to not cull litters until weaning, when pups are selected for continued observation. Many consider culling to potentially confound pup body weight gain and viability data. Others hold that behavioral observations are confounded by differences in litter sizes.) If culling is to be used, it is suggested that the a better reason (justification with a reference) be provided. Please also note that without providing a definition of a "runt", it is not possible to identify that preferential culling of runts will not occur (need to define what pup size at weaning age is considered to be a "runt").

Feed Consumption (page 8)

Please note that having the feed containers in the chambers means that the feed will be contaminated by the test material, and that there will be considerable differences in the oral level exposures as the result of differences in contamination of the feed and the amounts of feed consumed.

Body Weights (pages 8 and 9)

The dams are removed from housing in chambers on gestational day 20, but body weights are recorded on day 21 (exposure does not occur on day 21). It is suggested that weights be recorded on the last day of exposure (GD 20); this procedure will also allow for ease in changing from cages to boxes and eliminate the loss of some data for dams that may begin to deliver litters on GD 21, before weighing occurs.

Litter Data (page 9)

It is unclear why litter size and viability can be determined on day 0 postpartum, but weights for the dams and pups are not recorded until day 1 postpartum. Is there any reason for not collecting body weight and sex data, in addition to number and viability data, on day 0 postpartum? If not, why not adjust to days 0, 4, 7, 14 and 21 postpartum for weights or redefine day 0 as day 1 postpartum?

Reproduction - Pathology - Adult Rats (page 10)

The last necropsy is scheduled after the last litter is weaned in the respective generation. This means that there will be variation of approximately two weeks in the females regarding regression of mammary glands. It is suggested that it would be more appropriate to sacrifice the female rats on the day the litters are sacrificed. Although estrous cycling affects uterine weights, the number of implantation scars and the duration of the postpartum period also affect uterine weights. Justification should be provided to support the rational for sacrifice at different ages postpartum versus at comparable estrous stages.

Reproduction - Organ Weights - Adult Rats (page 10)

It is unclear why only the first 15 P1 and P2 animals have organ weights taken. Justification should be provided for selection of the first animals, rather than random selection of animals in a dosage group, as the potential for bias exists, based on when the animals mate and produce litters. It appears that the rats that mate first, thus producing the first litters, will be the rats that are evaluated for these parameters in the P2 generation, a potential bias, if reproductive performance of the P1 generation is affected by the agent.

Reproduction - Sperm Count, Motility and Morphology (page 11)

Justification needs to be provided for taking sperm from only the first 15 P1 and P2 males at termination (see previous comments). Also, it should be determined before study conduct whether sperm samples will be taken from the distal cauda epididymis or the proximal vas deferens for evaluation of percent progressively motile sperm and

possible evaluation of sperm morphology. Historical experience should be used to justify the region used for obtaining the sample.

Statistical Evaluation (page 14)

Duration of gestation - please define in text (assume it will be based on the difference between day 0 of gestation and day 0 postpartum).

Note: the comment on page 16 regarding dose-response relationships and whether the results are significant in the light of other biologic and pathologic findings if very good to include in the statistics section.

4. Any overall comments on the protocol?

In general, the protocol is well designed and clearly written. It generally includes the more recent recommendations by EPA for multigeneration studies. With the exception that blood level determination would be helpful, and that there are methodological difficulties in performing these types of studies that need to be stated so that the reason for certain procedures that are differently addressed when other routes are tested are evident, all comments made are merely fine-tuning.

Typographical Errors

Page 9, next to last line - "om" the - correct to "on" the.

Page 11, para 1, line 3 - low and middle (add "dose") groups.

Page 11, para 1, line 4 - use "that", rather than 'which" - "which" is generally used for time or preceded by a comma.

Page 12, para 1, line 3 - suggest modifying to "subsequent complete necropsy ...."

Page 12, para 3 - please note that high-dose is hyphenated in line 2 but is not hyphenated in line 4 (please check for consistency throughout).

Page 12, para 3, line 2 - please add "dose" after middle (middle dose groups)

Page 13, line 1 - please correct "studesign" to "study design"

Page 13, para 1, lines 1-2 - please modify to "Sexually mature, adult virgin females.....with male rats of the same strain (one male/female) at Charles River...." - This will clarify that one male rat does not mate all of the female rats, which is what the sentence currently states.

Page 13, para 1, line 5 - please change "males cage" to "male's cage"

Page 14, para 1, line 6 - please change "a 10% aqueous solution of sodium sulfide" to "an aqueous 10% solution of sodium sulfide" - the solution is 10% sodium sulfide and not 10% water.

Page 14, para 1, line 9 - please use an upper case for C-section.

Page 21 - TOTAL - 240 needs to be aligned.

Page 21 - Developmental Study - Uterine Examine - please change to Uterine Examination.

"VINYL CHLORIDE: COMBINED INHALATION TWO-GENERATION REPRODUCTION AND DEVELOPMENTAL TOXICITY STUDY IN CD RATS"

PEER REVIEWER COMMENT FORM

September 1995

Name Mildred S. Christian, Ph.D.

1. Are the study objectives clearly stated and appropriate?

(Yes) No Unsure

Why?

2. Is the study design appropriate for the study objectives?

Yes (No) Unsure

Why? See typed comments

It is unclear whether the housing is actually in the exposure chamber (see page 7 line 9) or in individual cages and in the chambers only during exposures. page 6 - Experimental design, line 1, page 1, Housing in

PAGE 2 -- PEER REVIEWER COMMENT FORM

Name Mildred S. Christian, Ph.D.

3. Are the methods selected appropriate for the study objectives?

Yes No Unsure

Why? See typed comments

4. Any overall comments on the protoool?

See typed comments

PAGE 3 -- PEER REVIEWER COMMENT FORM

Name Mildred S. Christian, Ph.D.

Select the appropriate category below (List recommended changes r reasons for not recommending:

A. Recommend ( )

B. Recommend with Required Changes (X)

1. Minor corrections regarding methodology
2. Add justification for culling and for different exposure intervals for P_1 gen, P_2 gen and pregnant rats. (i.e. daily, 7 days/week versus 5 days/week and interruption of exposure from GD 20 thru PP 4.

C. Not Recommended ( )

8/29/95

Signature **Dat**

CMA 115514

PAGE 4 -- PEER REVIEWER COMMENT FORM

Name Mildred S. Christian, Ph.D.

5. Any comments on ATSDR's peer review process?

This is an excellent procedure and adds confidence level to the research.

6. Any other comments?

Thank you for the opportunity to do this in a prospective, constructive mode.

approximately 0, 9.2, 92 and 1012 mg/kg/day assuming ventilation rates of 1 l/min/kg, 100 percent absorption and a 6 hour/day exposure. The high concentration of vinyl chloride was selected based on the oral equivalent of the limit dose of 1 mg/kg body weight/day. The high exposure of 1100 ppm is also expected to produce effects on the liver and other organ systems (ATSDR, 1993). The middle and low exposure levels were selected to provide a dose response for the observed effects and a no-observed-effect level, respectively. The calculations used to convert ppm to an oral dose equivalent are as follows:

Dose (mg/kg/day) = exposure concentration (mg/l) X minute volume/kg body wt X minutes of exposure X absorbed dose

Exposure concentration (mg/l) = (ppm X molecular wt)/24,450

Dose (mg/kg/day) = ppm X molecular wt X 1/24,450 X minute volume/kg body wt (1 l/min/kg) X minutes of exposure X absorbed dose

Where:

Dose = 1012, 92 or 9.2 mg/kg/day
Minute volume/kg body wt = 1 liter/min
Minutes of exposure/day = 360
Molecular wt = 62.5
Proportion absorbed = 100%
Conversion factor used in converting mg/l to ppm = 1/24,450

Example: 1012 mg/kg/day = 1100 ppm X 62.5 (molecular wt.) X 1/24,450 X 1 l/min/kg body wt X 360 min X 100% absorption

Reproduction - Experimental Design.

Groups of 30 male and 30 female rats will be exposed to 0, 10, 100 or 1100 ppm of the test material via inhalation, for 6 hours/day, 5 days/week prior to mating and 6 hours/day, 7 days/week during mating, gestation and lactation. The overall chronology of events and study design for this study are depicted in Tables 1 and 2, respectively. The treatment of the first generation parental (P1) rats will begin at approximately 6 weeks of age. After approximately 10

weeks of exposure (5 days/week, excluding holidays), P1 rats will be mated (one male to one female of the respective treatment group) to produce the F1 litters. Following weaning (3 weeks of age) of the F1 litters, 30 males and 30 females from each treatment group will be randomly selected and assigned to the respective treatment group to become the second generation parents (P2). After approximately 10 weeks of treatment following weaning of the last F1 litter, the P2 adults will be bred to produce the F2 litters. Exposures of P1 and P2 adults rats to the test material will continue until the adults are sent to necropsy. All rats will be housed continuously in exposure chambers following the initial exposure to the test material, except during late gestation and throughout lactation periods, when female rats will be housed outside of the exposure chambers. Maternal rats will not be exposed to the test material after day 20 of gestation (as calculated from day 0 of gestation via sperm-positive vaginal lavage) through the fourth day postpartum, in order to allow for parturition and initiation of lactation. During the lactation period, pups will not be placed in the exposure chambers, but will remain in the nesting boxes separated from the dam for approximately 7 hours/day on lactation days 5 through 21.

Reproduction - Breeding Procedure

Breeding of the P1 and P2 adults will commence after approximately 10 weeks of treatment. Each female will be placed with a single male from the same dose level (1:1 mating) until pregnancy occurs or two weeks have elapsed. During each breeding period, daily vaginal lavage samples will be evaluated for the presence of sperm as an indication of mating. The day on which sperm are detected or a vaginal plug is observed *in situ* will be considered day 0 of gestation. Sperm- and plug-positive females will then be separated and placed back into wire mesh, stainless steel cages. If mating has not occurred after two weeks, the animals will be separated without further opportunity for mating. For the P2 mating, cohabitation of male and female litter mates will be avoided.

Reproduction - Culling and Weaning

To reduce the variation in the growth of the pups, the F1 and F2 litters with a total number of pups exceeding eight will be culled on day 4 postpartum. Culled litters will be reduced to a total of eight pups, four males and four females, if possible. Pups to be culled will be selected using a computer generated randomization procedure. Litters with eight or fewer pups will not be culled. Preferential culling of runts will not be performed. Culled pups will be examined grossly for abnormalities and euthanized by the deposition of sodium pentobarbital (Succumb Butler, Columbus, OH) into the oral cavity. Weaning of all litters will be done 21 days after delivery. Weanlings not held for prospective generations or selected for necropsy will be examined grossly for abnormalities and euthanized by CO_2 inhalation.

Reproduction - Physical Observations

Each rat on study will be observed twice daily (a.m. and p.m.) for mortality, morbidity and moribundity as well as availability of feed and water. Changes in behavior or demeanor and indications of overt toxicity will be evaluated during the a.m. or p.m. observation. In addition, a thorough clinical examination will be conducted on all animals prior to the start of the study and weekly thereafter. This examination will include thorough evaluations of the skin and fur, mucous membranes, respiration, nervous system and behavior pattern. All adult rats found dead or in moribund condition will be submitted for a gross pathologic examination. Adult rats found dead after normal working hours will be refrigerated until a necropsy can be performed. All pups found dead or pups that are euthanized in moribund condition will be examined to the extent possible for defects and/or cause of death and preserved in neutral, phosphate-buffered 10% formalin. Cannibalized pups will be examined to the extent possible and discarded.

Reproduction - Body Weights and Feed Consumption

All P1 animals will have body weights and feed consumption (optional) recorded weekly during the 10-week pre-breeding treatment period, beginning on or before the first week of the study. Body weights for males will be recorded weekly throughout the course of the study. Sperm and plug positive

females will be weighed on Days 0, 7, 14 and 21 of gestation. Females that deliver litters will be weighed on Days 1, 4, 7, 14, and 21 of lactation. During breeding, feed consumption will not be measured in males or females due to cohousing. Following completion of the breeding periods, weekly feed consumption again will be measured in males. During gestation, feed consumption will be measured at weekly intervals in sperm and plug positive females. After parturition, feed consumption will be measured twice during the first and second week of lactation and at 2 - 3 day intervals during the last week of lactation. A similar schedule will be followed for the P2 generation.

Reproduction - Litter Data

All litters will be examined as soon as possible after delivery. The following parameters will be recorded for each litter: total litter size on the day of parturition (day 0), the number of live and dead pups on days 0, 1, 4, 7, 14, and 21 postpartum, and the sex and the weight of each pup on days 1, 4 (before and after culling), 7, 14, and 21 of lactation. Any visible physical abnormalities or demeanor changes in the neonates will be recorded during the lactation period.

Reproduction - Physical Maturational Landmarks

All F1 weanlings selected for mating will be observed daily for vaginal opening beginning on postnatal day 30 (Adams *et al.*, 1985) or preputial separation beginning on day 35 (Korenbrot *et al.*, 1977). If there is a treatment-related effect observed on the F1 sex ratio, age of vaginal opening or age of preputial separation, then anogenital distance will be measured on post natal day 4 for all F2 pups.

Reproduction - Estrous Cycling

Estrous cycle length and normality will be evaluated daily by vaginal lavage (Cooper et al., 1993) on the first 15 P1 and P2 females starting three weeks prior to mating and continuing throughout cohabitation.

Reproduction - Pathology - Adult Rats

A complete necropsy will be conducted by a team of trained individuals under the direct supervision of a veterinary pathologist on all P1 and P2 adults. The scheduled necropsy will be performed after the last litter of the respective generation has been weaned. Adult males will be fasted overnight, anesthetized with methoxyflurane and euthanized. Adult females will be necropsied on day 2 of diestrus whenever possible. This will be accomplished by monitoring (by vaginal lavage) for the occurrence of at least one estrous cycle after which time females found to be in day one of diestrus will be fasted overnight and necropsied on the following morning. The expected, subsequent stage of the estrous cycle (day 2 of diestrus) will be confirmed by vaginal lavage on the day of necropsy, prior to euthanasia. Based upon the results of these smears, exclusion of appropriate data parameters used for statistics will be performed for females not found to be in the appropriate stage of the estrous cycle (day 2 of diestrus) on the day of necropsy. The fasted females will be euthanized as described for the males. The eyes of both males and females will be examined *in situ* by gently pressing a moistened glass slide against the cornea and observing the eyes under fluorescent light. The uteri of all cohabited females will be examined for the presence and number of implantation sites. Tissues routinely collected (Table 3) will be saved from these rats and preserved in neutral, phosphate-buffered 10% formalin, with the following exceptions. The testes and epididymides will be preserved in Bouin's fixative. The lungs will be infused with formalin to their approximate normal inspiratory volume. The nasal cavity will be flushed with formalin via the pharyngeal duct to ensure rapid fixation of the tissue. Moribund rats and those dying spontaneously will be necropsied in a similar manner. However, body and organ weights will not be recorded.

Reproduction - Organ Weights - Adult Rats

The following organs on the first 15 P1 and P2 parental animals will be weighed at the scheduled terminal necropsy: uterus, ovaries, testes, single epididymis (total and cauda), seminal vesicles (with coagulating glands and their fluids), prostate, brain, liver, kidneys, lungs, adrenal glands, spleen, and thymus, and the organ-to-body weight ratios calculated.

uterine wts affected by implants?

Reproduction - Histology - Adult Rats

Histologic examination of potential target organs and reproductive tissues (Table 3) will be performed on the control and high dose groups. Examination of tissues from the low and middle groups will be limited to those tissues which demonstrate treatment-related histologic changes in the high dose group. Only the right ovary will be routinely processed for standard microscopic examination. The left ovary will be saved for possible oocyte quantification. If deemed necessary by the study sponsor, oocyte quantification will include evaluation of a minimum of ten sections, randomly selected from one completely sectioned ovary per female of the high-dose and control groups. Ovarian follicles will be placed into one of three categories as described by Plowchalk et al., (1993). The total number of follicles and the number of follicles in each of the three categories will be evaluated. Ovaries from the low and middle dose groups may be evaluated if treatment-related changes are observed in the high dose group. A complete set of tissues (excluding the left ovary for females), encompassing all organs listed in Table 3, will be prepared from all rats dying spontaneously or euthanized in a moribund condition and examined in an attempt to determine cause of death.

Reproduction - Sperm Count, Motility and Morphology

For the first 15 P1 and P2 males at termination, samples of sperm from the distal cauda epididymis or the proximal vas deferens will be collected for evaluation of percent progressively motile sperm and possible evaluation of sperm morphology. The entire right cauda epididymis will be weighed and then minced in saline to enumerate the total number of sperm (cauda reserves). Sperm motility and count will be determined with the use of the Hamilton-Thorn (HTM) Integrated Visual Optical System (IVOS) motility analyzer (Hamilton-Thorn Research, Beverly, Massachusetts). All samples for motility analyses will be video recorded or recorded digitally on disk and the recording kept as raw data. Sperm samples will be prepared for morphological evaluation and saved, but will not be evaluated unless deemed necessary by the study sponsor.

Reproduction - Pathology - Weanling Rats

At the time of weaning, 1 pup/sex/litter/dose from the F1 and F2 litters will be randomly selected for a complete necropsy by a team of trained individuals under the direct supervision of a veterinary pathologist. In order to control for variation in body and organ weight, all F1 and F2 pups selected for a complete necropsy will be euthanized at the same age. Pups will be anesthetized with methoxyflurane and euthanized. Terminal body weights will be recorded. Gross pathologic examination and preservation of tissue samples (Table 3) will be performed as described above for adults.

Reproduction - Organ Weights - Weanling Rats

For the first 15 male and female F1 and F2 pups that are examined macroscopically (one/sex/litter), the following organs will be weighed: ovaries, testes, brain, liver, kidneys, adrenal glands, spleen and thymus.

Reproduction - Histology - Weanling Rats

Organs that demonstrate treatment-related effects in weanlings will be examined microscopically in the control and high-dose groups. Examination of tissues from the low and middle dose groups will be limited to those tissues which demonstrate treatment-related histologic changes in the high dose group. Microscopic examination will also be made of all tissues showing gross pathologic changes.

Developmental - Experimental Design.

Groups of 30 adult time-mated female rats will be exposed to 0, 10, 100 or 1012 ppm of the test material via inhalation, for 6 hours/day, on gestation days 6 through 20. These exposure levels correspond to oral equivalent doses of approximately 0, 9.2, 92 and 1012 mg/kg/day assuming ventilation rates of 1 l/min/kg, 100 percent absorption and a 6 hour/day exposure. The high concentration of vinyl chloride was selected based on the oral equivalent of the limit dose of 1 mg/kg body weight/day. For further discussion of dose level selection refer to the "Exposure Concentrations" section of this protocol.

study design

The overall chronology and ~~studesign~~ for the developmental portion of this study is depicted in Tables 1 and 2, respectively.

Developmental - Breeding Procedure

Sexually mature, adult virgin females, approximately 10 weeks of age and weighing approximately 200 - 250 grams, will be naturally mated with one male of the same strain at the Charles River Breeding Laboratory. Females will be checked for plugs the following morning and those found with a vaginal plug will be removed from the males' cage. The day on which a vaginal plug is detected will be considered Day 0 of gestation. Day 0 body weights will be provided by Charles River Breeding Laboratory, and maintained in the study record. Rats will be shipped on Day 0 or 1 of gestation and will arrive at our laboratory on Day 1 or 2 of gestation.

Developmental - Maternal Observations

All animals will be observed daily during the study for alterations in behavior or demeanor as previously described under the Reproduction Study "Physical Observation" section, with the exception that a thorough weekly clinical examination will not be conducted. Any animal which dies, appears moribund or shows indications of early termination of pregnancy will be submitted for a complete necropsy as described for the Reproduction Study. Body weights will be recorded on gestation days 0, 6, 14 and 21.

On Day 21 of gestation, all surviving animals assigned to the developmental study will be euthanized by carbon dioxide inhalation and given a limited necropsy. Any obvious structural or pathologic changes noted in the adult will be recorded and the weight of the liver, kidneys and gravid uteri will be recorded. Liver, kidneys and gross lesions will be preserved in neutral, phosphate-buffered 10% formalin, but microscopic examination of tissues will not be conducted unless deemed necessary to interpret other observations made during the study or requested by the sponsors.

Developmental - Fetal Observations

At necropsy, the uterine horns will be exteriorized through an abdominal incision and the following data recorded: 1) the number and position of fetuses *in utero*, 2) the number of live and dead fetuses, 3) the number and position of resorptions, 4) the number of corpora lutea, 5) the sex and body weight of each fetus, and 6) any gross external alteration. The uteri of apparently non-pregnant animals will be stained with a 10% aqueous solution of sodium sulfide (Kopf et al., 1964) and examined for evidence of early resorptions. Corpora lutea will not be recorded for females that are not visibly pregnant at C-section or for females that are submitted for necropsy prior to Day 21. At least one-half of the fetuses in each litter, selected using a table of random numbers, will be examined immediately by dissection under a low power stereo-microscope for evidence of visceral alterations (Staples, 1974). The heads of rat fetuses examined by dissection will be removed, placed in Bouin's fixative and examined by the serial sectioning technique of Wilson (1965). All fetuses will then be preserved in alcohol, eviscerated and stained with alizarin red-S (Dawson, 1926). Skeletal examination will be conducted on all fetuses that were not given visceral examinations.

Statistical Evaluation.

Descriptive statistics (means and standard deviations) will be reported for feed consumption. Body weights, gestation/lactation body weight gains, organ weights, and sperm count per gram cauda epididymis and percent motile sperm will first be evaluated by Bartlett's test for equality of variances. Based upon the outcome of Bartlett's test, either a parametric or nonparametric analysis of variance (ANOVA) will be performed. If the ANOVA is significant, a Dunnett's test or the Wilcoxon Rank-Sum test with Bonferroni's correction will be performed.

Gestation length, average time to mating, number of corpora lutea, number of implants, litter size, age at vaginal opening and age at preputial separation will be analyzed using a nonparametric ANOVA. If the ANOVA is significant, the Wilcoxon Rank-Sum test with Bonferroni's correction will be performed. Statistical outliers will be identified by the method of Grubbs

(1969) and will be routinely excluded from analysis for feed consumption only. Outliers for other endpoints will only be excluded from analysis for documented, scientifically sound reasons. Fertility indices and pregnancy rate will be analyzed by the Fisher exact probability test and Bonferroni's correction will be used for multiple testing of groups in comparison to a single control. Evaluation of the neonatal sex ratio will be performed by the binomial distribution test. Survival indices will be analyzed using the litter as the experimental unit by the Wilcoxon test as modified by Haseman and Hoel (1974). Statistical evaluation of the frequency of pre-implantation loss, resorptions and fetal alterations among litters and the fetal population will be performed using a censored Wilcoxon test with Bonferroni's correction. Nonpregnant females, females pregnant following staining or females having totally resorbed litters will be excluded from the appropriate analyses.

The nominal alpha levels to be used are as follows:

Bartlett's Test (Winer, 1971)	α=0.01
Parametric ANOVA (Steel and Torrie, 1960)	α=0.10
Nonparametric ANOVA (Hollander and Wolfe, 1973)	α=0.10
Dunnett's Test (Winer, 1971)	α=0.05, two-sided
Wilcoxon Rank-Sum Test (Hollander and Wolfe, 1973)	α=0.05, two-sided with Bonferroni correction (Miller, 1966)
Fisher's Test (Siegel, 1956)	α=0.05, two-sided
Censored Wilcoxon Test (Haseman and Hoel, 1974)	α=0.05, two-sided
Outlier Test (Grubbs, 1969)	α=0.02, two-sided
Binomial Distribution Test (Steel and Torrie, 1960)	α=0.05, two-sided

Because numerous measurements are statistically compared in the same group of animals, the overall false positive rate (Type I errors) will be much greater than the cited alpha levels would suggest. Thus, the final

interpretation of numerical data will consider statistical analyses along with other factors such as dose-response relationships and whether the results are significant in the light of other biologic and pathologic findings.

Safety Precautions.

Standard safety precautions will be followed during the conduct of this study.

Quality Assurance.

Permanent records of all data generated during the course of this study, the protocol, any addenda to the protocol, and the final report will be available for inspection by the Quality Assurance Unit. All data generated including the protocol, addenda, and final report will be archived at Health and Environmental Sciences, The Dow Chemical Company, Midland, Michigan.