CHEMICAL MANUFACTURERS ASSOCIATION

June 9, 1995

Christopher T. DeRosa, Ph.D.
Director, Division of Toxicology
Agency for Toxic Substances and Disease Registry
1600 Clifton Road, N.E., Mailstop E29
Atlanta, Georgia 30333

Re: Vinyl Chloride

Dear Dr. DeRosa:

By letter dated May 4, you indicated that the Agency for Toxic Substances and Disease Registry (ATSDR) would be receptive to a proposal for a combined reproductive toxicity and developmental toxicity study to meet the identified data needs for vinyl chloride. In our conference call on May 16, Dr. William L. Breslin of the Dow Toxicology Research Laboratory described how we would propose to expand the protocol submitted by the Vinyl Chloride Panel on March 22 for a two-generation reproductive toxicity study by including an additional group of 30 female rats that would be mated and evaluated (including fetal evaluation) for the developmental parameters specified in the EPA guideline for inhalation developmental toxicity studies, 40 C.F.R. § 798.4350.

During the conference call, we indicated that the Panel would submit by June 9 a revised protocol to address the priority data needs identified by ATSDR for reproductive and developmental toxicity studies for vinyl chloride. The final protocol is enclosed.

My letter of November 28, 1994 indicated the Panel's intent to work with ATSDR toward the execution by May 31, 1995 of a memorandum of understanding (MOU) to address the data needs identified for vinyl chloride, consistent with the policy statement issued by ATSDR and EPA on November 18, 1994. Obviously, we will not be able to have an MOU in place by that deadline, but I believe that we are making significant progress. I understand that you will coordinate with EPA to extend, as appropriate, the time within which ATSDR will

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notify EPA of the vinyl chloride testing to be conducted under a voluntary research agreement between the Panel and ATSDR, which in turn will extend the time for EPA to decide whether such agreement adequately addresses the testing needs identified by EPA in its September 30, 1994 solicitation notice.

In this regard, we understand that ATSDR will shortly be meeting with EPA and that you will discuss with EPA staff the rationale for their interest in the neurotoxicity of vinyl chloride, as well as the possibility of satisfying EPA's identified testing need by expanding the enclosed protocol to address additional neurotoxicity endpoints. We also would be interested in any comments EPA staff might have on the enclosed protocol.

We will wait to hear from you as to ATSDR's and EPA's responses. In particular, we will need to know whether the enclosed protocol should be revised and resubmitted to ATSDR prior to its submission to the panel of peer reviewers that we understand ATSDR will be selecting.

Sincerely,

Hasmukh Shah

Hasmukh C. Shah, Ph.D.
Manager, Vinyl Chloride Panel

Enclosure

cc: W. Caffey Norman, Esq.

Preliminary Copy X
Final Copy ___

HEALTH AND ENVIRONMENTAL SCIENCES
THE DOW CHEMICAL COMPANY

PROTOCOL

THE TOXICOLOGY RESEARCH LABORATORY,
1803 BUILDING, MIDLAND, MICHIGAN 48674

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

DATE.

FILE NUMBER:

PROPOSED EXPERIMENTAL START DATE:

PROJECT NUMBER:

TASK NUMBER:

PROPOSED EXPERIMENTAL TERMINATION DATE:

OSD NUMBER:

COST CENTER:

ESTIMATED DATE FINAL REPORT:

SPONSOR:

CAS NUMBER:

SIGNATURES:

STUDY DIRECTOR: _____ /DATE

COINVESTIGATOR(S): _____ /DATE

INHALATION SPECIALIST: _____ /DATE

STUDY PATHOLOGIST: _____ /DATE

APPROVAL(S): _____ /DATE

SPONSOR: _____ /DATE

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INTRODUCTION

Objective.

The objectives of the combined inhalation two-generation reproduction and developmental toxicity study outlined in this protocol are to evaluate the effects of the test material on parental toxicity, reproductive capability, *in utero* development, and neonatal growth and survival in rats. This study will be conducted to meet the requirements of the Environmental Protection Agency (EPA): TSCA Test Guidelines (EPA, 1985), the Organisation for Economic Co-Operation and Development (OECD), Guidelines, for Testing of Chemicals, Section 4: Health Effects, (OECD, 1981), and the European Economic Community (EEC), Methods for the Determination of Toxicity (EEC, 1988).

Statement of GLP Compliance.

This study will be conducted in accordance with the Food and Drug Administration (FDA) Good Laboratory Practice Regulations for NonClinical Studies (FDA, 1988), the EPA TSCA Good Laboratory Practice Standards (EPA, 1990), the OECD Good Laboratory Practice Procedures (OECD, 1982), and the Standard Operating Procedures of The Toxicology Research Laboratory of The Dow Chemical Company.

In addition, in response to the Final Rules amending the U.S. Animal Welfare Act that were promulgated by the U.S. Department of Agriculture effective October 30, 1989, the Animal Care and Use Activity (ACUA) that is required for the conduct of this study has been reviewed and given full approval by the Institutional Animal Care and Use Committee (IACUC). The IACUC has determined that the proposed Activity is in full accordance with these Final Rules. The IACUC has assigned Activity No(s). Reproductive Toxicology 01 and 02 to this Animal Care and Use Activity.

MATERIALS AND METHODS

The test material used for this study will be obtained from a commercial supplier and identified by lot number. The purity of the test material will be determined and reported. A sample of the test material will be taken and stored in a manner consistent with the reference sample retention policy of this laboratory. The test material will be reanalyzed at approximately 6-month intervals to confirm purity and stability.

The test substance has the following properties:

Chemical Name:	Vinyl chloride
Synonyms:	Monochloroethylene, VC, VCM, vinyl chloride monomer
Molecular Formula:	C_2H_3Cl
Molecular Weight:	62.5
Structures:	CH_2=CHCl
Appearance:	Colorless gas
Vapor Pressure:	2,530 mm Hg at 20 °C
Saturated Atmosphere:	Gas at room temperature
Vapor Density:	2.16
Flash Point:	-77.75 °C (open cup)
Boiling Point:	-13.6 °C
Specific Gravity:	0.9121
Conversion Factors:	1 ppm = 2.60 mg/m^3 in air 1 mg/mm^3 = 0.39 ppm in air

Test Species and Husbandry.

Male and female CD rats (Charles River Breeding Laboratory, Kingston, NY or Portage, MI) approximately four weeks of age will be purchased for the reproduction phase of this study. An additional group of adult female time-mated CD rats (Charles River Breeding Laboratory, Kingston, NY or Portage, MI) approximately ten weeks of age will be purchased for the developmental portion of this study. Animals will be ordered to ensure that a sufficient

number of animals of acceptable health and weight are available to conduct the study as designed. This strain of rat has been selected because of its general acceptance and suitability for toxicity testing and the availability of a reliable commercial source. Upon arrival at the laboratory[1], all rats will be examined for health status by a veterinarian and acclimated to the laboratory environment (approximately two weeks for the reproduction study; 5 days for the developmental study), according to the Standard Operating Procedures of the Reproductive Toxicology Group. The animals will be randomly assigned by weight to the treatment groups to increase the probability of uniform group mean weights and standard deviations at the initiation of the study. Rats not placed on test will be removed from the test room and the disposition of these animals will be documented in the study file.

Identification of all rats on test will be accomplished by inserting a uniquely coded alphanumeric metal tag in one ear of each rat. In the event that an ear tag becomes dislodged during the course of the study, it will be replaced with one having the same alphanumeric code (i.e., a new alphanumeric code will not be assigned) and noted in the study file.

Rats will be housed singly in wire mesh, stainless steel cages in racks provided with cage board to minimize odor and aid in maintaining a clean environment. Prior to and following daily exposures during late gestation and throughout lactation, females will be housed in plastic nesting boxes provided with ground corn cob nesting material (further details provided in experimental design section). The animal rooms of the facility are designed to maintain humidity at approximately 40-60%, temperature at approximately 22 °C, photoperiod at 12 hrs light:12 hrs dark and air flow at 12-15 changes/hour. A feed crock and a pressure-activated stainless steel water nipple will be components of all cages. A basal diet of Purina Certified Rodent Chow No. 5002 (Purina Mills Inc., St. Louis, MO) will be provided *ad libitum* except during the six hour per day exposures, at which time feed will be withheld. Municipal drinking water will be available *ad libitum*

[1]Fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

throughout the prestudy and study periods. Analysis of the chow will be performed by Purina Mills Inc. to confirm that the diet provides adequate nutrition, and to quantify the levels of selected contaminants associated with the formulation process. Drinking water obtained from the City of Midland, MI will be analyzed for chemical parameters and biological contaminants by the City of Midland Water Department. In addition, specific analyses for chemical contaminants will be conducted at periodic intervals as stated in the Standard Operating Procedures of The Toxicology Research Laboratory, The Dow Chemical Company.

Exposure Chamber.

Animals will be housed and exposed to test material vapors in a 14.5 m^3 chamber (2.4 m wide x 2.4 m high x 2.4 m deep with a pyramidal top) under dynamic airflow conditions. Chamber airflows will be maintained at approximately 2900 liters/minute, which is sufficient to provide the normal concentration of oxygen to the animals.

Vapor Generating System.

The various concentrations of test material will be generated using a glass J-tube method (Miller et al., 1980). Liquid test material will be metered into a glass J-tube assembly and vaporized by a preheated stream of compressed air (up to 100 l/min) passing through the J-tube. Compressed air will be heated to the minimum extent necessary to facilitate complete vaporization of the test material. The compressed air and test material vapors will be diluted and mixed with room air to achieve the desired concentration of test material vapors at a total flow rate of 2900 l/min.

Chamber Monitoring.

The concentration of the test material in each chamber will be measured at least once per hour using a MIRAN 1A infrared spectrophotometer (Foxboro Analytical, Norwalk, CT). Analytical equipment will be calibrated using standards having a known test material vapor concentration contained in 90 liter SARAN* film gas bags prior to the first exposure and at least monthly

* Trademark of The Dow Chemical Company

thereafter. Daily checks of the analytical equipment will be performed prior to each exposure period using a single test material standard concentration. In addition, the amount of test material used each day will be recorded and the nominal concentrations (amount of the test material used/total chamber airflow) of test material will be calculated. Prior to the start of the study, each of the chambers to be used will be checked to ensure that a uniform distribution of vapors occurs within the breathing zone according to Standard Operating Procedures.

Airflow through each chamber will be determined at hourly intervals using a differential pressure transducer (Model C264, Setra Systems, Inc., Acton, MA) or a Universal Venturi tube (Series 180, BIF, 345 Harris Ave., Providence, RI). The manometer and differential pressure transducer will be calibrated with a gas meter (Singer Aluminum Diaphragm Meter, Model AL-1400, American Meter Division, Philadelphia, PA) prior to the start of the study. The Universal Venturi tubes have been calibrated at the factory.

Chamber temperatures will be measured with a thermometer or resistance temperature device (RTD) and relative humidities will be measured with relative humidity gauges or humidity sensors (HMP 112A, Vaisala, Helsinki, Finland) at least once each hour. Calibration of the hygrometers, RTD's and humidity sensors will be documented in the study file. The temperature and relative humidity in each chamber will be controlled by a system designed to maintain temperature at approximately 22±2°C and relative humidity at approximately 40-60%.

Output from the differential pressure transducer, RTD, humidity sensor and infrared spectrophotometer will be collected by the CAMILE* Data Acquisition and Control System.

Exposure Concentrations.

Rats will be exposed to target concentrations of 0, 10, 100 or 1100 ppm of the test material. These exposure levels correspond to oral equivalent doses of

* Trademark of SAGIAN Indianapolis, Indiana.

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) om 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.

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 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.

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.

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TABLE 1

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

CHRONOLOGY OF EVENTS

WEEKS ON STUDY	P1/Developmental	F1/P2	F2
1-10	Exposure of P1 males and females prior to first mating. Exposure (gestation days 6-20) of the dams for developmental toxicity evaluation Necropsy of developmental toxicity dams and fetal evaluations		
11-12	P1 mating period for F1 litters.		
14-15		F1 born and litters culled on day 4 post-partum to 8 pups each.	
17-18		F1 litters weaned on day 21 post-partum; offspring selected for P2 adults; 1 F1 pup/sex/dose/litter selected for necropsy; remaining pups euthanized.	
19-28	Necropsy P1 adults.	Exposure of P2 males and females prior to first mating.	
29-30		Mating period of P2 for F2 litters.	
32-33			F2 born and litters culled on day 4 post-partum to 8 pups each.
35-36			F2 litters weaned on day 21 post-partum; 1 F2 pup/sex/dose/litter selected for necropsy; remaining pups euthanized.
37		Necropsy P2 adults.	

TABLE 2

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

STUDY DESIGN

EXPOSURE LEVELS (PPM)	NO. NO. MALES	NO. FEMALES
0	30	30 Reproductive 30 Development
10	30	30 Reproductive 30 Development
100	30	30 Reproductive 30 Development
1000	30	30 Reproductive 30 Development
	TOTAL= 120	240

PARAMETERS

REPRODUCTION STUDY
Animal Observations
Body Weights (parental and neonatal)
Feed Consumption (optional)
Necropsy (parental and weanlings)
Organ Weights (parental and weanlings)
Gross Pathology (parental and weanlings)
Histopathology (parental and weanling)
Fertility Indices
Estrous Cycling and Sperm Analyses
Developmental Landmarks
Neonatal Survival

DEVELOPMENTAL STUDY
Animal Observations
Body Weights (maternal and fetal)
Feed Consumption (optional)
Necropsy
Limited Organ Weights
Gross Pathology
Uterine Examine
External, Visceral and Skeletal Examination

TABLE 3

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

TISSUES COLLECTED AND PRESERVED AT NECROPSY

ADRENALS	KIDNEYS*	PROSTATE*
AORTA	LACRIMAL/HARDERIAN GLANDS	RECTUM
AUDITORY SEBACEOUS GLANDS	LARYNX	SALIVARY GLANDS
BONE (INCLUDING JOINT)	LIVER*	SEMINAL VESICLES*
BONE MARROW	LUNGS*	SKELETAL MUSCLE
BRAIN (CEREBRUM, BRAINSTEM, CEREBELLUM)	MAMMARY GLAND*	SKIN
CECUM	MEDIASTINAL LYMPH NODE	SPINAL CORD (CERVICAL, THORACIC, LUMBAR)
CERVIX*	MEDIASTINAL TISSUES	SPLEEN
COAGULATING GLANDS*	MESENTERIC LYMPH NODE	STOMACH
COLON	MESENTERIC TISSUES	TESTES*
DUODENUM	NASAL TISSUES*	THYMUS
EPIDIDYMIDES*	ORAL TISSUES	THYROID GLAND
ESOPHAGUS	OVARIES*	TONGUE
EYES	OVIDUCTS*	TRACHEA*
GROSS LESIONS*	PANCREAS	URINARY BLADDER
HEART	PARATHYROID GLANDS	UTERUS*
ILEUM	PERIPHERAL NERVE	VAGINA*
JEJUNUM	PITUITARY*	

*TISSUE SELECTED FOR HISTOPATHOLOGIC EVALUATION.