CHEMICAL MANUFACTURERS ASSOCIATION

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Carlos Nuilo, B-2234, TX

March 23, 1995

Dear Vinyl Chloride Health Committee Members:

A March 22, 1995 letter to William Cibulus of ATSDR is enclosed for your review and files. I will contact Mr. Cibulus next week to schedule a meeting. If you have any questions, please call me at (202) 887-1192.

FYI

Sincerely,

Has Shah

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

Enclosures

R&S151505

2501 M Street, NW, Washington, DC 20037 Telephone 202-887-1100 Fax 202-887-1237

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CHEMICAL MANUFACTURERS ASSOCIATION

March 22, 1995

William Cibulas, Ph.D.
Chief, Research Implementation Branch
Division of Toxicology
Agency for Toxic Substances & Disease Registry
1600 Clifton Road, N.E.
Mailstop E29
Atlanta, Georgia 30333

Dear Dr. Cibulas:

On January 23, Dr. Christopher DeRosa responded to my letter of November 28, 1994 indicating the intent of the Chemical Manufacturers Association (CMA) Vinyl Chloride Panel to address certain data needs for vinyl chloride identified by the Agency for Toxic Substances and Disease Registry (ATSDR). Dr. DeRosa requested that the Panel take the lead role in developing the protocol for a two-generation reproductive toxicity study of vinyl chloride by the inhalation route.

We have prepared the enclosed protocol of such a study for ATSDR review. Pursuant to your telephone conversation with our counsel, Caffey Norman, we have not attempted to identify dose levels at this time. Dose levels would be determined after a review of existing literature or, if necessary, following a range-finding study, in either event in consultation with ATSDR.

You will note that in a few places information has been redacted from the enclosed protocol. The purpose of these deletions is to remove certain references that would limit the study to a particular laboratory. Such information will be included when a final protocol is submitted for review.

As Mr. Norman discussed with you, selecting dose levels and otherwise providing a completely final protocol for your review would require several additional weeks. We believe that it would be more useful if this time were devoted to considering ways that the enclosed protocol might be enhanced to include measures of

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# INTRODUCTION

Prior Toxicity Data.

(TO BE INSERTED)

Objective.

The objective of the two-generation inhalation reproduction study outlined in this protocol is to evaluate the effects of the test material on the reproductive capability 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 Practice.

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 .

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. Reproductive Toxicology 01 to this Animal Care and Use Activity.

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 for approximately two weeks . For the randomization procedure, the rats will be weighed and ranked according to body weight and those from the extremes of the distribution will be identified and removed from the population until only the number of animals required for the study remain. These rats 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 deotized 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

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

SARAN* film gas bags prior to the first exposure and at least monthly thereafter. Daily checks of the analytical equipment will be performed prior to each exposure period using a single the test material standard concentration. In addition, the amount of the test material used each day will be recorded and the nominal concentrations (amount of the test material used/total chamber airflow) of the 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.

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 presssure 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.

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* Trademark of The Dow Chemical Company
* Trademark of SAGIAN Indianapolis, Indiana.

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 either three estrous cycles or two weeks has 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.

*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 Beuthanasia-D Special (Schering Corporation, U.S.A., Kenilworth, NJ) 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.

*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. In addition, 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

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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.

*Estrous Cycling*

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

*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 euthanatized. Adult females will be necropsied on day 2 of diestrus whenever possible. This will be accomplished by monitoring (by vaginal lavage) for the occurence 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 cohabitated females will be examined for the presence and number of implantation sites. Tissues routinely collected (Table 2) 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

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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 videorecorded 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.

*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 euthanatized. Terminal body weights will be recorded. Gross pathologic examination and preservation of tissue samples (Table 2) will be performed as described above for adults.

*Organ Weights - Weanling Rats*

For all 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.

*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.

Statistical Evaluation.

Descriptive statistics (means and standard deviations) will be reported for feed consumption. Body weights, gestation/lactation body weight gains, organ

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(Grubbs, 1969)
Binomial Distribution Test $\alpha$=0.05, two-sided
(Steel and Torrie, 1960)

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 avaiable for inspection by . All data generated including the protocol, addenda, and final report will be archived at .

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TABLE 2

Two-Generation Inhalation Reproduction 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.