# P R O T O C O L

## CHRONIC INHALATION TOXICITY STUDIES ON $TiO_2$ AND $TiCl_4$

Approved for Pathology: _____
J. G. Aftosmis, DVM

Approved for Biochemistry: _____
J. R. Barnes, Ph.D.

Approved for Toxicology: _____
H. J. Trochimowicz, Sc.D.
Chief, Inhalation Toxicology

_____
J. R. Gibson, Ph.D.
Manager, Toxicology

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D R A F T

# PRELIMINARY PROTOCOL*
# FOR TWO YEAR INHALATION STUDY
# FOR TITANIUM DIOXIDE AND TITANIUM TETRACHLORIDE

## INTRODUCTION

This study is designed to evaluate the potential chronic toxicity and carcinogencity of titanium dioxide and titanium tetrachloride, respectively, in rats, by inhalation. The two test compounds will be started simultaneously utilizing one common control.

## MATERIALS TO BE TESTED

Titanium dioxide ($TiO_2$, Titanium Oxide) is a crystalline and amorphous powder with the following properties:

| | |
|---|---|
| Form: | White solid, Median Particle Size $\sim$1.0$\mu$ |
| Molecular Weight: | 79.9 |
| Melting Point: | Decomposes at 1640°C |
| Specific Gravity (water = 1): | 3.8-4.2 |
| Solubility: | Insoluble in water; soluble in sulfuric acid or alkali |

Titanium tetrachloride ($TiCl_4$) is a liquid at room temperature and has the following properties:

| | |
|---|---|
| Specific Density | 1.7 g/cc |
| Molecular Weight | 189.73 |
| Boiling Point at 760 mm Hg | 137°C |
| Color | APHA-40 |
| Vapor Pressure at 25°C | 15 mm Hg |
| Solubility | > 10% in water; reacts with acetone, ethanol, dimethylsulfoxide and vegetable oil |

EXPOSURES

Cost estimates are included for two test materials x 3 exposure levels/ test material sharing one common control. The exposure regimen will be 6 hours/ day x 5 days/week (except holidays) x 78 weeks and holding animals for an additional 26 weeks unless group size drops to 10/sex before that time, in which case the study will be terminated. In addition, an estimate is included for an additional 6 months exposure.

Animals are to receive food and water ad libitum except during exposure. Chamber atmospheres are to be analyzed at least hourly by an analytical method determined by us and acceptable to Pigments Department. The method should have a precision and accuracy of ± 5%. Analytical samples should be taken from all areas of the chamber where animals are exposed. The standard deviation of chamber concentrations for the entire study should not exceed 15% of the average concentration.

Test animals are to be randomized by regular rotation in the chamber daily. Control animals are to be treated exactly as test animals, except their chamber atmosphere will not contain any test material.

All exposures will be carried out during the same eight-hour period of the day. If exposures of some test groups are to be regularly carried out at other times, the diurnal cycles of these rats will be adjusted and maintained so that all rats are being exposed during the same part of this cycle.

The chamber used for these studies are to be dedicated to these studies throughout the duration of the exposure phase (78 weeks or longer) and are not to be used for exposure of other materials or animals during the portion of the day or week when not in use on this study.

The chambers and all surfaces that the test materials contact prior to exhausting from the chamber shall be constructed of materials which will not

EXPOSURES (Continued)

absorb, or react with, the test materials. The chambers shall be free of contamination from previous tests.

During exposure, the chambers should be maintained at 40-60% RH and 72-78°F. Air flow rates through the chamber shall be adequate to prevent any toxic effects from volatiles in the animal excreta and provide adequate air for normal respiration.

The exhaust from the chambers will be scrubbed through an appropriate solution for removal of test materials. The resulting solutions will be disposed of by Pigments as agreed to in discussions with Dr. R. Salemi.

The chambers should be operated in a one-pass flow-through mode as opposed to recirculation of the test atmosphere.

EXPOSURE LEVELS

The study will consist of three test levels/compound and one common control. Exposure levels will be determined by Haskell Laboratory after assessing the results of acute and 4-week range-finding studies now in progress. The levels selected should adequately assess the potential chronic toxicity and carcinogenic potential of these materials. Chamber concentrations of the test materials may be changed during the course of the study after consultation with the Pigments Department.

ATMOSPHERE GENERATION

Titanium Dioxide

Titanium dioxide is an amorphous and crystalline powder and will be generated as a dust. A glass cyclone head type dust generator, or its equivalent, will be used to disperse the dust particles into the chamber atmosphere.

ATMOSPHERE GENERATION (Continued)

Chamber atmospheric concentrations will be determined gravimetrically employing glass fiber filters. Particle size determinations of the chamber atmosphere will be determined periodically.

Titanium tetrachloride

Titanium tetrachloride has sufficient vapor pressure (15 mm Hg, at 25°C) to generate chronic exposure levels by nitrogen stream saturation utilizing fritted glass bublers and constant temperature baths. $TiCl_4$ hydrolyzes extensively in air resulting in chamber atmospheres consisting of $TiCl_4$ and its hydrolysis products. To minimize the latter, some degree of control over chamber temperature and humidity will be necessary.

The analytical method to be used for the chronic study has not yet been decided. At present, a specific ion electrode method for total chlorides is in use. Other methods to be explored include atomic absorption and a colorimetric determination for titanium.

TEST ANIMALS AND HOUSING

The test animal will be the Charles River-CD rat. This rat has an expected lifetime of two years in our laboratory.

One hundred and twenty (120) weanling rats of each sex will be used at each test level and in the control group.

Rats with grossly visible corneal opacities should not be used in this study. Light levels shall be kept low enough to avoid cataract formation.

The test animals may be housed in either Bioclean® rooms or in conventional animal rooms. Housing facility RH and temperature will be similar

TEST ANIMALS AND HOUSING (Continued)

to the conditions maintained during exposure. Air flow rates through the housing facility should be adequate to prevent any toxic effects due to volatiles from the animal excreta. Good animal husbandry practices shall be employed at all times.

Test groups shall be left in their respective exposure chambers for an appropriate time post-exposure to allow the test material to evaporate from their fur before placing them in their housing quarters. The control animals shall not be housed with any rats exposed to any test materials by any route. None of the test rats will be housed in rooms with animals on other tests.

Except during exposure periods, food and water will be available ad libitum. The rats are to be fed a balanced, nutritionally adequate, commercial laboratory diet.

SAFETY AND HOUSEKEEPING

Titanium Dioxide

Because of the nature of $TiO_2$, measures taken to insure unnecessary spreading of the fine dust particles in the work area will include wearing disposable clothing, jump suits, gloves, hats and shoe covers during loading and un-loading of the test animals. Respiratory protection should be worn during the un-loading process. Racks used for transporting rats to and from the exposure chamber will be covered with plastic sheeting if deemed necessary by study personnel.

Titanium tetrachloride

Liquid $TiCl_4$ hydrolyzes in contact with moist skin resulting in acid burn. $TiCl_4$ vapors cause irritation to the throat and bronchial passages resulting in coughing and/or wheezing.

SAFETY AND HOUSEKEEPING (Continued)

Respiratory protection and disposable clothing, jump suits, rubber gloves, hats, and shoe covers will be worn during loading and un-loading of the test animals or any time the possibility of contact with $TiCl_4$ liquid or vapor exists.

OBSERVATIONS AND RECORDS

The animals will be observed at least daily on exposure days for gross signs of toxicity and changes in appearance and demeanor during the period of exposure. Body weights will be individually recorded weekly for the first 3 months of the study and biweekly thereafter. This schedule will be altered if more or less frequent intervals are indicated. The time of appearance and location of all tumors that occur among both control and test animals will be recorded. Mortality records will be kept on all groups of animals. All animals which die or are sacrificed during the study will be necropsied and their tissues processed in accordance with the methods given in the Pathology Section of this protocol. Care will be exercised to minimize loss of tissue through cannibalism or autolysis. Animals in a moribund state will be sacrificed in extremis when death is imminent. Animals losing $\geq$ 10% body weight between weighings will be observed daily until weight gain is again normal or they are sacrificed.

CLINICAL LABORATORY EVALUATIONS

The hematologic, urine analytical, and blood chemistry indices listed below will be determined for 10 rats/sex for all test levels and the control at 3, 6, 12, 15, and 18 months of exposure. If possible, the same test animals should be used at each test period.

CLINICAL LABORATORY EVALUATIONS (Continued)

The indices to be determined are:

1. Hematology - red blood cell count, white blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and white cell differential.
2. Urinalysis - urine volume, osmolality, pH, sugar, proteins, blood, urobilinogen, bilirubin, and microscopic examination of pooled urine sediment.
3. Blood chemistry - gamma-glutamyl transpeptidase, alkaline phosphatase, bilirubin, lactic dehydrogenase, glutamic-pyruvic transferase, glutamic-oxaloacetic transferase, urea nitrogen and total protein.

PATHOLOGIC EXAMINATIONS

Gross and histopathologic evaluations will be conducted on 5 rats/sex/level at 3 months and 6 months of exposure, and 10 rats/sex/level at one year with the option for an 18 month sacrifice. In addition, all animals found dead or sacrificed in extremis (integrity of tissue permitting), and all survivors at two years will be examined grossly and histopathologically.

All sacrifices will be by chloroform inhalation. The trachea will be exposed and tied off caudal to thyroid. The animal then will be exsanguinated by severing the vena cava. The lungs and trachea will be removed as a unit for gross observations. After discarding the tied area and saving the upper part of trachea with thyroid, the lungs will be inflated to normal inspiratory volume by gravity infusion with Bouin's fixative. A gross pathologic examination of all internal organs will be made and observations recorded, even if the organ was grossly normal.

PATHOLOGIC EXAMINATIONS (Continued)

Representative specimens of the following organs and tissues will be taken from all animals and fixed in Bouin's fixative, unless autolysis has occured. The tissues marked * will be weighed. All tissues will be processed by conventional methods, embedded in paraplast or its functional equivalent, sectioned (4-6 μ), stained with hematoxylin-eosin, and histopathologically evaluated by light microscopy:

Adipose Tissue
*Adrenal Glands
All Gross Lesions, including tumors
Bone (femur)
Bone Marrow (sternal)
*Brain (cerebrum, cerebellum, stem)
Ear Canal with Ceruminal (Zymbal's) Glands
Epididymis
Esophagus
Exorbital Lacrimal Gland
Eyes
Gonads (*testes and ovaries)
*Heart
*Kidneys
Large Intestine (caecum and colon)
*Liver (minimum of 2 lobes)
*Lungs
Lymph Nodes (tracheobronchial, cervical and mesenteric)
Mammary Gland
Nasal turbinates
Pancreas
Peripheral (Sciatic) Nerve
*Pituitary Gland
Prostate
Salivary Gland
Skeletal Muscle
Skin - Neck
Small Intestine (duodenum, jejunum and ileum)
Spinal Cord
*Spleen
Stomach
Thymus
Thyroid Gland (with parathyroid)
Trachea
Urinary Bladder
Uterus

PATHOLOGIC EXAMINATIONS (Continued)

Additional histopathological evaulations will be conducted on all lesions suggestive of a tumor or a tumor-like process observed in all rats at necropsy.

ANIMAL HUSBANDRY

All animals will be fed Purina Lab Chow (or equivalent) and given water ad libitum except during exposure. Animals will be initially identified upon arrival by usual laboratory procedures and individually identified in the study by one other means -- earpunch and toeclip combination, for example.

REPORTS

A final report on the study describing procedures and results and interpreting the results will be sent to the Department within one year of the terminal sacrifice. The report content will conform to contemporary standards for this type study. It should include statistical analysis of tumor incidence and type and comparison of incidence and type with test controls and historical data for the rat used. Number of historical controls and time span covered will be included. The report will also contain an acceptable statistical analysis of 1) life span data for each group, and 2) time to appearance for tumors which occur spontaneously in the rat used.

Progress reports will be given monthly on a verbal basis and a comprehensive written summary at the end of the first year will be submitted to the Pigments Department.

MISCELLANEOUS

Haskell Laboratory will retain all original written records.

Any change in or addition to the protocol will be documented and copies sent to all Haskell personnel involved, as well as to the Pigments Department.