National Toxicology Program
Toxicity Report Series
Number 32

NTP Technical Report
on Toxicity Studies of

Methylene Bis(thiocyanate)

(CAS No. 6317-18-6)

Administered by Gavage
to F344/N Rats and B6C3F$_1$ Mice

Leo T. Burka, Ph.D., Study Scientist
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Post Office Box 12233
Research Triangle Park, NC 27709

NIH Publication 94-3381
December 1993

United States Department of Health and Human Services
Public Health Service
National Institutes of Health

Note to the Reader

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- the National Cancer Institute (NCI) of the National Institutes of Health;
- the National Institute of Environmental Health Sciences (NIEHS) of the National Institutes of Health;
- the National Center for Toxicological Research (NCTR) of the Food and Drug Administration; and
- the National Institute for Occupational Safety and Health (NIOSH) of the Centers for Disease Control.

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This NTP report on the toxicity studies of methylene bis(thiocyanate) is based primarily on 2-week gavage studies conducted in August 1988 and on 13-week gavage studies that began in April 1989 and ended in July 1989 at Hazleton Laboratories America, Inc., Rockville, MD.

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PEER REVIEW

The draft report on the toxicity studies of methylene bis(thiocyanate) was evaluated in June 1993 by the reviewers listed below. These reviewers serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determine if the design and conditions of these NTP studies are appropriate and ensure that this toxicity study report presents the experimental results and conclusions fully and clearly. The comments of the peer reviewers were reviewed by the NTP staff and were addressed in this toxicity study report.

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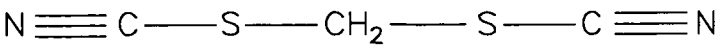
ABSTRACT

Methylene Bis(thiocyanate)



Molecular Formula	$C_3H_2N_2S_2$
CAS Number	6317-18-6
Molecular Weight	130.2
Synonyms	MBT methylene-bis-thiocyanate methylene bisthiocyanate methylene dithiocyanate

Methylene bis(thiocyanate) is used as a biocide in a number of applications. Its major use is in water cooling systems and paper mills as an inhibitor of algae, fungi, and bacteria. Methylene bis(thiocyanate) was selected for study because of the potential for human exposure to the compound and because of the interest in organothiocyanates as a chemical class. Toxicity studies of methylene bis(thiocyanate) (approximately 98% pure) were conducted with male and female F344/N rats and B6C3F$_1$ mice; the compound was administered to the animals by gavage in an aqueous methyl cellulose vehicle for 2 weeks or 13 weeks. In addition to these studies, the genetic toxicity of methylene bis(thiocyanate) was evaluated by determining mutagenicity in *Salmonella typhimurium* with and without S9 activation and frequency of micronucleated normochromatic erythrocytes in the peripheral blood of mice.

In the 2-week studies, groups of five rats and five mice per sex were administered methylene bis(thiocyanate) at concentrations of 0, 10, 20, 40, 80, and 160 mg/kg body weight. All animals in the two highest dose groups (80 and 160 mg/kg) died by Day 2 of the studies. Except for one female rat, all animals receiving 40 mg/kg methylene bis(thiocyanate) also died before the end of the studies. Few significant gross lesions were observed in the 80 and 160 mg/kg groups. Clinical observations were similar to those reported for cyanide toxicity and included dyspnea, tremors, and ataxia. The stomach, which was identified as the target organ in rats and mice surviving for at least 24 hours,

had necrotic inflammatory lesions of the mucosal surface of both the glandular and nonglandular portions.

In the 13-week studies, groups of 10 rats and 10 mice per sex were administered methylene bis(thiocyanate) at concentrations of 0, 1, 2, 4, 8, and 16 mg/kg body weight. In the rat study, deaths occurred in the 2, 4, 8, and 16 mg/kg groups, while in the mouse study, deaths occurred only in the 8 and 16 mg/kg groups. As in the 2-week studies, the stomach was identified as the primary target organ. However, the lower doses administered in the 13-week studies resulted in gastric effects that were limited to the forestomach and consisted primarily of squamous mucosal hyperplasia and hyperkeratosis. Rats receiving the higher doses of methylene bis(thiocyanate) developed a mild anemia, and sperm motility was decreased in male rats receiving 4 or 8 mg/kg.

Methylene bis(thiocyanate) was not mutagenic in *S. typhimurium*, with or without S9 activation. The frequencies of micronucleated normochromatic erythrocytes in the peripheral blood of dosed and control mice were similar.

Chemical disposition studies of [^{14}C]-labeled methylene bis(thiocyanate) were conducted in male F344 rats. In these studies, more than 90% of the administered radioactivity was eliminated in 48 hours. However, as the dose was increased from 0.2 to 1 to 10 mg/kg, greater percentages of the administered radioactivity remained in the tissues. Blood cyanide concentrations were increased shortly after the administration of 10 mg/kg [^{14}C]-methylene bis(thiocyanate) but were similar to control values 2 hours after dosing.

Overall, the toxic effects of methylene bis(thiocyanate) were consistent with those of an irritant chemical administered by gavage. There was also some indication that the release of cyanide may result in acute toxicity at the higher dose levels used in these studies. The no-observed-adverse-effect level for forestomach lesions in the 13-week studies was 4 mg/kg for male rats and 2 mg/kg for female rats and male and female mice.

INTRODUCTION

Physical Properties, Production, Use, and Exposure

Methylene bis(thiocyanate) is a yellow to light orange solid that melts at 105° to 107° C (Konnert and Britton, 1971). It has limited solubility in water (<1 mg/mL) but is soluble in organic solvents (Keith and Walters, 1987). Methylene bis(thiocyanate) is used as a biocide in a number of applications. Its major use is in water cooling systems and paper mills as an inhibitor of algae, fungi, and bacteria. It has also been used as a preservative in latex emulsions and cutting oils (Wehner and Hinz, 1971). The toxic mechanism responsible for the biocidal activity of methylene bis(thiocyanate) is not well understood; however, a suggested mechanism is competitive inhibition of cell respiration. During this process, the thiocyanate moiety inactivates the electron transport system by complexing with the ferric ion of cytochrome (Maas-Diepeveen and van Leeuwen, 1988).

Based on data submitted to the United States International Trade Commission (USITC) by two producers of methylene bis(thiocyanate), it is estimated that more than 4.5×10^3 kg of the chemical was produced in 1978 (USITC, 1979). In 1973, 2.0×10^6 kg of 10% methylene bis(thiocyanate) was consumed for biocide applications, and 75% of this amount was used by the paper industry (Biocides USA, 1974). More recent estimates are not available.

According to a National Occupational Hazard Survey, 1,670 workers may have been occupationally exposed to methylene bis(thiocyanate) between 1972 and 1974 (NIOSH, 1974). Additionally, 24,231 workers, including 995 women, were potentially exposed to the chemical in 1983 (NIOSH, 1992). No threshold-limit value has been set for methylene bis(thiocyanate). Humans may be exposed through the use of the compound as a slimicide in process water used in the production of paper and paperboard.

No information was found on the environmental occurrence of methylene bis(thiocyanate). The compound hydrolyzes rapidly above pH 8 and may degrade in the environment (Maas-Diepeveen and van Leeuwen, 1988). Hydrogen cyanide (HCN) is produced from methylene bis(thiocyanate) under alkaline conditions in the presence of a reducing agent; alkali alone causes other reactions to occur which prevent the formation of HCN (Wehner and Hinz, 1971).

Absorption, Disposition, Metabolism, and Excretion

There are no published reports on the absorption, disposition, metabolism, or excretion of methylene bis(thiocyanate) in humans or animals. However, the metabolism and mechanism of toxicity for several organothiocyanates similar to methylene bis(thiocyanate) have been studied (Ohkawa *et al.*, 1972; Habig *et al.*, 1975). Reaction of these organothiocyanates with glutathione, either with or without catalysis from glutathione-S-transferases, results in the formation of HCN. Tissue concentrations of HCN are increased after administration of organothiocyanates to mice, and the toxic response is similar to that observed after HCN intoxication.

Toxicity

HUMAN TOXICITY

In a study of dermatitis cases in workers involved in the manufacture of polyester yarns in which methylene bis(thiocyanate) was present in a spin finish formulation, skin sensitization patch tests with methylene bis(thiocyanate) were positive in 2 of 10 cases (Burrows and Campbell, 1980); patch tests in 2 of 23 controls were also positive. The authors suggested that the positive test results were more likely due to an irritant effect of methylene bis(thiocyanate) than to sensitization.

The skin sensitization potential of Cytox® 3522, a formulation of 10% methylene bis(thiocyanate) in a water-hydrocarbon dispersion, has also been investigated. In a standard skin patch study, 476 individuals were exposed to a 1% solution of Cytox® 3522 in petroleum ether, and no positive reactions were elicited (Andersen and Hamann, 1983). Cytox® 3522 was also included in a study of the skin sensitization potential of several biocides (Andersen and Veien, 1985). In this study, after 46 individuals were exposed to a 1% solution of Cytox® 3522, which was from a different batch than that used in the 1983 study, it was evident that the material was an irritant. The concentration was reduced to 0.2% for the remaining 1,606 individuals tested, and five positive reactions were elicited. Neither the concentration nor the presence of methylene bis(thiocyanate) in the formulation was verified by the authors. Lack of skin sensitization by Cytox ® 3522 in paper mill workers exposed to methylene bis(thiocyanate) has been verified in another study (Jäppinen and Eskelinen, 1987).

ANIMAL TOXICITY

Cytox® 3522 had a strong sensitization potential in guinea pigs when tested using the Guinea Pig Maximization Test and the Open Epicutaneous Test (Andersen and Hamann, 1983). In the Guinea Pig Maximization Test, a 5% solution of Cytox® 3522 in propylene glycol was used for intradermal induction, and challenge doses were given as petroleum ether solutions of Cytox® 3522. Positive responses were noted in all 20 animals challenged with a 1% solution of Cytox® 3522 (0.1% methylene bis(thiocyanate)), in 12 of 20 animals challenged with a 0.5% solution, and in 3 of 20 animals challenged with a 0.1% solution. In the Open Epicutaneous Test, Cytox® 3522 in an acetone solution was applied to the flank skin of guinea pigs. Positive responses were noted in animals challenged with a 10% solution of Cytox® 3522 following induction with a 30% solution.

Little additional mammalian toxicity data are available on methylene bis(thiocyanate). Wehner and Hinz (1971) reported an acute oral LD_{50} of 55 mg/kg for young male albino rats. No deleterious effects were noted in rats administered 100 ppm methylene bis(thiocyanate) in feed for 30 days (Wehner and Hinz, 1971). The results of this study are questionable because methylene bis(thiocyanate) was found to be unstable in feed in the early stages of the NTP studies. Subcutaneous administration of 10 mg/kg methylene bis(thiocyanate) to rabbits resulted in the rapid onset of severe seizures and shortness of breath (Taubmann, 1930); administration of 20 mg/kg produced a similar response followed by death. Subcutaneous administration of 3 mg/kg resulted in a 1.1° C drop in body temperature but apparently caused no seizures.

In short-term toxicity studies of methylene bis(thiocyanate) in rainbow trout, an LC_{50} of 84 µg/L was reported following 14 days of exposure, and an LC_{50} of 65 µg/L was reported after 60 days of exposure (Maas-Diepeveen and van Leeuwen, 1988). No histopathologic investigation on the nature of the toxicity was reported.

DEVELOPMENTAL TOXICITY

The teratogenicity of methylene bis(thiocyanate) has been assessed using the embryolarval test with rainbow trout (Maas-Diepeveen and van Leeuwen, 1988). Egg samples were placed in aquaria containing various concentrations of methylene bis(thiocyanate) 3 hours after fertilization and were kept in the aquaria until hatching 28 days later. The no-effect level for teratogenicity was 32 µg/L. At a concentration of 100 µg/L, congenital effects

including scoliotic caudal peduncle, reduced caudal fin, and anomalous adipose fin were induced.

GENETIC TOXICITY

Methylene bis(thiocyanate) was not mutagenic in *Salmonella typhimurium* in the presence or absence of S9 (Zeiger *et al.*, 1992).

Study Rationale and Design

Methylene bis(thiocyanate) was nominated for toxicity and carcinogenicity testing by the National Cancer Institute because of its potential for human exposure and its structure (the compound was one of several organothiocyanates in a class study). Because ingestion is a likely route of exposure for methylene bis(thiocyanate), oral administration was chosen as the route for these toxicity studies. Gavage in a methyl cellulose vehicle was selected as the route of administration after it was determined that methylene bis(thiocyanate) rendered drinking water unpalatable and was unstable in feed. The studies performed included clinical pathology, histopathology, and reproductive system evaluations and chemical disposition studies. Methylene bis(thiocyanate) was also evaluated for mutagenicity in *Salmonella typhimurium*.

MATERIALS AND METHODS

Procurement and Characterization of Methylene Bis(thiocyanate)

Methylene bis(thiocyanate) was obtained in one lot (Lot 2908) from Albright & Wilson, Incorporated (Richmond, VA). Identity and purity analyses were performed by Midwest Research Institute (MRI; Kansas City, MO). The chemical, a yellow powder, was identified as methylene bis(thiocyanate) by infrared, ultraviolet/visible, and nuclear magnetic resonance spectroscopy; the spectra were consistent with the structure of methylene bis(thiocyanate), the available literature reference (*Sadtler Standard Spectra*), and the spectra obtained from a previously analyzed lot of methylene bis(thiocyanate) (Lot 001557).

The results of elemental analyses for carbon, hydrogen, nitrogen, and sulfur agreed with theoretical values. Karl Fischer analysis indicated a water content of 0.81% ± 0.03%, and functional group titration indicated a purity of 100.5% ± 0.3%. Analysis by thin-layer chromatography indicated a major spot, a trace impurity, and a very slight trace impurity using an acetone:hexane (60:40) solvent system and a major spot and a trace impurity using a toluene:acetonitrile (96:4) solvent system. Gas chromatography by two systems indicated no impurities greater than 1.32% relative to the major peak. Cumulative analytical data based on elemental analysis, Karl Fischer water analysis, and thin-layer and gas chromatography indicated a purity of approximately 98%.

No stability studies were performed on Lot 2908 by MRI; however, accelerated stability studies conducted on Lot 001557 with gas chromatography indicated that methylene bis(thiocyanate) is stable as a bulk chemical for 2 weeks at temperatures up to 60° C when stored capped with a nitrogen headspace and protected from light. At the study laboratory, the bulk chemical was stored capped with a nitrogen headspace, refrigerated, and protected from light. Bulk chemical reanalyses performed by the study laboratory using gas chromatography showed consistent purity levels throughout the studies.

Dose Formulations

Dose formulations were prepared by stirring 2 g increments of methylene bis(thiocyanate) into a 0.6% (w/v) methyl cellulose solution in deionized water. After methylene bis(thiocyanate) was added, formulations were alternately stirred and sonicated for 1 hour and then stirred for an additional 15 minutes. Dose formulations were prepared weekly during the 2-week studies and once every 2 weeks during the 13-week studies.

Homogeneity analyses were conducted by MRI on dose formulations of 16 and 35 mg methylene bis(thiocyanate)/g methyl cellulose solution (0.6% w/v). Formulations were tested on the day of preparation as well as after 1 to 4 days of storage at 5° C. Analyses with gas chromatography indicated that the maximum variation for each dose formulation was less than 3%. Analyses conducted by the study laboratory before the studies began confirmed the homogeneity of the dose formulations. Stability studies conducted by MRI with gas chromatography indicated that 0.35 mg/g methylene bis(thiocyanate) in a 0.6% methyl cellulose solution was stable for 3 weeks when stored in sealed glass containers in the dark at 5° C and for 3 hours when stored open to air and light at room temperature. The dose formulations used in the 2-week and 13-week studies were stored in amber bottles in the dark at 5° C.

At the study laboratory, dose formulations and animal room samples were analyzed periodically using gas chromatography. All dose formulations used for dosing were within 10% of the target concentrations. In the 2-week studies, four of five animal room samples for rats and three of five animal room samples for mice were within 10% of the target concentrations. In the 13-week studies, 15 of 19 animal room samples for rats and 12 of 15 animal room samples for mice were within 10% of the target concentrations. The results of referee analyses performed by MRI on dose formulations used in the 13-week studies were also within 10% of the target concentrations.

Toxicity Study Designs

BASE STUDIES

Male and female F344/N rats and B6C3F$_1$ mice used in the 2-week and 13-week studies were obtained from Taconic Laboratory Animals and Services (Germantown, NY). Rats and mice were shipped to the study laboratory at approximately 4 to 5 weeks of age and quarantined for 10 to 14 days. The animals were about 6 weeks of age when the studies

began. At the end of the 13-week studies, blood samples were taken from five sentinel rats and five control mice per sex, and the sera were analyzed for viral antibody titers (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b); all results were negative. Additional details concerning study design and performance are listed in Table 1.

In the 2-week studies, five rats and five mice per sex were administered methylene bis(thiocyanate) in a 0.6% (w/v) methyl cellulose solution at a concentration of 0 (vehicle control), 10, 20, 40, 80, or 160 mg/kg body weight for 12 dosing days. Doses used in the 13-week studies were based on the results of the 2-week studies. In the 13-week studies, 10 rats and 10 mice per sex were administered methylene bis(thiocyanate) in a 0.6% (w/v) methyl cellulose solution at a concentration of 0, 1, 2, 4, 8, or 16 mg/kg body weight 5 days per week, excluding weekends and holidays, for 13 weeks; additional rats (10 males and 10 females per dose group) were used in a supplemental clinical pathology study.

For all studies, rats were housed five per cage by sex and mice were housed individually. Animal rooms were maintained at 67° to 75° F and 35% to 70% relative humidity, with 12 hours of fluorescent light per day and approximately 18 to 21 air changes per hour. Feed and water were available *ad libitum.*

Complete necropsies were performed on all animals in the 2-week and 13-week base studies. For rats and mice, the heart, liver (with gallbladder for mice), lungs, right kidney, thymus, and right testis were removed and weighed. Organs and tissues were examined for gross lesions and fixed in 10% neutral buffered formalin. Tissues to be examined microscopically were trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

For the 2-week studies, histopathologic examinations were performed on all tissues showing evidence of gross lesions. For the 13-week studies, complete histopathologic examinations were performed on the following animals:

- all animals that died before the end of the studies,
- all vehicle control animals,
- all animals in the highest dose group with at least 60% survivors,
- and all animals in the higher dose groups.

The specific dose groups receiving complete histopathologic examinations are listed in Table 1. Target organs were identified, and these organs plus gross lesions were examined

in the lower dose groups to a no-observed-effect level. Organs examined microscopically are listed in Table 1.

Upon completion of the laboratory pathologist's histologic evaluation, the slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were sent to an independent pathology laboratory where quality assessment was performed. Results were reviewed and evaluated by the NTP Pathology Working Group (PWG); the final diagnoses represent a consensus of contractor pathologists and the PWG. Details of these review procedures have been described by Maronpot and Boorman (1982) and Boorman *et al.* (1985).

SUPPLEMENTAL EVALUATIONS

A summary of the disposition and metabolism studies of methylene bis(thiocyanate) is presented in Appendix E.

Clinical Pathology

In the 13-week studies, hematology and clinical chemistry evaluations were performed on 10 male and 10 female rats per dose group at Weeks 1 and 3 and on base-study rats at the end of the study (Week 13). For these evaluations, rats were anesthetized with a CO_2:O_2 gas mixture, and blood was collected from the retroorbital sinus. Blood for hematology analyses was placed in tubes containing sodium EDTA, and blood for clinical chemistry evaluations was placed in similar tubes devoid of anticoagulant. The latter blood samples were allowed to clot at room temperature; the samples were then centrifuged and serum was removed.

Hematologic determinations were made with a Coulter Counter® Model S-Plus IV System (Coulter Electronics, Inc., Hialeah, FL). The parameters evaluated are listed in Table 1. Differential leukocyte counts and morphologic evaluation of blood cells were determined by light microscopic examination of blood films stained with Wright-Giemsa. Smears for reticulocyte determination were prepared from blood stained by incubating equal volumes of whole blood and new methylene blue for at least 20 minutes; smears were examined by light microscopy using a Miller disc for reticulocyte quantitation.

Excluding sorbitol dehydrogenase (SDH) and bile acids, all clinical chemistry endpoints were determined on a Hitachi® 737 chemistry analyzer (Boehringer Mannheim, Indianapolis, IN) with reagents obtained from the manufacturer. SDH and bile acids were measured on a Baker CentrifiChem® analyzer (Baker Instruments, Allentown, PA) with reagents obtained from Sigma Diagnostics® (St. Louis, MO). Clinical chemistry parameters evaluated are listed in Table 1.

Sperm Motility and Vaginal Cytology Evaluations

At the end of the 13-week studies, sperm motility and vaginal cytology evaluations were performed on all surviving base-study rats in the 0, 2, 4, and 8 mg/kg groups and all surviving mice in the 0, 4, 8, and 16 mg/kg groups. The parameters evaluated are listed in Table 1. Methods were similar to those described by Morrissey *et al.* (1988). Briefly, for the 12 days prior to sacrifice, the vaginal vaults of 10 females of each species and dose group were lavaged, and the aspirated lavage fluid and cells were stained with toluidine blue. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (*i.e.*, diestrus, proestrus, estrus, or metestrus).

Sperm motility was evaluated at necropsy in the following manner. The left testis and epididymis were weighed. The tail of the epididymis (cauda epididymis) was then removed from the corpus epididymis and weighed. Test yolk (rats) or modified Tyrode's buffer (mice) was applied to slides, and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on each of two slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers.

Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Cauda were finely minced and swirled, and the tissue was incubated and then heat fixed. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in 10% dimethyl sulfoxide in phosphate-buffered saline. Homogenization-resistant spermatid nuclei were counted using a hemacytometer.

TABLE 1 Experimental Design and Materials and Methods in the 2-Week and 13-Week Gavage Studies of Methylene Bis(thiocyanate)

	2-Week Studies	13-Week Studies
EXPERIMENTAL DESIGN		
Study Laboratory	Hazleton Laboratories America, Inc., Rockville, MD	Same as 2-week studies
Strain and Species	F344/N rats B6C3F$_1$ mice	Same as 2-week studies
Animal Source	Taconic Laboratory Animals and Services, Germantown, NY	Same as 2-week studies
Size of Study Groups	Five males and five females	Base Studies: 10 males and 10 females Clinical Pathology Study: 10 male and 10 female rats
Doses/Duration of Dosing	0, 10, 20, 40, 80, or 160 mg/kg for 12 dosing days	Base Studies: 0, 1, 2, 4, 8, or 16 mg/kg, 5 days per week for 13 weeks Clinical Pathology Study: 0, 1, 2, 4, 8, or 16 mg/kg, 5 days per week for 3 weeks
Dose Volume	Rats: 5 mL/kg body weight Mice: 10 mL/kg body weight	Same as 2-week studies
Date of First Dose	Rats: 9 August 1988 Mice: 8 August 1988	Rats: 24 April 1989 Mice: 21 April 1989
Date of Last Dose	Rats: 24 August 1988 Mice: 23 August 1988	Rats: 23 or 24 July 1989 Mice: 20 or 21 July 1989
Date of Necropsy	Rats: 25 August 1988 Mice: 24 August 1988	Rats: 24 July 1989 or 25 July 1989 Mice: 21 July 1989 or 22 July 1989
Type and Frequency of Observation	Observed twice daily for mortality and morbidity. Clinical observations were performed daily. Individual body weights were recorded prior to dosing, weekly thereafter, and at necropsy.	Observed twice daily for mortality and morbidity. Clinical observations were recorded weekly. Individual body weights were recorded prior to dosing, weekly thereafter, and at necropsy.

TABLE 1 Experimental Design and Materials and Methods in the 2-Week and 13-Week Gavage Studies of Methylene Bis(thiocyanate) (continued)

2-Week Studies	13-Week Studies
EXPERIMENTAL DESIGN (continued)	
Necropsy and Histopathologic Examinations Complete necropsies were performed on all animals in the base studies. Histopathologic examinations were performed on all tissues showing evidence of gross lesions.	Complete necropsies were performed on all animals in the base studies. Complete histopathologic examinations were performed on the following animals: all animals that died before the end of the studies; all vehicle control animals; male rats receiving 4 mg/kg or greater; female rats receiving 8 mg/kg or greater; and male and female mice receiving 16 mg/kg. The following tissues were examined microscopically in these animals: adrenal glands, brain (three sections), esophagus, femur with marrow, gallbladder (mice only), gross lesions, heart, intestines (large: cecum, colon, rectum; small: duodenum, jejunum, ileum), kidneys, liver, lungs/mainstem bronchi, lymph nodes (mandibular, mesenteric), mammary gland, muscle, nasal cavity and turbinates (three sections), ovaries, pancreas, parathyroid glands, pituitary gland, preputial or clitoral glands, prostate gland, salivary glands, spleen, stomach (forestomach and glandular stomach), testes (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, uterus, and vagina. The forestomach, nasal cavity, and trachea were identified as targets of methylene bis(thiocyanate) toxicity in rats and mice; these tissues plus gross lesions were examined microscopically in the lower dose groups.
Supplemental Evaluations None	Clinical Pathology Study: Hematology and clinical chemistry evaluations were performed on male and female rats designated for clinical pathology testing at Weeks 1 and 3 and on base-study rats at the end of the study (Week 13). Ten rats per dose group were evaluated. Hematology parameters evaluated included hematocrit (Hct), hemoglobin (Hgb) concentration, erythrocyte (RBC) count, reticulocyte count, mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), platelet count, and leukocyte (WBC) count and differential. Clinical chemistry parameters evaluated included urea nitrogen (UN), creatinine, total protein, albumin, alkaline phosphatase (AP), alanine aminotransferase (ALT), creatine kinase (CK), sorbitol dehydrogenase (SDH), and bile acids.

TABLE 1 Experimental Design and Materials and Methods in the 2-Week and 13-Week Gavage Studies of Methylene Bis(thiocyanate) (continued)

2-Week Studies	13-Week Studies
EXPERIMENTAL DESIGN (continued)	
Supplemental Evaluations (continued)	
	Sperm Motility and Vaginal Cytology: Sperm motility and vaginal cytology evaluations were performed at the end of the 13-week studies. Rats in the vehicle control, 2, 4, and 8 mg/kg groups and mice in the vehicle control, 4, 8, and 16 mg/kg groups were evaluated. Males were evaluated for necropsy body and reproductive tissue weights, epididymal spermatozoal data, and spermatogenesis. Females were evaluated for necropsy body weight, estrous cycle length, and the percent of cycle spent in the various stages.
ANIMAL MAINTENANCE	
Time Held Before Study	
Rats: 14 days Mice: 13 days	Rats: 13 days Mice: 10 days
Age When Study Began	
6 weeks	Same as 2-week studies
Age When Killed	
8 weeks	19 weeks
Method of Animal Distribution	
Animals were weighed and randomized using a computer program.	Same as 2-week studies
Diet	
NIH-07 Open Formula Meal and Pellets (Zeigler Brothers, Inc., Gardners, PA) and water were available *ad libitum.*	Same as 2-week studies
Animal Room Environment	
Rats were housed five per cage by sex, and mice were housed individually. The temperature was maintained at 67° to 71° F and relative humidity at 57% to 69%, with approximately 18 air changes per hour. Fluorescent light was provided for 12 hours per day.	Rats were housed five per cage by sex, and mice were housed individually. The temperature was maintained at 70° to 75° F and relative humidity at 35% to 70%, with approximately 21 air changes per hour. Fluorescent light was provided for 12 hours per day.

Genetic Toxicity Studies

SALMONELLA TYPHIMURIUM MUTAGENICITY TEST PROTOCOL

Testing was performed as reported by Zeiger *et al.* (1992). Methylene bis(thiocyanate) was sent to the laboratory as a coded aliquot. It was incubated with the *Salmonella typhimurium* tester strains (TA97, TA98, TA100, TA1535, and TA1537) either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with *l*-histidine and *d*-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and of at least five doses of methylene bis(thiocyanate). The high dose was limited by toxicity.

PERIPHERAL BLOOD MICRONUCLEUS TEST PROTOCOL

A detailed discussion of this assay is presented in MacGregor *et al.* (1990). Blood samples were collected from all surviving B6C3F_1 mice at the end of the 13-week methylene bis(thiocyanate) study. Smears were prepared immediately and fixed in absolute methanol. The slides were later stained with acridine orange, a chromatin-specific fluorescent dye. The slides were then coded and scanned to determine the frequency of micronuclei in 2,000 normochromatic erythrocytes in each of five mice per group. The criteria of Schmid (1976) were used to define micronuclei.

Statistical Methods

ANALYSIS OF CONTINUOUS VARIABLES

During the 13-week studies, two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which are approximately normally distributed, were analyzed with the parametric multiple comparisons procedures of Williams (1971, 1972) or Dunnett (1955). Clinical chemistry and hematology data, which typically have skewed distributions, were analyzed with the nonparametric multiple comparisons methods of Shirley (1977) or Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used

to assess the significance of dose-response trends and to determine whether a trend-sensitive test (Williams, Shirley) was more appropriate for pairwise comparisons than a test capable of detecting departures from monotonic dose response (Dunnett, Dunn). If the P-value from Jonckheere's test was greater than or equal to 0.10, Dunn's or Dunnett's test was used rather than Shirley's or Williams' test.

The outlier test of Dixon and Massey (1951) was employed to detect extreme values. No value selected by the outlier test was eliminated unless it was at least twice the next largest value or at most half of the next smallest value. In addition, values indicated by the laboratory report as being inadequate due to technical problems were eliminated from the analysis.

ANALYSIS OF VAGINAL CYTOLOGY DATA

Because the data are proportions (the proportion of the observation period that an animal was in a given estrous stage), an arcsine transformation was used to bring the data into closer conformance with normality assumptions. Treatment effects were investigated by applying a multivariate analysis of variance (Morrison, 1976) to the transformed data to test for the simultaneous equality of measurements across dose levels.

ANALYSIS OF MUTAGENICITY IN *SALMONELLA TYPHIMURIUM*

A positive response in the *Salmonella typhimurium* assay was defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any strain/activation combination. An equivocal response was defined as an increase in revertants that was not dose related, not reproducible, or of insufficient magnitude to support a determination of mutagenicity. A negative response was obtained when no increase in revertant colonies was observed following chemical treatment. There was no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

ANALYSIS OF PERIPHERAL BLOOD MICRONUCLEUS DATA

The frequency of micronucleated NCEs was analyzed by a statistical software package (Integrated Laboratory Systems, Research Triangle Park, NC) that employed a one-tailed trend test across dose groups and a *t*-test for pairwise comparisons of each dosed group to the concurrent control.

Quality Assurance

The animal studies of methylene bis(thiocyanate) were performed in compliance with United States Food and Drug Administration Good Laboratory Practices regulations (21 CFR, Part 58). The Quality Assurance Unit of Hazleton Laboratories America, Inc., performed audits and inspections of protocols, procedures, data, and reports throughout the course of the studies.

RESULTS

2-Week Gavage Study in F344/N Rats

All male and female rats in the two highest dose groups (80 and 160 mg/kg) died by Day 2 of the study (Table 2). All males and four females in the 40 mg/kg groups and two males and one female in the 20 mg/kg groups also died before the end of the 2-week study. At the end of the study, the final mean body weights and body weight gains of rats in the 20 mg/kg groups were notably lower than those of the vehicle control groups (Table 2). Additionally, the surviving female rat in the 40 mg/kg group lost weight during the study.

TABLE 2 Survival and Body Weights of F344/N Rats in the 2-Week Gavage Study of Methylene Bis(thiocyanate)

Dose (mg/kg)	Survival[1]	Mean Body Weight (grams)			Final Weight Relative to Controls[2] (%)
		Initial	Final	Change	
MALE					
0	5/5	143	211	68	
10	5/5	144	208	64	99
20	3/5[3]	137	145	8	69
40	0/5[4]	140	—	—	—
80	0/5[5]	140	—	—	—
160	0/5[5]	137	—	—	—
FEMALE					
0	5/5	107	140	33	
10	5/5	107	139	32	99
20	4/5[6]	107	129	22	92
40	1/5[7]	106	95	-11	68
80	0/5[8]	107	—	—	—
160	0/5[5]	107	—	—	—

[1] Number surviving at 2 weeks/number of animals per group. For groups with no survivors, no final mean body weights or body weight changes are given.
[2] (Dose group mean/control group mean) x 100.
[3] Day of death: 4, 7.
[4] Day of death: 1, 1, 2, 7, 8.
[5] All animals died on Day 1.
[6] Day of death: 4.
[7] Day of death: 2, 4, 8, 9.
[8] Day of death: 1, 1, 1, 2, 2.

Clinical signs of toxicity noted after the first day of dosing in male rats in the 20 mg/kg group and male and female rats in the 40 mg/kg groups included dyspnea, hunched posture, ataxia, and prostration.

Significant differences from the control values for absolute and relative organ weights were limited to rats receiving 20 mg/kg methylene bis(thiocyanate), and most of these differences could be attributed to low final mean body weights. In the 20 mg/kg groups, the absolute thymus weights of males and females and the relative thymus weights of males were significantly decreased.

All rats in the 80 and 160 mg/kg groups died by Day 2 of the study and had few gross lesions at necropsy. Two of five females in the 80 mg/kg group had lesions of the glandular stomach; microscopic examination showed that these lesions corresponded to focal superficial mucosal erosion and hemorrhage. Most animals in the 20 and 40 mg/kg groups, including both rats dying early and those surviving to the end of the study, had gross lesions of the glandular and nonglandular stomach (forestomach). Microscopically, the gross lesions corresponded to severe proliferative, necrotizing, and inflammatory changes of the glandular stomach and forestomach.

Proliferative lesions including mild to moderate hyperplasia and hyperkeratosis of the squamous epithelium were observed in the forestomach. Hyperplasia was characterized by uniform thickening of all cell layers of the squamous epithelium, with papillary downgrowths of the basal layer in the more severe cases. Hyperkeratosis consisting of increased thickness of the superficial keratin layer was associated with hyperplastic areas. Hyperplasia and hyperkeratosis were distributed in multifocal areas throughout the forestomach mucosa, alternating with multifocal areas of moderate to marked necrosis and associated inflammation. Necrosis varied from superficial mucosal erosion to large craterlike necrotic areas extending into the submucosa. Inflammatory changes generally correlated in location and severity with necrosis and varied from acute exudation of fluid and neutrophils to chronic fibrovascular proliferation (granulation tissue).

Microscopic lesions of the glandular stomach in rats in the 20 and 40 mg/kg groups consisted of moderate to marked mucosal necrosis, hemorrhage, and inflammation.

As was observed in the forestomach lesions, mucosal necrosis varied from superficial erosion to deep ulceration extending into the submucosa and was accompanied by both acute and chronic inflammatory changes.

In the 40 mg/kg groups, gastric lesions occurred with equal incidence and severity in both the glandular and nonglandular compartments. In rats in the 20 mg/kg groups, the forestomach was the primary target tissue. At the lowest dose of 10 mg/kg, no significant treatment-related lesions of the stomach were identified. Gross lesions were observed in several other organs, primarily in rats that died early, and were usually identified microscopically as congestion. These lesions were not considered directly related to compound administration.

Based on mortality, clinical signs of toxicity, microscopic findings, and decreased body weight gains, the doses selected for the 13-week study in rats were 1, 2, 4, 8, and 16 mg methylene bis(thiocyanate)/kg body weight.

13-Week Gavage Study in F344/N Rats

Five males and four females in the 8 mg/kg groups and five males and six females in the 16 mg/kg (high-dose) groups died before the end of the study (Table 3). In addition, two males each in the 2 and 4 mg/kg groups and one female in the 4 mg/kg group died before the end of the study. The final mean body weights and body weight gains of surviving rats in all dose groups were similar to or greater than those of the vehicle control groups (Table 3 and Figure 1). Clinical signs of toxicity were generally limited to rats in the 4, 8, and 16 mg/kg groups and included abnormal respiratory sounds, chromodacryorrhea, hunched posture, and dyspnea.

TABLE 3 Survival and Body Weights of F344/N Rats in the 13-Week Gavage Study of Methylene Bis(thiocyanate)

Dose (mg/kg)	Survival[1]	Mean Body Weight (grams)			Final Weight Relative to Controls[2] (%)
		Initial	Final	Change	
MALE					
0	10/10	130	318	189	
1	10/10	134	345	211	108
2	8/10[3]	128	335	208	105
4	8/10[4]	126	353	229	111
8	5/10[5]	128	331[6]	206	104
16	5/10[7]	125	340	213	107
FEMALE					
0	10/10	105	196	91	
1	10/10	106	205	99	105
2	10/10	102	195	93	100
4	9/10[8]	106	195	90	100
8	6/10[9]	106	196	93	100
16	4/10[10]	103	188	86	96

[1] Number surviving at 13 weeks/number of animals per group.
[2] (Dose group mean/control group mean) x 100.
[3] Week of death: 2, 9.
[4] Week of death: 4, 10.
[5] Week of death: 1, 4, 7, 9, 13.
[6] n=6; one rat in this group died after final mean body weight was determined.
[7] Week of death: 4, 4, 5, 8, 8.
[8] Week of death: 5.
[9] Week of death: 4, 5, 7, 13.
[10] Week of death: 2, 3, 4, 5, 6, 8.

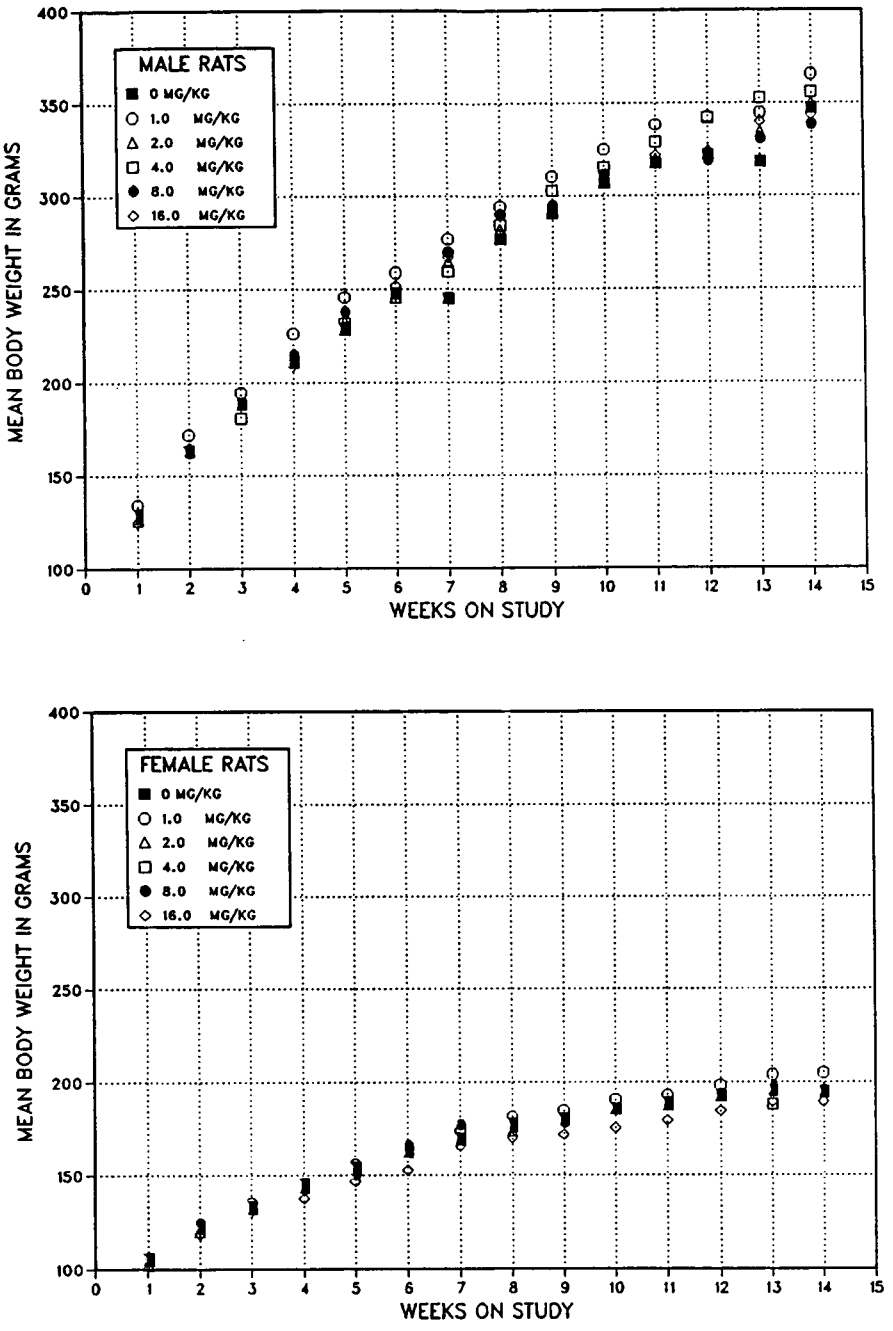


FIGURE 1 Body Weights of F344/N Rats Administered Methylene Bis(thiocyanate) by Gavage for 13 Weeks

In general, significant hematology changes were observed in rats in the 4, 8, and 16 mg/kg groups (Tables 4 and B1). A mild, dose- and time-related anemia occurred in male rats receiving 8 or 16 mg/kg, as evidenced by decreases in erythrocyte count, hemoglobin (Hgb) concentration, and hematocrit (Hct). The anemia was present at Week 1 in the 16 mg/kg group but did not occur until Week 3 in the 8 mg/kg group. Decreased Hct at Week 3 and decreased Hgb concentration at Week 13 indicated a developing anemia in male rats in the 4 mg/kg group. The anemia was weakly responsive at most, with mild increases in reticulocyte counts occurring in male rats in the 16 mg/kg group at Weeks 3 and 13. Considering the mild nature of the anemia, the reticulocyte response may be appropriate. Similar changes indicative of anemia also occurred in female rats in the 8 and 16 mg/kg groups, especially at Week 1. However, the severity of the anemia was less than that in the male rats, and changes did not occur at all time points. In general, the anemias in both the male and female rats were normocytic and normochromic. A dose-related thrombocytosis, evidenced by increased platelet counts, also occurred in male and female rats in the 8 and 16 mg/kg groups at Weeks 1 and 13. The platelet count was also increased in female rats in the 4 mg/kg group at Week 1. Mild increases in leukocyte and segmented neutrophil counts occurred in male and female rats in the 16 mg/kg groups at Week 1. Also at Week 1, the lymphocyte count was increased in male rats the 16 mg/kg group and the number of segmented neutrophils was increased in female rats in the 8 mg/kg group.

Significant changes in clinical chemistry parameters occurred in male and female rats at all time points in the study of methylene bis(thiocyanate) (Table B2). Urea nitrogen (UN) was decreased in female rats in the 16 mg/kg group at Week 1, in females in the 4, 8, and 16 mg/kg groups at Week 3, and in male rats in the 4, 8, and 16 mg/kg groups at Week 13. Decreased UN values have been related to nonrenal causes, and factors such as decreased protein intake, hepatic insufficiency, and anabolic steroid treatment have been reported to decrease UN concentrations. In male rats, total protein and albumin were decreased in the 8 and 16 mg/kg groups at all time points and in the 2 and 4 mg/kg groups at Week 3. The female rats were not as greatly affected as the males; total protein and albumin were decreased only in female rats in the 16 mg/kg group at Week 3, and mild decreases in total protein occurred in the 8 and 16 mg/kg groups at Week 13.

TABLE 4 Selected Hematology Data for F344/N Rats In the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

	Dose (mg/kg)					
	0	1	2	4	8	16
MALE						
n						
Week 1	10	10	9	9	10	10
Week 3	9	8	9	8	8	7
Week 13	10	10	6	7	4	5
Hematocrit (%)						
Week 1	42.6 ± 0.4	42.5 ± 0.2	42.2 ± 0.4	41.9 ± 0.5	42.4 ± 1.0	40.1 ± 0.4**
Week 3	46.7 ± 0.6	45.7 ± 0.6	44.9 ± 0.7	44.8 ± 0.4*	44.3 ± 0.3**	39.8 ± 2.0**
Week 13	46.3 ± 0.4	46.0 ± 0.4	46.5 ± 1.0	45.1 ± 0.3	43.4 ± 0.1**	41.6 ± 1.4**
Hemoglobin (g/dL)						
Week 1	14.5 ± 0.1	14.4 ± 0.1	14.2 ± 0.1	14.2 ± 0.2	14.5 ± 0.3	13.6 ± 0.2**
Week 3	15.7 ± 0.2	15.5 ± 0.2	15.1 ± 0.2	15.2 ± 0.1	14.9 ± 0.1**	13.7 ± 0.4**
Week 13	16.0 ± 0.1	16.0 ± 0.1	16.1 ± 0.4	15.6 ± 0.1*	14.9 ± 0.0**	14.1 ± 0.5**
Erythrocytes (10^6/μL)						
Week 1	7.27 ± 0.09	7.25 ± 0.05	7.16 ± 0.09	7.12 ± 0.07	7.25 ± 0.17	6.84 ± 0.09**
Week 3	8.01 ± 0.10	7.86 ± 0.11	7.71 ± 0.12	7.74 ± 0.10	7.82 ± 0.06	7.10 ± 0.35**
Week 13	9.04 ± 0.06	9.05 ± 0.06	9.16 ± 0.18	8.91 ± 0.07	8.35 ± 0.06**	7.79 ± 0.27**
Reticulocytes (10^6/μL)						
Week 1	0.33 ± 0.04	0.32 ± 0.02	0.32 ± 0.02	0.36 ± 0.03	0.35 ± 0.03	0.36 ± 0.04
Week 3	0.17 ± 0.01	0.19 ± 0.02	0.17 ± 0.02	0.19 ± 0.01	0.19 ± 0.02	0.24 ± 0.02*
Week 13	0.20 ± 0.02	0.19 ± 0.02	0.19 ± 0.02	0.17 ± 0.01	0.28 ± 0.04	0.32 ± 0.03*
Platelets (10^3/μL)						
Week 1	1095.0 ± 20.0	1074.0 ± 50.0	1112.0 ± 19.0	1136.0 ± 26.0	1252.0 ± 31.0**	1323.0 ± 27.0**
Week 3	971.8 ± 23.9	1006.4 ± 26.0	952.6 ± 18.5	976.6 ± 17.7	918.1 ± 38.3	980.0 ± 93.3
Week 13	751.9 ± 40.2	823.8 ± 22.7	705.5 ± 57.5	820.9 ± 18.1	918.3 ± 21.5**	987.4 ± 45.4**
FEMALE						
n						
Week 1	10	10	10	10	10	9
Week 3	9	10	9	9	8	7
Week 13	10	8	10	9	6	4
Hematocrit (%)						
Week 1	42.2 ± 1.1	42.5 ± 0.5	42.4 ± 0.3	42.8 ± 0.7	41.6 ± 1.1*	39.2 ± 0.4**
Week 3	46.5 ± 0.2	46.8 ± 0.4	46.2 ± 0.3	45.8 ± 0.5	45.6 ± 0.5	45.0 ± 0.9
Week 13	44.8 ± 0.8	45.4 ± 0.3	45.4 ± 0.5	44.7 ± 0.7	42.9 ± 1.4	43.2 ± 1.1
Hemoglobin (g/dL)						
Week 1	14.8 ± 0.2	14.8 ± 0.2	14.8 ± 0.1	15.0 ± 0.3	14.4 ± 0.4*	13.8 ± 0.1**
Week 3	16.0 ± 0.1	16.2 ± 0.2	16.0 ± 0.1	16.0 ± 0.2	15.9 ± 0.2	15.6 ± 0.3
Week 13	15.6 ± 0.2	15.5 ± 0.1	15.5 ± 0.2	15.3 ± 0.2	14.6 ± 0.4*	14.6 ± 0.4*
Erythrocytes (10^6/μL)						
Week 1	7.32 ± 0.19	7.35 ± 0.10	7.38 ± 0.06	7.45 ± 0.13	7.21 ± 0.19*	6.82 ± 0.07**
Week 3	7.88 ± 0.05	7.88 ± 0.08	7.85 ± 0.05	7.79 ± 0.10	7.76 ± 0.10	7.77 ± 0.13
Week 13	8.39 ± 0.15	8.48 ± 0.04	8.50 ± 0.10	8.34 ± 0.13	7.97 ± 0.24	7.91 ± 0.21*

TABLE 4 Selected Hematology Data for F344/N Rats in the 13-Week Gavage Study of Methylene Bis(thiocyanate) (continued)

	Dose (mg/kg)					
	0	1	2	4	8	16
FEMALE (continued)						
Reticulocytes (10^6/μL)						
Week 1	0.22 ± 0.02	0.22 ± 0.02	0.21 ± 0.01	0.21 ± 0.02	0.23 ± 0.02	0.23 ± 0.03
Week 3	0.10 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.02	0.09 ± 0.02
Week 13	0.20 ± 0.01	0.21 ± 0.01	0.19 ± 0.01	0.19 ± 0.02	0.22 ± 0.03	0.27 ± 0.02
Platelets (10^3/μL)						
Week 1	959.2 ± 27.6	980.8 ± 23.7	1013.0 ± 33.6	1102.9 ± 20.4**	1146.1 ± 34.5**	1284.8 ± 21.4**
Week 3	964.6 ± 59.0	1007.1 ± 24.3	986.9 ± 20.0	1015.0 ± 19.4	1022.1 ± 19.3	980.0 ± 47.0
Week 13	750.1 ± 47.6	769.8 ± 12.1	671.5 ± 57.6	785.3 ± 22.2	917.2 ± 81.2**	972.3 ± 28.4**

[1] Mean ± standard error. Statistical tests were performed on unrounded data.
* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test.
** Significantly different (P≤0.01) from the control group by Shirley's test.

Treatment-related decreases in alanine aminotransferase (ALT) and alkaline phosphatase (AP) activities occurred in various dose groups at various time points in male and female rats (Table B2). The decreases in AP activities in female rats in the 8 and 16 mg/kg groups were particularly consistent, occurring at all time points. Decreases in serum AP activity have been noted in animals with decreased food intake. However, changes in enzyme production, metabolism (inhibition), structure, or catabolism/excretion, which result in decreased serum enzyme activity, could not be ruled out. Total bile acid concentration was significantly increased in female rats in the 16 mg/kg group at Week 1. This increase suggests that a transient cholestasis occurred in the high-dose female rats. However, the results for bile acid were not supported by a concomitant increase in AP activity, and bile acid concentrations were not increased in male rats.

At the end of the 13-week study of methylene bis(thiocyanate), very mild increases in absolute and relative liver weights were noted in female rats in the 8 and 16 mg/kg groups. In addition, the relative heart and lung weights of high-dose males were slightly but significantly increased. Complete organ weight data for rats are presented in Appendix A, Table A1.

No consistent gross observations were noted at necropsy. In a few animals that died early, fluid within or reddening of the trachea or various abnormalities of the nonglandular

stomach (forestomach) were seen. Microscopic findings related to methylene bis(thiocyanate) exposure were identified in the forestomach and upper respiratory tract of both male and female rats (Table 5).

TABLE 5 Incidence and Severity of Selected Histopathologic Lesions in F344/N Rats in the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

	Dose (mg/kg)					
	0	1	2	4	8	16
MALE						
Forestomach						
Hyperplasia						
early deaths	—	—	0/2	0/2	1/5 (2.0)	4/5 (2.0)
survivors	0/10	0/10	0/8	0/8	0/5	5/5 (1.8)
Hyperkeratosis						
early deaths	—	—	0/2	0/2	1/5 (2.0)	2/5 (3.0)
survivors	0/10	0/10	0/8	0/8	1/5 (1.0)	5/5 (2.4)
Ulceration						
early deaths	—	—	0/2	0/2	0/5	0/5
survivors	0/10	0/10	0/8	0/8	0/5	1/5 (3.0)
Nasal cavity						
Acute exudate						
early deaths	—	—	0/2	1/2 (2.0)	5/5 (2.2)	5/5 (2.0)
survivors	0/10	0/10	0/8	0/8	0/5	0/5
Epithelial necrosis						
early deaths	—	—	0/2	1/2 (3.0)	3/5 (2.3)	4/5 (3.0)
survivors	0/10	0/10	0/8	0/8	0/5	0/5
Trachea						
Acute exudate						
early deaths	—	—	0/2	0/2	3/5 (3.3)	2/5 (3.5)
survivors	0/10	0/10	0/8	0/8	0/5	0/5
Epithelial necrosis						
early deaths	—	—	0/2	0/2	3/5 (3.3)	2/5 (2.5)
survivors	0/10	0/10	0/8	0/8	0/5	0/5
FEMALE						
Forestomach						
Hyperplasia						
early deaths	—	—	—	0/1	3/4 (1.3)	2/6 (2.0)
survivors	0/10	0/10	0/10	1/9 (1.0)	3/6 (1.7)	3/4 (2.0)
Hyperkeratosis						
early deaths	—	—	—	0/1	1/4 (2.0)	2/6 (2.0)
survivors	0/10	0/10	0/10	0/9	2/6 (2.0)	2/4 (2.5)
Ulceration						
early deaths	—	—	—	0/1	0/4	0/6
survivors	0/10	0/10	0/10	0/9	0/6	1/4 (3.0)

TABLE 5 Incidence and Severity of Selected Histopathologic Lesions in F344/N Rats in the 13-Week Gavage Study of Methylene Bis(thiocyanate) (continued)

	Dose (mg/kg)					
	0	1	2	4	8	16
FEMALE (continued)						
Nasal cavity						
Acute exudate						
early deaths	—	—	—	1/1 (2.0)	4/4 (2.8)	5/6 (2.2)
survivors	0/10	1/10 (1.0)	0/10	1/9 (1.0)	2/6 (1.5)	1/4 (2.0)
Epithelial necrosis						
early deaths	—	—	—	0/1	4/4 (3.0)	5/6 (2.8)
survivors	0/10	1/10 (1.0)	0/10	0/9	2/6 (2.0)	0/4
Trachea						
Acute exudate						
early deaths	—	—	—	0/1	1/4 (1.0)	1/6 (4.0)
survivors	0/10	0/10	1/10 (2.0)	0/9	0/6	0/4
Epithelial necrosis						
early deaths	—	—	—	0/1	0/4	1/6 (4.0)
survivors	0/10	0/10	1/10 (2.0)	0/9	0/6	0/4

[1] Average severity (in parentheses) is based on the number of animals with lesions: 1=minimal, 2=mild, 3=moderate, and 4=marked.

Forestomach lesions were present in male and female rats in the two highest dose groups (8 and 16 mg/kg) and occurred in animals that died early as well as in those surviving to the end of the study (Table 5). Hyperplasia and hyperkeratosis of the squamous epithelium were present and were similar to those observed in the 2-week study. In contrast to the findings in the 2-week study, however, hyperplasia and hyperkeratosis were only minimal to mild in severity and were more localized in distribution. The limiting ridge was typically involved, with scattered focal thickenings occurring elsewhere on the mucosal surface. Less frequently associated with the proliferative lesions were minimal to moderate inflammatory cell infiltrates of mixed leukocytes in the submucosa. Ulceration of the forestomach mucosa was observed in only one high-dose male and one high-dose female rat that survived to the end of the study.

Upper respiratory tract lesions consisted of necrotizing inflammation of the trachea and nasal cavity. In a few instances, these lesions were present in rats surviving to the end of the study, but most were observed in animals that died early; in these animals, the lesions were severe and were considered to have contributed to the cause of death. In both the nasal cavity and the trachea, acute purulent to fibrinopurulent exudate of mild to

marked severity was observed in the airway lumen as well as in the mucosa and submucosa, and the exudate always occurred in association with extensive necrosis of the epithelium (Table 5). Inflammation and necrosis were present in all sections of the nasal cavity but were more severe in the posterior sections, suggesting reflux from the nasopharynx. Upper respiratory effects were attributed to direct irritation due to the local contact of methylene bis(thiocyanate) with these tissues during or after the gavage procedure.

Inflammatory lung lesions were observed in both control and treated rats in the 13-week methylene bis(thiocyanate) study. Lung lesions consisted of increased amounts of peribronchial lymphoid tissue and multifocal mononuclear inflammatory cell infiltration in alveolar lumens and around blood vessels of the lung parenchyma. The incidence and severity of lung lesions in treated rats were not increased compared to the controls; therefore, lung changes were not attributed to exposure to methylene bis(thiocyanate).

Sperm morphology and vaginal cytology evaluations were performed on rats in the 0 (vehicle control), 2, 4, and 8 mg/kg groups (Appendix C). The only change which appeared consistent and dose related was a modest decrease in sperm motility in males in the 4 and 8 mg/kg groups. No significant findings were noted in females.

2-Week Gavage Study in B6C3F$_1$ Mice

All mice in the three highest dose groups (40, 80, and 160 mg/kg) died by Day 4 of the 2-week study of methylene bis(thiocyanate) (Table 6). At the end of the study, the final mean body weight and body weight gain of female mice in the 20 mg/kg group were notably lower than those of the vehicle control group. The increased final mean body weight of males in the 10 mg/kg group was not considered toxicologically significant because the final mean body weight of vehicle control males was low (due to the marked weight loss of one mouse).

TABLE 6 Survival and Body Weights of B6C3F$_1$ Mice in the 2-Week Gavage Study of Methylene Bis(thiocyanate)

Dose (mg/kg)	Survival[1]	Mean Body Weight (grams)			Final Weight Relative to Controls[2] (%)
		Initial	Final	Change	
MALE					
0	5/5	22.8	24.1	1.3	
10	5/5	23.0	26.5	3.5	110
20	5/5	22.7	23.8	1.1	99
40	0/5[3]	23.1	—	—	—
80	0/5[4]	22.6	—	—	—
160	0/5[4]	22.9	—	—	—
FEMALE					
0	5/5	19.4	21.9	2.5	
10	5/5	19.0	20.9	1.9	95
20	5/5	19.0	19.6	0.6	89
40	0/5[5]	19.2	—	—	—
80	0/5[4]	19.3	—	—	—
160	0/5[4]	19.2	—	—	—

[1] Number surviving at 2 weeks/number of animals per group. For groups with no survivors, no final mean body weights or body weight changes are given.
[2] (Dose group mean/control group mean) x 100.
[3] Day of death: 1, 2, 2, 4, 4.
[4] All animals died on Day 1.
[5] Day of death: 1, 2, 2, 3, 4.

Generally, clinical signs of toxicity noted after the first day of dosing were limited to male and female mice in the 40 mg/kg groups; findings included ataxia, dyspnea, hunched posture, tremors, low body temperature, and prostration.

At the end of the study, the absolute and relative liver weights of male mice in the 20 mg/kg group, the absolute liver weight of males in the 10 mg/kg group, and the relative liver weight of females in the 20 mg/kg group were significantly increased. In addition, the absolute testis weight of male mice in the 20 mg/kg group, the absolute and relative kidney weights of female mice in the 10 mg/kg group, and the absolute kidney weight of females in the 20 mg/kg group were significantly decreased.

All mice in the two highest dose groups (80 and 160 mg/kg) died on Day 1 of the study, and no significant gross observations were made at necropsy. All mice in the 40 mg/kg groups died by Day 4; most of these mice had various gross lesions of the glandular and nonglandular stomach which corresponded microscopically to acute hemorrhage, inflammation, and necrosis of the squamous and glandular mucosa. These lesions were similar in morphology to those observed in the 2-week rat study. Gross lesions of the stomach were noted less frequently in treated mice that survived to the end of the study. Corresponding microscopic effects in these animals were limited to the nonglandular stomach (forestomach) of mice in the 20 mg/kg groups, primarily males, and consisted of mild to moderate hyperplasia and hyperkeratosis of the squamous epithelium. These forestomach lesions were multifocal to diffuse in distribution and were variably accompanied by focal mucosal ulcerations and inflammatory cell infiltration.

Based on mortality, clinical signs of toxicity, microscopic findings, and organ and body weight differences, the doses selected for the 13-week study in mice were 1, 2, 4, 8, and 16 mg methylene bis(thiocyanate)/kg body weight.

13-Week Gavage Study in B6C3F$_1$ Mice

One male mouse in the 8 mg/kg group and three males and one female in the 16 mg/kg (high-dose) groups died before the end of the 13-week study (Table 7). The final mean body weights and body weight gains of female mice in the 8 mg/kg group and male and female mice in the high-dose groups were lower than those of the vehicle control groups (Table 7 and Figure 2).

Clinical signs of toxicity attributed to methylene bis(thiocyanate) treatment were minimal in the 13-week study in mice. Hypothermia, dyspnea, and hypoactivity were noted in one high-dose male at Week 10, and rales were noted in another high-dose male at Week 13.

TABLE 7 Survival and Body Weights of B6C3F$_1$ Mice in the 13-Week Gavage Study of Methylene Bis(thiocyanate)

Dose (mg/kg)	Survival[1]	Mean Body Weight (grams)			Final Weight Relative to Controls[2] (%)
		Initial	Final	Change	
MALE					
0	10/10	23.5	38.3	14.8	
1	10/10	24.2	38.7	14.6	101
2	10/10	23.8	37.5	13.7	98
4	10/10	24.0	36.8	12.8	96
8	9/10[3]	23.9	37.7	13.8	98
16	7/10[4]	24.0	34.0	10.1	89
FEMALE					
0	10/10	20.1	35.9	15.8	
1	10/10	19.5	36.5	17.0	102
2	10/10	19.4	36.3	16.9	101
4	10/10	19.9	34.7	14.7	97
8	10/10	19.9	32.8	13.0	91
16	9/10[5]	19.6	32.1	12.6	89

[1] Number surviving at 13 weeks/number of animals per group.
[2] (Dose group mean/control group mean) x 100.
[3] Week of death: 9.
[4] Week of death: 9, 10, 12.
[5] Week of death: 3.

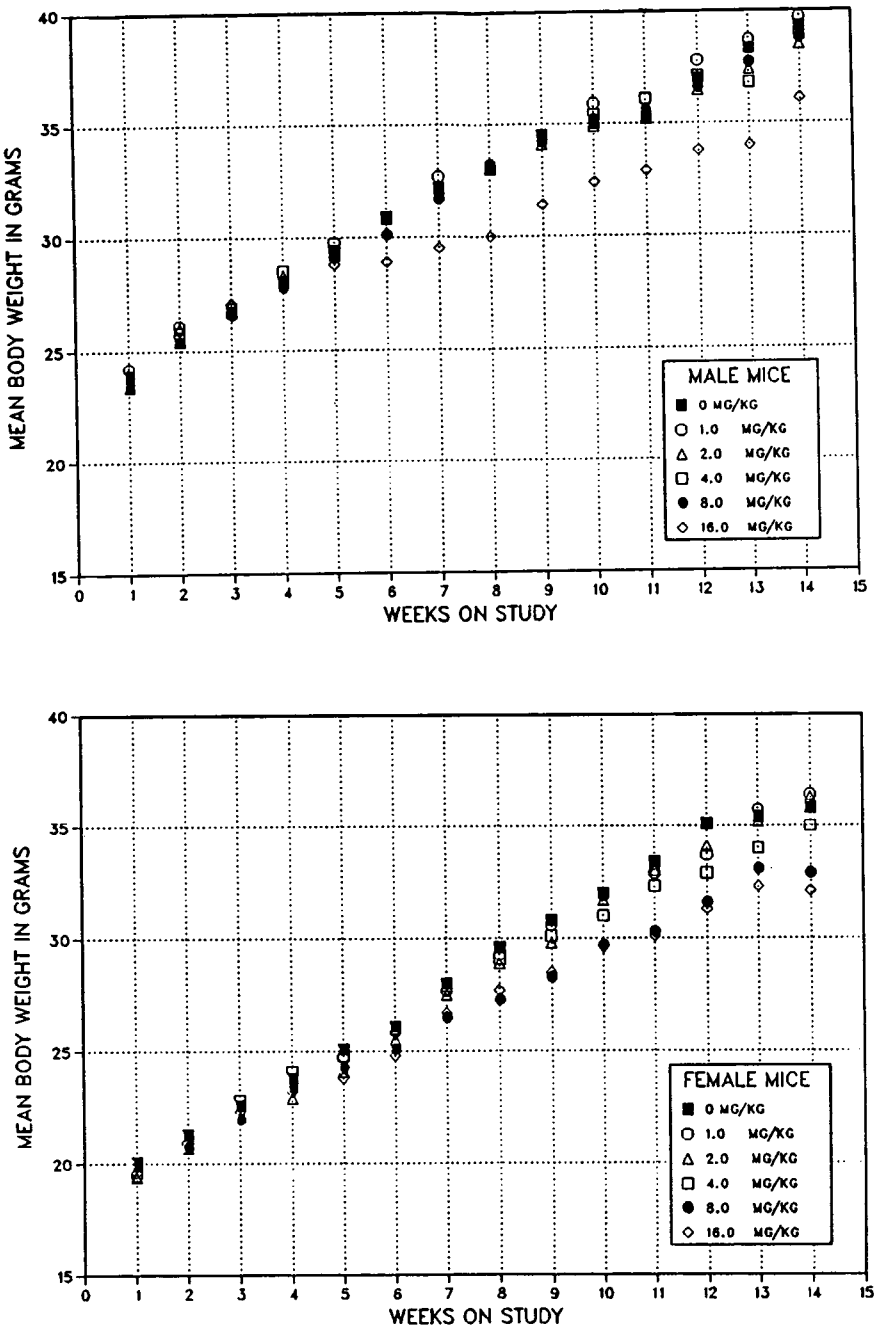


FIGURE 2 Body Weights of B6C3F$_1$ Mice Administered Methylene Bis(thiocyanate) by Gavage for 13 Weeks

The absolute and relative liver weights of mice in all treated groups were slightly increased, and the majority of these increases were significant (Table 8). In addition, the absolute thymus weight of high-dose females was significantly decreased, while the relative kidney weight of high-dose males and the relative heart weight of females in the 8 mg/kg group were significantly increased. There were no histopathologic findings to explain the observed weight changes. Complete organ weight data for mice are presented in Appendix A, Table A2.

TABLE 8 Liver Weights and Liver-Weight-to-Body-Weight Ratios for B6C3F$_1$ Mice in the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

	Dose (mg/kg)					
	0	1	2	4	8	16
MALE						
n	10	10	10	10	9	7
Necropsy body wt	39.3 ± 0.8	39.7 ± 0.8	38.6 ± 0.4	38.9 ± 1.2	38.8 ± 0.6	36.1 ± 1.3
Liver						
Absolute	1.757 ± 0.040	1.830 ± 0.034	1.792 ± 0.031	1.827 ± 0.058	1.936 ± 0.056*	2.009 ± 0.065**
Relative	44.70 ± 0.48	46.11 ± 0.27	46.51 ± 0.78	47.02 ± 0.66	49.90 ± 0.92**	55.81 ± 1.09**
FEMALE						
n	10	10	10	10	10	9
Necropsy body wt	35.9 ± 1.0	36.4 ± 0.7	36.3 ± 0.6	35.0 ± 0.9	32.9 ± 0.5*	32.1 ± 0.5**
Liver						
Absolute	1.529 ± 0.045	1.686 ± 0.047*	1.681 ± 0.036*	1.672 ± 0.038*	1.609 ± 0.033	1.683 ± 0.028*
Relative	42.68 ± 0.61	46.30 ± 0.98*	46.26 ± 0.48*	47.92 ± 1.04**	48.91 ± 0.99**	52.42 ± 0.63**

[1] Liver weights and body weights are given in grams; relative liver weights (liver-weight-to-body-weight ratios) are given as mg liver weight/g body weight (mean ± standard error).
* Significantly different (P≤0.05) from the control group by Dunnett's test.
** Significantly different (P≤0.01) from the control group by Dunnett's test.

No gross lesions that could be attributed to exposure to methylene bis(thiocyanate) were observed at necropsy. Microscopic lesions related to methylene bis(thiocyanate) exposure were present in the forestomach and upper respiratory tract of both male and female mice (Table 9).

TABLE 9 Incidence and Severity of Selected Histopathologic Lesions in B6C3F$_1$ Mice in the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

	Dose (mg/kg)					
	0	1	2	4	8	16
MALE						
Forestomach						
Hyperplasia						
early deaths	—	—	—	—	0/1	2/3 (1.0)
survivors	0/10	0/10	0/10	7/10 (1.0)	9/9 (1.0)	7/7 (1.3)
Hyperkeratosis						
early deaths	—	—	—	—	0/1	2/3 (1.0)
survivors	0/10	0/10	0/10	1/10 (1.0)	0/9	2/7 (1.5)
Nasal cavity						
Acute exudate						
early deaths	—	—	—	—	0/1	2/3 (2.5)
survivors	0/10	0/10	0/10	0/10	0/9	0/7
Epithelial necrosis						
early deaths	—	—	—	—	0/1	1/3 (2.0)
survivors	0/10	0/10	0/10	0/10	0/9	0/7
Trachea						
Acute exudate						
early deaths	—	—	—	—	1/1 (3.0)	0/3
survivors	0/10	0/10	0/10	0/10	0/9	0/7
Epithelial necrosis						
early deaths	—	—	—	0/1	1/1 (4.0)	0/3
survivors	0/10	0/10	1/10 (2.0)	0/9	0/9	0/7
FEMALE						
Forestomach						
Hyperplasia						
early deaths	—	—	—	—	—	1/1 (1.0)
survivors	0/10	0/10	0/10	4/10 (1.0)	10/10 (1.0)	9/9 (1.1)
Hyperkeratosis						
early deaths	—	—	—	—	—	1/1 (2.0)
survivors	0/10	0/10	0/10	1/10 (1.0)	7/10 (1.0)	9/9 (1.6)
Nasal cavity						
Acute exudate						
early deaths	—	—	—	—	—	1/1 (3.0)
survivors	0/10	0/10	0/10	0/10	0/10	0/9
Epithelial necrosis						
early deaths	—	—	—	—	—	1/1 (3.0)
survivors	0/10	0/10	0/10	0/10	0/10	0/9
Trachea						
Acute exudate						
early deaths	—	—	—	—	—	1/1 (1.0)
survivors	0/10	0/10	0/10	0/10	0/10	1/9 (1.0)
Epithelial necrosis						
early deaths	—	—	—	—	—	1/1 (1.0)
survivors	0/10	0/10	0/10	0/10	0/10	1/9 (2.0)

[1] Average severity (in parentheses) is based on the number of animals with lesions: 1=minimal, 2=mild, 3=moderate, and 4=marked.

The forestomach lesions in mice consisted of minimal to mild thickening of all layers of the squamous epithelium (hyperplasia), with an increased amount of keratin on the surface (hyperkeratosis). These forestomach lesions were morphologically similar to but generally less extensive than those in mice in the 2-week study. In most cases, the lesions involved the limiting ridge and surrounding area. These effects were seen in animals that died early and in those surviving to the end of the study (Table 9). For both male and female mice, 2 mg/kg was determined to be the no-observed-adverse-effect level for forestomach lesions.

Upper respiratory tract lesions consisted of nasal or tracheal inflammation and necrosis (Table 9). As in the 13-week rat study, these lesions were found almost exclusively in mice that died early and were considered to have contributed to the cause of death. In both the nasal cavity and the trachea, mild to marked, acute purulent to fibrinopurulent exudate was observed in the airway, mucosa, and submucosa. With the exception of exudate in the nasal cavity of a single high-dose male, the exudate was always associated with extensive necrosis of the epithelium. Inflammation and necrosis of the nasal cavity were more severe in the posterior ventral sections (nasopharyngeal region) but were found in all regions. Upper respiratory effects were attributed to direct irritation due to chemical deposition on these tissues during or after the gavage procedure.

Sperm motility and vaginal cytology evaluations were performed on mice in the 0 (vehicle control), 4, 8, and 16 mg/kg groups. No significant findings were noted for males or females (Appendix C).

Genetic Toxicity Studies

Methylene bis(thiocyanate) (0.01 to 166 µg/plate) was not mutagenic in *Salmonella typhimurium* strain TA97, TA98, TA100, TA1535, or TA1537 when tested using a preincubation protocol with and without S9 metabolic activation (Table D1; Zeiger *et al.*, 1992). Analysis of peripheral blood samples obtained from male and female mice after 13 weeks of dosing with methylene bis(thiocyanate) showed no significant differences in the frequency of micronucleated normochromatic erythrocytes between dosed and control mice (Table D2).

Disposition and Metabolism Studies

Two single-dose studies were conducted in male F344 rats to assess the disposition and metabolism of methylene bis(thiocyanate). For the distribution studies, groups of three rats were administered radiolabeled methylene bis(thiocyanate) at doses of 0.2, 1, or 10 mg/kg. Over 90% of the radioactivity was eliminated in the 48 hours after dosing; the primary route of elimination was urine. The only significant dose-related effect on routes of elimination was decreased elimination in feces at the highest dose. The percentage of the administered dose remaining in the tissues was higher for the 10 mg/kg dose than for the two lower doses. Most of the difference was due to an approximately 10-fold increase in the percentage of the radioactivity remaining in stomach tissue following administration of 10 mg/kg radiolabeled methylene bis(thiocyanate).

Metabolism studies were conducted to determine the effect of a 10 mg/kg dose of methylene bis(thiocyanate) on blood cyanide and thiocyanate concentrations. An increase in blood cyanide was observed at 30 minutes and 1 hour after dosing, but the concentration was similar to the control value at the 2-hour time point and thereafter. An increase in blood thiocyanate was also observed, with the peak concentration occurring approximately 2 hours after methylene bis(thiocyanate) administration. These studies are presented in Appendix E.

DISCUSSION

In the 2-week studies, all animals administered 80 or 160 mg/kg methylene bis(thiocyanate) died by the second day of dosing. These results confirm the LD_{50} reported for rats by Wehner and Hinz (1971) and indicate that the acute LD_{50} for mice should be approximately the same as that for rats. The current study design was based on a reported oral LD_{50} of 161 mg/kg for rats (NIOSH, 1986). This value has apparently been deleted, as it does not appear in more recent searches of RTECS.

In the 2-week studies, few significant gross lesions were observed in the 80 and 160 mg/kg groups. Clinical observations were similar to those reported for cyanide toxicity (Egekeze and Oehme, 1979). In the cyanide metabolite study presented in this report, administration of 10 mg/kg methylene bis(thiocyanate) resulted in the concentration of approximately 0.3 µg cyanide/mL blood 30 minutes after dosing (Appendix E). Because the minimum lethal blood cyanide concentration has been reported to be 2.6 to 2.9 µg/mL (Egekeze and Oehme, 1979), it is not unreasonable to expect lethal cyanide concentrations in the 80 and 160 mg/kg groups. The stomach, identified as the target organ in rats and mice surviving 24 hours, had necrotic inflammatory lesions of the mucosal surface of both the glandular and nonglandular compartments. A proliferative response of the squamous epithelium of the forestomach (hyperplasia and hyperkeratosis) was also present in these animals. These effects were attributed to the direct toxicity of methylene bis(thiocyanate) to gastric mucosal cells.

In the 13-week studies, methylene bis(thiocyanate) administration resulted in deaths in male rats in the 2, 4, 8, and 16 mg/kg groups and in female rats in the 4, 8, and 16 mg/kg groups. Mortality in mice was somewhat less, with deaths in males in the 8 and 16 mg/kg groups and in only one female in the 16 mg/kg group. As was the case in the 2-week studies, the stomach was identified as a target organ. However, in contrast to the necrotic and proliferative effects on both the glandular and nonglandular stomach in the 2-week studies, the lower doses administered in the 13-week studies resulted in gastric effects limited to the forestomach and consisting primarily of squamous mucosal hyperplasia and hyperkeratosis. Another treatment-related effect observed in the 13-week studies with both rats and mice was necrotizing inflammation of the nasal cavity and trachea. This lesion was seen almost exclusively in animals that died early and was considered to have contributed to death by upper respiratory obstruction. Reflux of gastric

contents into the pharynx, perhaps secondary to forestomach lesions, or accidental pharyngeal deposition of methylene bis(thiocyanate) during the gavage procedure is presumed to be the cause of the upper respiratory lesions, as suggested by their localization in the upper trachea and the more posterior nasal sections.

Inflammatory lung lesions unrelated to methylene bis(thiocyanate) exposure were present in control and treated rats from the 13-week study. Morphologically identical lesions of undetermined etiology have been noted in other toxicity studies (NTP, 1992); an infectious etiology is suspected, although routine serologic tests for rodent respiratory pathogens have been negative.

Hematology results indicated a normocytic, normochromic, poorly responsive to nonresponsive anemia in both male and female rats. Anemias of this type may result from a mild bone marrow suppression. In such cases, anemias develop from nutrient deficiency or poor utilization of nutrients, from decreased bone marrow stimulation, or from structural or functional bone marrow damage. There was no obvious nutritional deficiency in this study, at least insofar as weight gain is a measure of nutritional sufficiency, or other obvious explanation for the observed anemia. The dose-related thrombocytosis (increased platelet counts) seen at Weeks 1 and 13, but not at Week 3, in the two highest dose groups of male and female rats is also difficult to explain. Two possible causes of increased platelet counts are a physiologic response (*e.g.*, to exercise, epinephrine), and rebound thrombocytosis following recovery from a thrombocytopenia, bone marrow suppression, or both.

Clinical chemistry results indicated a decrease in total protein and albumin in male rats in the two highest dose groups at all time points and in lower dose groups at Week 3. Decreased albumin concentration, often resulting in a concomitant decrease in total protein, is a common dysproteinemia. Albumin can be lost in protein-losing nephropathies or enteropathies, and because albumin is synthesized in the liver, hypoalbuminemia is a common finding with chronic hepatic disease. There was no histopathologic evidence of nephropathy or hepatic disease, however.

Methylene bis(thiocyanate) appears to have at least two modes of toxicity. At high doses, the release of cyanide may be more rapid than the conversion of cyanide to thiocyanate by rhodanese; thus, cyanide toxicity is observed. Methylene bis(thiocyanate) is also a reactive

chemical; toxicity (irritation) has been observed at the site of application in dermal sensitization studies (Burrows and Campbell, 1980; Anderson and Veien, 1985). Likewise, the stomach, the site of application in the current studies, was the primary target organ.

In a study of several organothiocyanates, Ohkawa *et al.* (1972) found that cyanide release was associated with the reaction of the organothiocyanate with glutathione (GSH). The rate of cyanide release was increased by the presence of GSH transferases, but some cyanide release was measured with GSH alone. The reaction can be illustrated as follows:

$$RCH_2SCN + GSH \rightarrow RCH_2SSG + HCN$$

$$RCH_2SSG + GSH \rightarrow RCH_2SH + GSSG$$

The stoichiometry of the reaction is such that 1 mole of organothiocyanate reacts with 2 moles of GSH and releases 1 mole of cyanide. For methylene bis(thiocyanate), the group represented by R in the reaction scheme is SCH; thus, the reaction could occur on both sides of the molecule, with the result that 4 moles of GSH react with 1 mole of methylene bis(thiocyanate) and release 2 moles of HCN. By this argument, it would appear that methylene bis(thiocyanate) could rapidly deplete tissue GSH, and this depletion may be part of the toxicity of methylene bis(thiocyanate) not attributable to HCN. Similar reactions could occur with nucleophiles other than GSH (*e.g.*, in proteins), leading to toxic effects. Additionally, GSH depletion could lead to even greater reaction with tissue nucleophiles. Some evidence of this reaction can be found in the disposition study summarized in Appendix E. As the dose was increased from 0.2 to 10 mg/kg, an increasing percentage of the dose remained in the animals after 48 hours. This trend was seen in most tissues but was especially remarkable in the stomach, where a greater than 10-fold increase in the *percentage* of the administered dose remained. This indicates that there was a greater than 100-fold increase in the *amount* of material derived from methylene bis(thiocyanate) in the stomach following a 10 mg/kg dose compared to a 1 mg/kg dose. (The stomach contents accounted for very little radioactivity.) GSH depletion and increased reaction with tissue nucleophiles would be consistent with these results. It is not known whether the remaining radioactivity is covalently bound or extractable.

In summary, gavage doses of 80 mg/kg methylene bis(thiocyanate) or greater were acutely lethal, possibly due to cyanide release from the thiocyanate. Deaths also occurred at lower doses for which the stomach and upper respiratory tract were target organs. This toxicity may be due to the reactivity of methylene bis(thiocyanate). The no-observed-adverse-effect level for forestomach lesions was 4 mg/kg for male rats and 2 mg/kg for female rats and male and female mice. The only reproductive effect observed was decreased sperm motility in male rats in the 8 mg/kg group. Methylene bis(thiocyanate) was not genotoxic.

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APPENDIX A

Organ Weights and Organ-Weight-to-Body-Weight Ratios

TABLE A1 Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

	Vehicle Control	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg
MALE						
n	10	10	8	8	5	5
Necropsy body wt	347 ± 7	365 ± 9	350 ± 8	356 ± 8	339 ± 10	344 ± 9
Heart						
Absolute	0.983 ± 0.024	1.072 ± 0.027*	1.009 ± 0.016	1.039 ± 0.030	0.984 ± 0.028	1.084 ± 0.037
Relative	2.83 ± 0.03	2.94 ± 0.05	2.88 ± 0.02	2.92 ± 0.06	2.91 ± 0.07	3.15 ± 0.05**
Right kidney						
Absolute	1.180 ± 0.032	1.224 ± 0.033	1.146 ± 0.029	1.193 ± 0.033	1.162 ± 0.064	1.218 ± 0.032
Relative	3.40 ± 0.06	3.35 ± 0.04	3.28 ± 0.05	3.35 ± 0.06	3.42 ± 0.11	3.54 ± 0.08
Liver						
Absolute	12.293 ± 0.347	13.078 ± 0.401	12.260 ± 0.427	12.950 ± 0.306	12.820 ± 0.766	12.802 ± 0.583
Relative	35.41 ± 0.73	35.75 ± 0.38	34.98 ± 0.65	36.41 ± 0.53	37.74 ± 1.21	37.21 ± 1.34
Lungs						
Absolute	1.376 ± 0.059[2]	1.696 ± 0.065*	1.624 ± 0.094	1.651 ± 0.116	1.528 ± 0.054	1.700 ± 0.138
Relative	3.95 ± 0.11[2]	4.65 ± 0.16	4.65 ± 0.27	4.62 ± 0.27	4.53 ± 0.22	4.93 ± 0.32*
Right testis						
Absolute	1.387 ± 0.034	1.418 ± 0.023[2]	1.428 ± 0.035	1.446 ± 0.022[3]	1.392 ± 0.044	1.470 ± 0.042[4]
Relative	4.00 ± 0.07	3.84 ± 0.07[2]	4.08 ± 0.04	4.09 ± 0.12[3]	4.12 ± 0.15	4.28 ± 0.12[4]
Thymus						
Absolute	0.301 ± 0.013	0.364 ± 0.021*	0.345 ± 0.013	0.332 ± 0.015	0.274 ± 0.009	0.347 ± 0.016
Relative	0.87 ± 0.04	1.00 ± 0.06	0.98 ± 0.03	0.94 ± 0.05	0.81 ± 0.05	1.01 ± 0.03
FEMALE						
n	10	10	10	9	6	4
Necropsy body wt	195 ± 4	205 ± 3	195 ± 2	194 ± 5	196 ± 6	189 ± 9
Heart						
Absolute	0.663 ± 0.016	0.665 ± 0.014	0.682 ± 0.012	0.681 ± 0.014	0.683 ± 0.022	0.640 ± 0.037
Relative	3.40 ± 0.05	3.24 ± 0.04	3.51 ± 0.07	3.52 ± 0.06	3.50 ± 0.11	3.38 ± 0.08
Right kidney						
Absolute	0.673 ± 0.022	0.698 ± 0.012	0.670 ± 0.015	0.680 ± 0.020	0.707 ± 0.013	0.668 ± 0.028
Relative	3.45 ± 0.07	3.40 ± 0.03	3.44 ± 0.06	3.51 ± 0.11	3.63 ± 0.12	3.53 ± 0.05
Liver						
Absolute	6.211 ± 0.223	6.666 ± 0.155	6.416 ± 0.097	6.059 ± 0.193	7.203 ± 0.404*	7.103 ± 0.265
Relative	31.81 ± 0.65	32.53 ± 0.65	33.00 ± 0.48	31.23 ± 0.66	36.89 ± 1.99**	37.59 ± 1.08**
Lungs						
Absolute	1.049 ± 0.025	1.063 ± 0.020	1.107 ± 0.035	1.052 ± 0.030	1.025 ± 0.036	1.045 ± 0.050
Relative	5.39 ± 0.09	5.19 ± 0.07	5.70 ± 0.19	5.42 ± 0.07	5.26 ± 0.22	5.52 ± 0.14
Thymus						
Absolute	0.253 ± 0.008	0.251 ± 0.010	0.238 ± 0.005[2]	0.236 ± 0.008	0.234 ± 0.033	0.234 ± 0.020
Relative	1.30 ± 0.04	1.22 ± 0.04	1.23 ± 0.03[2]	1.22 ± 0.05	1.18 ± 0.15	1.23 ± 0.05

[1] Organ weights and body weights are given in grams; relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight (mean ± standard error).
[2] n=9.
[3] n=7.
[4] n=3.
* Significantly different (P≤0.05) from the control group by Dunnett's test.
** Significantly different (P≤0.01) from the control group by Dunnett's test.

TABLE A2 Organ Weights and Organ-Weight-to-Body-Weight Ratios for B6C3F$_1$ Mice in the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

	Vehicle Control	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg
MALE						
n	10	10	10	10	9	7
Necropsy body wt	39.3 ± 0.8	39.7 ± 0.8	38.6 ± 0.4	38.9 ± 1.2	38.8 ± 0.6	36.1 ± 1.3
Heart						
Absolute	0.175 ± 0.009	0.174 ± 0.005	0.175 ± 0.007	0.184 ± 0.011	0.192 ± 0.008	0.171 ± 0.014
Relative	4.46 ± 0.24	4.40 ± 0.15	4.54 ± 0.19	4.73 ± 0.23	4.96 ± 0.19	4.78 ± 0.40
Right kidney						
Absolute	0.316 ± 0.011	0.334 ± 0.007	0.320 ± 0.005	0.313 ± 0.010	0.314 ± 0.006	0.331 ± 0.011
Relative	8.03 ± 0.17	8.42 ± 0.12	8.31 ± 0.13	8.07 ± 0.18	8.12 ± 0.13	9.21 ± 0.21**
Liver						
Absolute	1.757 ± 0.040	1.830 ± 0.034	1.792 ± 0.031	1.827 ± 0.058	1.936 ± 0.056*	2.009 ± 0.065**
Relative	44.70 ± 0.48	46.11 ± 0.27	46.51 ± 0.78	47.02 ± 0.66	49.90 ± 0.92**	55.81 ± 1.09**
Lungs						
Absolute	0.246 ± 0.020	0.221 ± 0.016	0.222 ± 0.015	0.285 ± 0.018	0.283 ± 0.007	0.214 ± 0.018
Relative	6.26 ± 0.49	5.61 ± 0.45	5.78 ± 0.41	7.38 ± 0.52	7.31 ± 0.17	5.98 ± 0.50
Right testis						
Absolute	0.124 ± 0.002	0.126 ± 0.003	0.129 ± 0.002	0.125 ± 0.002	0.123 ± 0.002	0.122 ± 0.003
Relative	3.17 ± 0.07	3.18 ± 0.07	3.34 ± 0.04	3.23 ± 0.07	3.19 ± 0.07	3.39 ± 0.08
Thymus						
Absolute	0.055 ± 0.003	0.057 ± 0.004	0.050 ± 0.002	0.054 ± 0.005	0.056 ± 0.002	0.044 ± 0.001
Relative	1.40 ± 0.07	1.43 ± 0.09	1.31 ± 0.04	1.37 ± 0.09	1.44 ± 0.05	1.23 ± 0.07
FEMALE						
n	10	10	10	10	10	9
Necropsy body wt	35.9 ± 1.0	36.4 ± 0.7	36.3 ± 0.6	35.0 ± 0.9	32.9 ± 0.5*	32.1 ± 0.5**
Heart						
Absolute	0.134 ± 0.003	0.145 ± 0.002	0.137 ± 0.002	0.137 ± 0.002	0.141 ± 0.005	0.132 ± 0.002
Relative	3.75 ± 0.09	3.99 ± 0.09	3.78 ± 0.07	3.94 ± 0.12	4.28 ± 0.15**	4.12 ± 0.10
Right kidney						
Absolute	0.228 ± 0.005	0.238 ± 0.008	0.230 ± 0.003	0.233 ± 0.006	0.225 ± 0.004	0.220 ± 0.004
Relative	6.38 ± 0.12	6.53 ± 0.17	6.34 ± 0.11	6.70 ± 0.23	6.84 ± 0.12	6.85 ± 0.10
Liver						
Absolute	1.529 ± 0.045	1.686 ± 0.047*	1.681 ± 0.036*	1.672 ± 0.038*	1.609 ± 0.033	1.683 ± 0.028*
Relative	42.68 ± 0.61	46.30 ± 0.98*	46.26 ± 0.48*	47.92 ± 1.04**	48.91 ± 0.99**	52.42 ± 0.63**
Lungs						
Absolute	0.202 ± 0.010	0.204 ± 0.009	0.194 ± 0.008	0.199 ± 0.009	0.211 ± 0.015	0.179 ± 0.005
Relative	5.64 ± 0.23	5.63 ± 0.29	5.35 ± 0.23	5.73 ± 0.30	6.38 ± 0.38	5.58 ± 0.16
Thymus						
Absolute	0.060 ± 0.003	0.066 ± 0.003	0.068 ± 0.003	0.063 ± 0.003	0.060 ± 0.002	0.050 ± 0.003*
Relative	1.68 ± 0.08	1.80 ± 0.05	1.88 ± 0.08	1.79 ± 0.07	1.81 ± 0.06	1.55 ± 0.09

[1] Organ weights and body weights are given in grams; relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight (mean ± standard error).

* Significantly different (P≤0.05) from the control group by Dunnett's test.

** Significantly different (P≤0.01) from the control group by Dunnett's test.

APPENDIX B

Hematology and Clinical Chemistry Results

TABLE B1 Hematology Data for F344/N Rats in the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

	Vehicle Control	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg
MALE						
n						
Week 1	10	10	9	9	10	10
Week 3	9	8	9	8	8	7
Week 13	10	10	6	7	4	5
Hematocrit (%)						
Week 1	42.6 ± 0.4	42.5 ± 0.2	42.2 ± 0.4	41.9 ± 0.5	42.4 ± 1.0	40.1 ± 0.4**
Week 3	46.7 ± 0.6	45.7 ± 0.6	44.9 ± 0.7	44.8 ± 0.4*	44.3 ± 0.3**	39.8 ± 2.0**
Week 13	46.3 ± 0.4	46.0 ± 0.4	46.5 ± 1.0	45.1 ± 0.3	43.4 ± 0.1**	41.6 ± 1.4**
Hemoglobin (g/dL)						
Week 1	14.5 ± 0.1	14.4 ± 0.1	14.2 ± 0.1	14.2 ± 0.2	14.5 ± 0.3	13.6 ± 0.2**
Week 3	15.7 ± 0.2	15.5 ± 0.2	15.1 ± 0.2	15.2 ± 0.1	14.9 ± 0.1**	13.7 ± 0.4**
Week 13	16.0 ± 0.1	16.0 ± 0.1	16.1 ± 0.4	15.6 ± 0.1*	14.9 ± 0.0**	14.1 ± 0.5**
Erythrocytes (10^6/μL)						
Week 1	7.27 ± 0.09	7.25 ± 0.05	7.16 ± 0.09	7.12 ± 0.07	7.25 ± 0.17	6.84 ± 0.09**
Week 3	8.01 ± 0.10	7.86 ± 0.11	7.71 ± 0.12	7.74 ± 0.10	7.82 ± 0.06	7.10 ± 0.35**
Week 13	9.04 ± 0.06	9.05 ± 0.06	9.16 ± 0.18	8.91 ± 0.07	8.35 ± 0.06**	7.79 ± 0.27**
Reticulocytes (10^6/μL)						
Week 1	0.33 ± 0.04	0.32 ± 0.02	0.32 ± 0.02	0.36 ± 0.03	0.35 ± 0.03	0.36 ± 0.04
Week 3	0.17 ± 0.01	0.19 ± 0.02	0.17 ± 0.02	0.19 ± 0.01	0.19 ± 0.02	0.24 ± 0.02*
Week 13	0.20 ± 0.02	0.19 ± 0.02	0.19 ± 0.02	0.17 ± 0.01	0.28 ± 0.04	0.32 ± 0.03*
Nucleated erythrocytes (10^3/μL)						
Week 1	0.10 ± 0.03	0.13 ± 0.05	0.08 ± 0.03	0.08 ± 0.03	0.14 ± 0.03	0.08 ± 0.05
Week 3	0.00 ± 0.00	0.02 ± 0.02	0.01 ± 0.01	0.05 ± 0.03	0.01 ± 0.01	0.03 ± 0.02
Week 13	0.01 ± 0.01	0.02 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.05
Mean cell volume (fL)						
Week 1	58.6 ± 0.3	58.7 ± 0.3	58.9 ± 0.4	58.9 ± 0.2	58.5 ± 0.2	58.6 ± 0.3
Week 3	58.2 ± 0.3	58.1 ± 0.3	58.2 ± 0.4	58.0 ± 0.3	56.7 ± 0.6*	56.1 ± 0.4**
Week 13	51.2 ± 0.2	50.9 ± 0.1	50.8 ± 0.2	50.6 ± 0.2	52.0 ± 0.5	53.5 ± 0.2
Mean cell hemoglobin (pg)						
Week 1	19.9 ± 0.1	19.9 ± 0.1	19.9 ± 0.1	19.9 ± 0.2	20.0 ± 0.1	19.8 ± 0.1
Week 3	19.6 ± 0.1	19.7 ± 0.1	19.6 ± 0.2	19.7 ± 0.1	19.1 ± 0.2	19.6 ± 0.9
Week 13	17.7 ± 0.1	17.6 ± 0.1	17.6 ± 0.1	17.4 ± 0.1	17.9 ± 0.2	18.2 ± 0.0*
Mean cell hemoglobin concentration (g/dL)						
Week 1	34.0 ± 0.1	34.0 ± 0.1	33.7 ± 0.2	33.8 ± 0.3	34.2 ± 0.2	33.9 ± 0.2
Week 3	33.6 ± 0.1	33.9 ± 0.1	33.6 ± 0.1	34.0 ± 0.1	33.7 ± 0.2	34.9 ± 1.5
Week 13	34.5 ± 0.1	34.7 ± 0.1	34.7 ± 0.1	34.5 ± 0.1	34.3 ± 0.1	33.9 ± 0.2*
Platelets (10^3/μL)						
Week 1	1095.0 ± 20.0	1074.0 ± 50.0	1112.0 ± 19.0	1136.0 ± 26.0	1252.0 ± 31.0**	1323.0 ± 27.0**
Week 3	971.8 ± 23.9	1006.4 ± 26.0	952.6 ± 18.5	976.6 ± 17.7	918.1 ± 38.3	980.0 ± 93.3
Week 13	751.9 ± 40.2	823.8 ± 22.7	705.5 ± 57.5	820.9 ± 18.1	918.3 ± 21.5**	987.4 ± 45.4**
Leukocytes (10^3/μL)						
Week 1	9.20 ± 0.47	10.34 ± 0.59	9.29 ± 0.50	10.31 ± 0.34	10.08 ± 0.48	11.03 ± 0.32**
Week 3	7.68 ± 0.52	8.45 ± 0.94	6.80 ± 0.46	9.55 ± 0.45	8.95 ± 0.81	7.07 ± 0.65
Week 13	10.01 ± 0.70	11.00 ± 0.41	11.20 ± 1.09	11.36 ± 0.70	11.13 ± 0.69	12.12 ± 1.20
Segmented neutrophils (10^3/μL)						
Week 1	0.84 ± 0.06	1.65 ± 0.19**	0.97 ± 0.11	1.06 ± 0.12	1.09 ± 0.11	1.35 ± 0.10**
Week 3	0.73 ± 0.10	0.98 ± 0.14	0.74 ± 0.08	1.10 ± 0.16	1.08 ± 0.22	0.94 ± 0.11
Week 13	1.70 ± 0.24	1.82 ± 0.12	1.40 ± 0.54	1.73 ± 0.25	2.13 ± 0.39	2.12 ± 0.33

TABLE B1 Hematology Data for F344/N Rats in the 13-Week Gavage Study of Methylene Bis(thiocyanate) (continued)

	Vehicle Control	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg
MALE (continued)						
Lymphocytes (10^3/μL)						
Week 1	8.26 ± 0.45	8.62 ± 0.50	8.16 ± 0.50	9.13 ± 0.29	8.84 ± 0.53	9.51 ± 0.28*
Week 3	6.76 ± 0.48	7.28 ± 0.80	5.92 ± 0.44	8.15 ± 0.32	7.56 ± 0.68	5.89 ± 0.63
Week 13	8.13 ± 0.52	9.02 ± 0.36	9.65 ± 0.58*	9.49 ± 0.61	8.90 ± 1.01	9.78 ± 0.85
Monocytes (10^3/μL)						
Week 1	0.09 ± 0.03	0.03 ± 0.02	0.16 ± 0.04	0.08 ± 0.05	0.08 ± 0.03	0.16 ± 0.05
Week 3	0.13 ± 0.03	0.19 ± 0.06	0.13 ± 0.05	0.20 ± 0.05	0.26 ± 0.05	0.21 ± 0.10
Week 13	0.05 ± 0.03	0.11 ± 0.03	0.08 ± 0.04	0.09 ± 0.06	0.08 ± 0.05	0.14 ± 0.06
Eosinophils (10^3/μL)						
Week 1	0.02 ± 0.01	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.05 ± 0.02	0.00 ± 0.00
Week 3	0.03 ± 0.02	0.01 ± 0.01	0.02 ± 0.02	0.10 ± 0.03	0.05 ± 0.02	0.03 ± 0.02
Week 13	0.12 ± 0.04	0.04 ± 0.02	0.08 ± 0.04	0.04 ± 0.02	0.03 ± 0.03	0.04 ± 0.04
FEMALE						
n						
Week 1	10	10	10	10	10	9
Week 3	9	10	9	9	8	7
Week 13	10	8	10	9	6	4
Hematocrit (%)						
Week 1	42.2 ± 1.1	42.5 ± 0.5	42.4 ± 0.3	42.8 ± 0.7	41.6 ± 1.1*	39.2 ± 0.4**
Week 3	46.5 ± 0.2	46.8 ± 0.4	46.2 ± 0.3	45.8 ± 0.5	45.6 ± 0.5	45.0 ± 0.9
Week 13	44.8 ± 0.8	45.4 ± 0.3	45.4 ± 0.5	44.7 ± 0.7	42.9 ± 1.4	43.2 ± 1.1
Hemoglobin (g/dL)						
Week 1	14.8 ± 0.2	14.8 ± 0.2	14.8 ± 0.1	15.0 ± 0.3	14.4 ± 0.4*	13.8 ± 0.1**
Week 3	16.0 ± 0.1	16.2 ± 0.2	16.0 ± 0.1	16.0 ± 0.2	15.9 ± 0.2	15.6 ± 0.3
Week 13	15.6 ± 0.2	15.5 ± 0.1	15.5 ± 0.2	15.3 ± 0.2	14.6 ± 0.4*	14.6 ± 0.4*
Erythrocytes (10^6/μL)						
Week 1	7.32 ± 0.19	7.35 ± 0.10	7.38 ± 0.06	7.45 ± 0.13	7.21 ± 0.19*	6.82 ± 0.07**
Week 3	7.88 ± 0.05	7.88 ± 0.08	7.85 ± 0.05	7.79 ± 0.10	7.76 ± 0.10	7.77 ± 0.13
Week 13	8.39 ± 0.15	8.48 ± 0.04	8.50 ± 0.10	8.34 ± 0.13	7.97 ± 0.24	7.91 ± 0.21*
Reticulocytes (10^6/μL)						
Week 1	0.22 ± 0.02	0.22 ± 0.02	0.21 ± 0.01	0.21 ± 0.02	0.23 ± 0.02	0.23 ± 0.03
Week 3	0.10 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.02	0.09 ± 0.02
Week 13	0.20 ± 0.01	0.21 ± 0.01	0.19 ± 0.01	0.19 ± 0.02	0.22 ± 0.03	0.27 ± 0.02
Nucleated erythrocytes (10^3/μL)						
Week 1	0.03 ± 0.02	0.01 ± 0.01	0.09 ± 0.05	0.01 ± 0.01	0.05 ± 0.03	0.03 ± 0.02
Week 3	0.00 ± 0.00	0.04 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00[2]	0.02 ± 0.02
Week 13	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.05 ± 0.03**
Mean cell volume (fL)						
Week 1	57.6 ± 0.3	57.8 ± 0.1	57.5 ± 0.2	57.4 ± 0.2	57.7 ± 0.2	57.6 ± 0.2
Week 3	59.0 ± 0.4	59.4 ± 0.3	58.9 ± 0.3	58.8 ± 0.3	58.8 ± 0.3	57.8 ± 0.5
Week 13	53.4 ± 0.1	53.6 ± 0.1	53.4 ± 0.1	53.6 ± 0.1	53.8 ± 0.2*	54.7 ± 0.8*
Mean cell hemoglobin (pg)						
Week 1	20.3 ± 0.3	20.1 ± 0.1	20.1 ± 0.1	20.2 ± 0.1	19.9 ± 0.1	20.3 ± 0.2
Week 3	20.4 ± 0.1	20.6 ± 0.1	20.4 ± 0.2	20.5 ± 0.1	20.4 ± 0.2	20.0 ± 0.2
Week 13	18.6 ± 0.4	18.3 ± 0.1	18.2 ± 0.1	18.3 ± 0.1	18.3 ± 0.1	18.5 ± 0.1

TABLE B1 Hematology Data for F344/N Rats in the 13-Week Gavage Study of Methylene Bis(thiocyanate) (continued)

	Vehicle Control	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg
FEMALE (continued)						
Mean cell hemoglobin concentration (g/dL)						
Week 1	35.3 ± 0.5	34.8 ± 0.1	34.9 ± 0.1	35.1 ± 0.2	34.6 ± 0.1	35.3 ± 0.3
Week 3	34.5 ± 0.1	34.6 ± 0.1	34.7 ± 0.1	34.8 ± 0.1	34.8 ± 0.2	34.6 ± 0.2
Week 13	34.9 ± 0.8	34.1 ± 0.2	34.1 ± 0.1	34.1 ± 0.1	34.1 ± 0.1	33.8 ± 0.3
Platelets (10^3/μL)						
Week 1	959.2 ± 27.6	980.8 ± 23.7	1013.0 ± 33.6	1102.9 ± 20.4**	1146.1 ± 34.5**	1284.8 ± 21.4**
Week 3	964.6 ± 59.0	1007.1 ± 24.3	986.9 ± 20.0	1015.0 ± 19.4	1022.1 ± 19.3	980.0 ± 47.0
Week 13	750.1 ± 47.6	769.8 ± 12.1	671.5 ± 57.6	785.3 ± 22.2	917.2 ± 81.2**	972.3 ± 28.4**
Leukocytes (10^3/μL)						
Week 1	9.64 ± 0.32	9.78 ± 0.38	9.84 ± 0.24	11.15 ± 0.62	10.29 ± 0.39	11.57 ± 0.56*
Week 3	10.19 ± 0.43	10.57 ± 0.48	10.33 ± 0.40	10.76 ± 0.38	10.43 ± 0.80	11.76 ± 1.49
Week 13	10.39 ± 0.67	8.69 ± 0.30	10.44 ± 0.66	9.30 ± 0.25	9.27 ± 0.95	9.58 ± 0.33
Segmented neutrophils (10^3/μL)						
Week 1	0.63 ± 0.07	0.70 ± 0.08	0.84 ± 0.11	0.86 ± 0.10	1.45 ± 0.54**	0.89 ± 0.13*
Week 3	0.83 ± 0.09	0.88 ± 0.11	0.80 ± 0.10	0.81 ± 0.15	0.84 ± 0.10	2.59 ± 1.04
Week 13	2.21 ± 0.62	1.24 ± 0.16	1.74 ± 0.17	1.66 ± 0.23	2.02 ± 0.52	1.55 ± 0.19
Lymphocytes (10^3/μL)						
Week 1	8.78 ± 0.34	9.02 ± 0.41	8.84 ± 0.25	10.17 ± 0.52	8.62 ± 0.67	10.58 ± 0.58
Week 3	9.17 ± 0.36	9.49 ± 0.44	9.24 ± 0.39	9.54 ± 0.34	9.34 ± 0.75	8.84 ± 1.52
Week 13	8.07 ± 0.42	7.36 ± 0.22	8.51 ± 0.60	7.42 ± 0.24	7.10 ± 0.63	7.85 ± 0.33
Monocytes (10^3/μL)						
Week 1	0.16 ± 0.03	0.06 ± 0.03	0.15 ± 0.06	0.10 ± 0.06	0.19 ± 0.07	0.09 ± 0.04
Week 3	0.14 ± 0.05	0.14 ± 0.05	0.09 ± 0.03	0.32 ± 0.09	0.23 ± 0.04	0.24 ± 0.08
Week 13	0.01 ± 0.01	0.03 ± 0.02	0.07 ± 0.03*	0.03 ± 0.02	0.15 ± 0.05**	0.10 ± 0.00**
Eosinophils (10^3/μL)						
Week 1	0.05 ± 0.02	0.00 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.02	0.02 ± 0.02
Week 3	0.03 ± 0.02	0.07 ± 0.04	0.14 ± 0.05	0.06 ± 0.03	0.04 ± 0.02	0.10 ± 0.04
Week 13	0.10 ± 0.05	0.06 ± 0.02	0.11 ± 0.06	0.14 ± 0.04	0.02 ± 0.02	0.08 ± 0.05

[1] Mean ± standard error. Statistical tests were performed on unrounded data.
[2] n=7.
* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test.
** Significantly different (P≤0.01) from the control group by Shirley's test.

TABLE B2 Clinical Chemistry Data for F344/N Rats In the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

	Vehicle Control	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg
MALE						
n						
Week 1	10	10	10	10	10	10
Week 3	10	8	10	9	9	7
Week 13	10	10	8	8	5	5
Urea nitrogen (mg/dL)						
Week 1	16.6 ± 1.0	15.9 ± 0.6	16.2 ± 0.8	16.5 ± 0.4	16.8 ± 0.9	14.3 ± 0.7
Week 3	18.1 ± 0.7	19.6 ± 0.6	18.8 ± 0.9	17.6 ± 0.7	17.2 ± 0.6	15.7 ± 1.1
Week 13	25.6 ± 0.6	23.5 ± 0.3*	23.1 ± 0.6*	20.6 ± 0.4**	21.0 ± 0.6**	16.8 ± 1.0**
Creatinine (mg/dL)						
Week 1	0.57 ± 0.02	0.57 ± 0.02	0.56 ± 0.02	0.58 ± 0.01	0.54 ± 0.02	0.57 ± 0.02
Week 3	0.59 ± 0.02	0.60 ± 0.00	0.59 ± 0.02	0.63 ± 0.02	0.61 ± 0.01	0.60 ± 0.02
Week 13	0.68 ± 0.02	0.75 ± 0.02	0.71 ± 0.01	0.70 ± 0.00	0.66 ± 0.02	0.66 ± 0.02
Total protein (g/dL)						
Week 1	5.9 ± 0.1	5.7 ± 0.1[2]	5.9 ± 0.1	5.8 ± 0.1	5.6 ± 0.1*	5.5 ± 0.1**
Week 3	6.4 ± 0.1	6.1 ± 0.1	6.0 ± 0.1**	6.0 ± 0.1**	6.0 ± 0.1**	5.6 ± 0.1**
Week 13	6.9 ± 0.1	7.1 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.5 ± 0.1*	6.1 ± 0.1**
Albumin (g/dL)						
Week 1	4.7 ± 0.1	4.6 ± 0.1[2]	4.7 ± 0.1	4.6 ± 0.1	4.4 ± 0.1*	4.3 ± 0.1**
Week 3	5.1 ± 0.1	4.9 ± 0.1	4.8 ± 0.1**	4.9 ± 0.1**	4.8 ± 0.1**	4.5 ± 0.1**
Week 13	5.4 ± 0.1	5.4 ± 0.0	5.4 ± 0.1	5.2 ± 0.1	5.1 ± 0.1**	4.8 ± 0.1**
Alanine aminotransferase (IU/L)						
Week 1	45 ± 2	43 ± 1	47 ± 2	43 ± 1	41 ± 1[2]	41 ± 1
Week 3	45 ± 1	42 ± 1*	42 ± 1*	43 ± 1	41 ± 1*	40 ± 2*
Week 13	89 ± 10	83 ± 8	73 ± 4	64 ± 4	60 ± 5*[3]	46 ± 5**
Alkaline phosphatase (IU/L)						
Week 1	639 ± 16	653 ± 15	661 ± 24	634 ± 10	574 ± 11**[2]	481 ± 17**
Week 3	481 ± 16	514 ± 18	508 ± 13	524 ± 8	474 ± 16	413 ± 28
Week 13	297 ± 12	249 ± 7**	255 ± 11*	220 ± 5**	193 ± 7**	157 ± 6**
Creatine kinase (IU/L)						
Week 1	310 ± 42	344 ± 31[2]	292 ± 25	324 ± 32	323 ± 30	294 ± 44
Week 3	293 ± 43	234 ± 26	280 ± 23	313 ± 25	250 ± 18	482 ± 183
Week 13	195 ± 22	203 ± 22	286 ± 36[4]	214 ± 56	197 ± 30	157 ± 30
Sorbitol dehydrogenase (IU/L)						
Week 1	9 ± 1	9 ± 1	11 ± 1	10 ± 1	9 ± 1	11 ± 1
Week 3	7 ± 1[2]	8 ± 1	5 ± 0	8 ± 1	7 ± 0	7 ± 1
Week 13	13 ± 1	12 ± 1	11 ± 1	12 ± 1	12 ± 2	9 ± 1
Bile acids (μmol/L)						
Week 1	17.60 ± 4.62	14.40 ± 4.43	12.10 ± 2.02	13.80 ± 3.68	8.60 ± 1.68	28.20 ± 5.56
Week 3	16.30 ± 2.92	16.50 ± 4.58	11.50 ± 2.07	7.00 ± 1.52	9.00 ± 1.89	19.14 ± 6.59
Week 13	7.10 ± 2.01	7.60 ± 3.15	8.88 ± 3.11	10.50 ± 3.74	3.80 ± 1.24	18.80 ± 5.59

TABLE B2 Clinical Chemistry Data for F344/N Rats In the 13-Week Gavage Study of Methylene Bis(thiocyanate) (continued)

	Vehicle Control	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg
FEMALE						
n						
Week 1	10	10	10	10	10	9
Week 3	9	10	10	9	9	7
Week 13	10	10	10	9	6	4
Urea nitrogen (mg/dL)						
Week 1	21.0 ± 0.6	19.5 ± 0.5	20.8 ± 0.7	21.1 ± 0.8	22.8 ± 2.8	18.2 ± 0.5*
Week 3	21.8 ± 0.7	19.9 ± 0.8	20.3 ± 0.7	18.4 ± 0.5**	19.9 ± 0.7*	18.3 ± 0.5**
Week 13	18.8 ± 0.3	21.6 ± 0.6**	20.4 ± 0.4	18.2 ± 1.1	17.8 ± 1.3	20.8 ± 0.6
Creatinine (mg/dL)						
Week 1	0.56 ± 0.02	0.56 ± 0.02	0.57 ± 0.02	0.59 ± 0.02	0.55 ± 0.02	0.56 ± 0.02
Week 3	0.64 ± 0.02	0.62 ± 0.01	0.62 ± 0.01	0.60 ± 0.00	0.63 ± 0.02	0.60 ± 0.02
Week 13	0.71 ± 0.01	0.73 ± 0.02	0.72 ± 0.02[2]	0.66 ± 0.02*	0.65 ± 0.02*	0.68 ± 0.03
Total protein (g/dL)						
Week 1	5.6 ± 0.1	5.7 ± 0.1	5.6 ± 0.1	5.8 ± 0.1	5.3 ± 0.2	5.5 ± 0.1
Week 3	6.1 ± 0.1	6.2 ± 0.1	6.1 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	5.5 ± 0.1**
Week 13	6.8 ± 0.1	7.1 ± 0.1	6.8 ± 0.1	6.6 ± 0.1	6.0 ± 0.3*	5.9 ± 0.1*
Albumin (g/dL)						
Week 1	4.6 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.4 ± 0.1	4.5 ± 0.1
Week 3	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	4.2 ± 0.1**
Week 13	5.3 ± 0.1	5.7 ± 0.1	5.4 ± 0.1	5.2 ± 0.1	4.8 ± 0.3	4.9 ± 0.1
Alanine aminotransferase (IU/L)						
Week 1	38 ± 2	39 ± 1	37 ± 1	40 ± 1	43 ± 6	34 ± 1
Week 3	39 ± 2	40 ± 1	37 ± 1	38 ± 1	36 ± 1	40 ± 4
Week 13	47 ± 2	52 ± 3	45 ± 3	44 ± 3	36 ± 3*	39 ± 1*
Alkaline phosphatase (IU/L)						
Week 1	550 ± 10	572 ± 14	540 ± 14	528 ± 26	417 ± 33**	402 ± 10**
Week 3	443 ± 11	460 ± 13	422 ± 11	381 ± 18*	381 ± 9**	277 ± 32**
Week 13	213 ± 5	213 ± 7	191 ± 5**	184 ± 7**	161 ± 18**	150 ± 7**
Creatine kinase (IU/L)						
Week 1	368 ± 36	352 ± 36	369 ± 43	398 ± 64	279 ± 37	276 ± 37
Week 3	276 ± 39	283 ± 40	248 ± 25	220 ± 29	232 ± 29	205 ± 30
Week 13	150 ± 26	150 ± 22	167 ± 36	156 ± 21	114 ± 12	147 ± 44
Sorbitol dehydrogenase (IU/L)						
Week 1	9 ± 1	9 ± 1	9 ± 2	9 ± 1	9 ± 1	9 ± 1
Week 3	15 ± 1	15 ± 2[2]	15 ± 2	19 ± 2	13 ± 2	15 ± 3
Week 13	12 ± 1	12 ± 1	11 ± 1	11 ± 1	11 ± 1	11 ± 2
Bile acids (μmol/L)						
Week 1	12.60 ± 2.68	13.40 ± 2.26	15.00 ± 3.16	8.00 ± 0.71	16.50 ± 3.52	28.50 ± 2.51**[5]
Week 3	11.11 ± 2.54	8.90 ± 2.09	9.40 ± 1.51	17.67 ± 4.43	19.56 ± 4.65	22.00 ± 5.86
Week 13	11.50 ± 2.62	16.30 ± 2.04	18.20 ± 3.56	17.11 ± 4.13	21.50 ± 5.16	24.75 ± 5.76

[1] Mean ± standard error. Statistical tests were performed on unrounded data.
[2] n=9.
[3] n=4.
[4] n=7.
[5] n=8.
* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test.
** Significantly different (P≤0.01) from the control group by Dunn's or Shirley's test.

APPENDIX C

Reproductive Tissue Evaluations and Estrous Cycle Characterization

TABLE C1 Summary of Reproductive Tissue Evaluations in Male F344/N Rats in the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

Study Parameters	Vehicle Control	2 mg/kg	4 mg/kg	8 mg/kg
n	10	8	8	5
Weights (g)				
Necropsy body weight	347 ± 7	350 ± 8	356 ± 8	339 ± 10
Left epididymis	0.445 ± 0.010	0.465 ± 0.010	0.507 ± 0.010**	0.458 ± 0.012
Left cauda epididymis	0.181 ± 0.006	0.197 ± 0.013	0.204 ± 0.015[2]	0.192 ± 0.014
Left testis	1.45 ± 0.02	1.46 ± 0.04	1.51 ± 0.03	1.44 ± 0.03
Spermatid measurements				
Spermatid heads (10^7/g testis)	11.55 ± 0.56	9.83 ± 0.27*	12.07 ± 0.37	11.92 ± 0.68
Spermatid heads (10^7/testis)	16.66 ± 0.67	14.27 ± 0.34*	18.26 ± 0.72	17.21 ± 1.14
Spermatid count (mean/10^{-4}mL suspension)	83.30 ± 3.37	71.34 ± 1.68*	91.31 ± 3.58	86.05 ± 5.72
Epididymal spermatozoal measurements				
Motility (%)	82.07 ± 0.74	79.64 ± 1.42	77.56 ± 1.33*	71.68 ± 0.78**
Concentration (10^6/g caudal epididymal tissue)	425.5 ± 16.7	415.9 ± 31.4	450.0 ± 60.0[2]	463.2 ± 34.2

[1] Data are presented as mean ± standard error. Differences from the control group for necropsy body, cauda epididymal, and testis weights and epididymal spermatozoal concentrations are not significant by Dunn's test.
[2] n=7.
* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test.
** Significantly different (P≤0.01) from the control group by Dunnett's or Shirley's test.

TABLE C2 Summary of Estrous Cycle Characterization in Female F344/N Rats in the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

Study Parameters	Vehicle Control	2 mg/kg	4 mg/kg	8 mg/kg
n	10	10	8	6
Necropsy body weight (g)	195 ± 4	195 ± 2	194 ± 5[2]	196 ± 6
Estrous cycle length (days)	4.75 ± 0.17	4.90 ± 0.28	4.81 ± 0.19[3]	4.50 ± 0.18[4]
Estrous stages (% of cycle)				
Diestrus	35.8	30.0	46.3	39.7
Proestrus	16.7	12.5	12.0	7.7
Estrus	25.8	38.3	25.0	33.3
Metestrus	21.7	19.2	16.7	19.2

[1] Necropsy body weights and estrous cycle lengths are presented as mean ± standard error. Differences from the control group for necropsy body weights and estrous cycle lengths are not significant by Dunn's test. By multivariate analysis of variance, dosed groups do not differ significantly from the controls in the relative length of time spent in the estrous stages.
[2] n=9.
[3] Estrous cycle longer than 12 days or unclear in one of nine animals.
[4] Estrous cycle longer than 12 days or unclear in one of seven animals.

TABLE C3 Summary of Reproductive Tissue Evaluations in Male B6C3F$_1$ Mice in the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

Study Parameters	Vehicle Control	4 mg/kg	8 mg/kg	16 mg/kg
n	10	10	9	7
Weights (g)				
Necropsy body weight	39.3 ± 0.8	38.9 ± 1.2	38.8 ± 0.6	36.1 ± 1.3*
Left epididymis	0.050 ± 0.003	0.048 ± 0.002	0.051 ± 0.002	0.047 ± 0.002
Left cauda epididymis	0.019 ± 0.001	0.017 ± 0.001	0.019 ± 0.001	0.017 ± 0.001
Left testis	0.123 ± 0.002	0.121 ± 0.003	0.115 ± 0.002	0.123 ± 0.006
Spermatid measurements				
Spermatid heads (10^7/g testis)	19.27 ± 0.74	20.15 ± 0.69	19.61 ± 0.62	20.19 ± 1.83
Spermatid heads (10^7/testis)	2.37 ± 0.09	2.44 ± 0.10	2.25 ± 0.07	2.42 ± 0.14
Spermatid count (mean/10^{-4}mL suspension)	73.98 ± 2.75	76.25 ± 3.12	70.28 ± 2.26	75.64 ± 4.48
Epididymal spermatozoal measurements				
Motility (%)	82.23 ± 1.76	72.02 ± 5.31	80.04 ± 1.47	78.89 ± 2.69
Concentration (10^6/g caudal epididymal tissue)	886.0 ± 133	802.3 ± 122	831.7 ± 52.6	859.6 ± 142

[1] Data are presented as mean ± standard error. Differences from the control group for testis, epididymal, and cauda epididymal weights, spermatid measurements, and epididymal spermatozoal measurements are not significant by Dunn's test.
* Significantly different (P≤0.05) from the control group by Shirley's test.

TABLE C4 Summary of Estrous Cycle Characterization in Female B6C3F$_1$ Mice in the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

Study Parameters	Vehicle Control	4 mg/kg	8 mg/kg	16 mg/kg
n	10	10	10	8
Necropsy body weight (g)	35.9 ± 1.0	35.0 ± 0.9	32.9 ± 0.5*	32.1 ± 0.5**[2]
Estrous cycle length (days)	5.05 ± 0.05	4.65 ± 0.13	4.65 ± 0.15	4.81 ± 0.16[3]
Estrous stages (% of cycle)				
Diestrus	28.3	28.3	26.7	30.6
Proestrus	21.7	20.8	16.7	21.3
Estrus	37.5	35.8	39.2	33.3
Metestrus	12.5	15.0	17.5	14.8

[1] Necropsy body weights and estrous cycle lengths are presented as mean ± standard error. Differences from the control group for estrous cycle lengths are not significant by Dunn's test. By multivariate analysis of variance, dosed groups do not differ significantly from the controls in the relative length of time spent in the estrous stages.
[2] n=9.
[3] Estrous cycle longer than 12 days or unclear in one of nine animals.
* Significantly different (P≤0.05) from the control group by Shirley's test.
** Significantly different (P≤0.01) from the control group by Shirley's test.

APPENDIX D

Genetic Toxicology

TABLE D1 Mutagenicity of Methylene Bis(thiocyanate) in *Salmonella typhimurium*[1]

Strain	Dose (µg/plate)	Revertants/plate[2] -S9 Trial 1	-S9 Trial 2	+ hamster S9 10%	+ hamster S9 30%	+ rat S9 10%	+ rat S9 30%
TA100	0.00	116 ± 4.3	120 ± 4.2	94 ± 4.5	116 ± 4.5	115 ± 6.7	148 ± 12.3
	0.01	117 ± 4.3					
	0.03	116 ± 1.0	122 ± 9.7				
	0.10	107 ± 3.2	116 ± 6.9				
	0.30	106 ± 10.2	107 ± 7.5				
	1.00	117 ± 9.2	107 ± 6.9	105 ± 18.5		110 ± 12.7	161 ± 7.1
	1.60		124 ± 8.4				
	3.00			96 ± 4.7	126 ± 14.3	119 ± 5.7	139 ± 16.9
	10.00			117 ± 4.7	94 ± 8.4	122 ± 5.0	134 ± 12.3
	33.00			138 ± 9.2	117 ± 6.0	130 ± 3.2	140 ± 1.7
	66.00						141 ± 11.3
	100.00			Toxic	128 ± 10.4	Toxic	
	166.00				0 ± 0.0[3]		
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control[4]		315 ± 8.1	499 ± 13.5	727 ± 23.4	435 ± 20.0	530 ± 9.3	361 ± 16.1
TA1535	0.00	22 ± 0.9	18 ± 1.8	6 ± 1.5	9 ± 1.2	8 ± 1.7	12 ± 2.0
	0.01						
	0.03	24 ± 0.3	19 ± 1.2				
	0.10	22 ± 2.4	19 ± 0.9				
	0.30	19 ± 1.2	14 ± 2.1				
	1.00	12 ± 1.5	15 ± 2.0	6 ± 1.8	11 ± 4.0	10 ± 0.9	12 ± 1.5
	1.60	14 ± 2.6	13 ± 1.2				
	3.00			4 ± 1.5	12 ± 2.6	7 ± 1.5	12 ± 0.9
	10.00			6 ± 0.9	9 ± 3.2	10 ± 2.2	9 ± 1.0
	33.00			11 ± 1.3	11 ± 1.7	7 ± 1.5	14 ± 2.2
	66.00						
	100.00			Toxic	16 ± 1.9	Toxic	17 ± 2.0
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		308 ± 5.1	400 ± 23.4	220 ± 19.1	624 ± 44.9	251 ± 8.9	193 ± 23.5
TA1537	0.00	6 ± 1.5			7 ± 1.5		12 ± 3.8
	0.01						
	0.03	7 ± 1.2					
	0.10	7 ± 2.9					
	0.30	7 ± 0.6					
	1.00	5 ± 1.8			6 ± 0.9		7 ± 0.9
	1.60	11 ± 2.7					
	3.00				4 ± 1.2		10 ± 1.0
	10.00				3 ± 0.7		9 ± 0.3
	33.00				6 ± 1.2		7 ± 0.3
	66.00						
	100.00				5 ± 0.9		6 ± 0.0
Trial summary		Negative			Negative		Negative
Positive control		414 ± 7.0			37 ± 2.9		42 ± 0.9

TABLE D1 Mutagenicity of Methylene Bis(thiocyanate) in *Salmonella typhimurium* (continued)

Strain	Dose (µg/plate)	Revertants/plate -S9 Trial 1	-S9 Trial 2	+ hamster S9 10%	+ hamster S9 30%	+ rat S9 10%	+ rat S9 30%
TA97	0.00	146 ± 5.2	133 ± 1.2	126 ± 6.7	164 ± 7.8	165 ± 3.8	181 ± 4.6
	0.01						
	0.03	155 ± 4.0	145 ± 3.7				
	0.10	161 ± 7.0	141 ± 22.9				
	0.30	177 ± 8.0	154 ± 8.5				
	1.00	186 ± 2.0	158 ± 5.8	113 ± 14.0	174 ± 3.7	169 ± 18.6	195 ± 4.2
	1.60	160 ± 6.7	172 ± 6.6				
	3.00			137 ± 15.3	187 ± 7.7	181 ± 17.3	199 ± 2.3
	10.00			157 ± 8.9	163 ± 10.0	140 ± 4.9	187 ± 11.3
	33.00			139 ± 6.1	163 ± 4.6	166 ± 12.7	197 ± 5.8
	66.00						
	100.00			Toxic	188 ± 5.5	Toxic	199 ± 12.3
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		554 ± 76.6	679 ± 34.4	345 ± 50.9	327 ± 9.0	509 ± 26.4	432 ± 14.9
TA98	0.00	15 ± 2.2	16 ± 0.9	18 ± 2.7	33 ± 4.3	28 ± 5.3	22 ± 1.2
	0.01	15 ± 1.8					
	0.03	12 ± 3.3	16 ± 1.2				
	0.10	19 ± 1.5	15 ± 3.4				
	0.30	13 ± 0.7	16 ± 0.7				
	1.00	20 ± 3.5	15 ± 0.9	24 ± 3.9		30 ± 2.3	26 ± 0.9
	1.60		20 ± 2.9				
	3.00			20 ± 3.0	38 ± 1.5	27 ± 2.7	25 ± 2.3
	10.00			18 ± 1.5	30 ± 2.5	22 ± 2.3	21 ± 3.0
	33.00			19 ± 2.0	32 ± 2.0	23 ± 1.2	30 ± 2.7
	66.00						26 ± 1.9
	100.00			Toxic	37 ± 0.3	Toxic	
	166.00				0 ± 0.0[3]		
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		439 ± 9.0	585 ± 32.7	589 ± 65.6	217 ± 18.8	349 ± 9.5	79 ± 2.6

[1] Study performed at SRI, International. The detailed protocol and these data are presented in Zeiger *et al.* (1992); 0 µg/plate is the solvent control.

[2] Revertants are presented as mean ± standard error from three plates.

[3] Slight toxicity.

[4] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA1537 and TA97), and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE D2 Frequency of Micronuclei in Peripheral Blood Erythrocytes of B6C3F$_1$ Mice in the 13-Week Gavage Study of Methylene Bis(thiocyanate)[1]

Dose (mg/kg)	Number Examined	Micronucleated NCEs/1,000 NCEs[2]
MALE		
0	5	4.7 ± 0.6
1	5	6.4 ± 0.8
2	5	3.6 ± 0.8
4	5	3.3 ± 1.0
8	5	4.5 ± 0.4
16	5	4.6 ± 1.0
		P=0.656[3]
FEMALE		
0	5	3.5 ± 0.7
1	5	2.3 ± 0.8
2	5	2.7 ± 0.1
4	5	2.3 ± 0.6
8	5	3.5 ± 0.8
16	5	3.0 ± 0.8
		P=0.347

[1] A detailed description of the protocol is presented by MacGregor *et al.* (1990). NCEs = normochromatic erythrocytes.
[2] Two thousand normochromatic erythrocytes were scored per animal. Data are presented as mean ± standard error.
[3] Significance of micronucleated NCEs/1,000 NCEs tested by a one-tailed trend test.

APPENDIX E

Disposition and Metabolism Studies

DISPOSITION AND METABOLISM STUDIES

Introduction

Two studies were conducted with male F344 rats to assess the disposition and metabolism of methylene bis(thiocyanate). An initial dose-response study was performed to determine the effect of dose on the rate and route of excretion of the compound. In this study, male rats were given single oral doses of 0.2 to 10 mg/kg [^{14}C]-labeled methylene bis(thiocyanate) by gavage. After administration, tissue and excreta of rats in each dose group were analyzed for radioactivity; the highest dose not affecting the rate or route of excretion (10 mg/kg) was selected for use in the subsequent cyanide metabolite study.

The cyanide metabolite study was conducted to determine whether blood levels of cyanide increase as a result of methylene bis(thiocyanate) metabolism. In this study, male rats were given single oral doses of 10 mg/kg [^{14}C]-labeled methylene bis(thiocyanate) by gavage. Rats were killed 30 minutes to 24 hours after dosing, and blood samples were collected and analyzed for cyanide, plasma thiocyanate, and radioactivity. Because cyanide is rapidly converted to thiocyanate by means of the rhodanese enzymes, it was expected that cyanide would be present only at the earlier time points following dosing.

Materials and Methods

CHEMICAL ANALYSES AND DOSE FORMULATIONS

Both radiolabeled (at the methylene carbon) and unlabeled methylene bis(thiocyanate) were obtained for use in the dose-response and cyanide metabolite studies. Analysis of the [^{14}C]-labeled methylene bis(thiocyanate) with high-performance liquid chromatography (HPLC) indicated a radiochemical purity of 98%. Additionally, analyses of the unlabeled compound by HPLC and gas chromatography indicated a purity greater than 99%.

Dose formulations were prepared by mixing the appropriate amount of [^{14}C]-methylene bis(thiocyanate) with a water:ethanol (7:3) solution. Similar formulations of unlabeled methylene bis(thiocyanate) were also prepared; the formulations of unlabeled compound were used to dilute the radiolabeled solutions to achieve the desired specific activities. Dose formulations were administered by gavage.

STUDY DESIGNS

Dose-Response Study

In the dose-response study, groups of three male F344 rats were given single oral doses of 0.2, 1, or 10 mg/kg [^{14}C]-labeled methylene bis(thiocyanate). Immediately after dosing, the rats were placed in glass metabolism cages equipped with traps containing ethylene glycol or Carbosorb to trap organic volatiles or CO_2, respectively. Urine, feces, and trapping solutions were collected 6, 12, 24, and 48 hours after dosing and analyzed for radioactivity. Sample collection was stopped after 48 hours because 80% or more of the radioactivity had been eliminated by this time point. Rats were then killed and necropsied, and the radioactivity of tissue samples was determined using a Packard® tissue oxidizer.

Cyanide Metabolite Study

In the cyanide metabolite study, groups of three male F344 rats were given single oral doses of 10 mg/kg [^{14}C]-labeled methylene bis(thiocyanate); three rats each were then killed 30 minutes or 1, 2, 4, 6, 12, or 24 hours after dosing, and blood samples were collected and analyzed for cyanide, plasma thiocyanate, and radioactivity.

To prepare for the cyanide analysis, blood samples were placed in an aerator immediately after collection. In the aerator, each sample was mixed with deionized water and octanol, and sulfuric acid was added to convert the cyanide present in the sample to hydrogen cyanide. The hydrogen cyanide in the sample was then swept into a sodium hydroxide trapping solution using dry nitrogen gas, and the entire trapping solution was removed for cyanide analysis. Plasma samples for thiocyanate analysis were mixed with trichloroacetic acid and centrifuged; 1 mL supernatant samples were then removed for analysis.

Cyanide and thiocyanate levels were determined spectrophotometrically; methods were adapted from Lundquist *et al.* (1985). Briefly, prepared samples were mixed with acetic acid and sodium hypochlorite and then allowed to stand for 1 minute. To develop color, a barbituric acid-pyridine reagent was added to the samples, which were diluted with water. After a 5-minute development period, absorbance was measured at 583 nm using a Beckman® DU-40 spectrophotometer. Thiocyanate response was linear over a range of 0.1 to 0.9 µg SCN/mL; plasma samples were diluted to fall within this range. Cyanide response was linear over a range of 40 to 400 ng CN/mL. The radioactivity of blood samples was determined using a Packard® tissue oxidizer.

ANALYSIS OF DATA

Radioactivity was expressed as the percentage of the dose administered (tissues) or as the cumulative percentage of the dose administered (excreta) in terms of ^{14}C equivalents. Blood cyanide and plasma thiocyanate were expressed as the observed concentrations less the control concentrations; control concentrations were determined to be 98 ng/mL for blood cyanide and 2.05 µg/mL for plasma thiocyanate. For all data, the means and standard deviations for each dose group were calculated.

Results

DOSE-RESPONSE STUDY

At all dose levels, radioactivity was excreted primarily in the urine of rats; concentrations of radioactivity in the urine after 48 hours accounted for 52% to 63% of the doses administered (Table E1). The other major routes of excretion were the feces (14% to 28%) and expired CO_2 (9% to 11%). Total radioactivity found in tissues accounted for about 3% to 7% of the doses administered, while total radioactivity found in excreta accounted for about 92% to 99% of the doses administered.

Generally, the percentages of administered radioactivity excreted in urine and expired CO_2 were not affected by dose (Table E1). However, the percentages of radioactivity found in the tissues and feces of rats at the high-dose level (10 mg/kg) were notably different from the amounts found at the two lower dose levels (0.2 and 1 mg/kg).

CYANIDE METABOLITE STUDY

The results of the cyanide metabolite study are summarized in Table E2. Analysis of blood samples indicated that radioactivity, expressed as a percentage of the dose administered, was greatest at the earliest time point (30 minutes) after administration; at this time point, the level of radioactivity in the blood accounted for about 16% of the [^{14}C]-labeled dose administered. At each subsequent time point, the percentage of radioactivity in the blood decreased, and by the final time point (24 hours), only about 1% of the administered dose remained in the blood.

In the cyanide metabolite study, blood cyanide concentration was greatest at the earliest time point after dosing (Table E2); at this time point, blood cyanide was increased threefold (to 296 ng/mL) relative to the determined control value of 98 ng/mL. As expected, blood cyanide levels decreased at the later time points, and by the 2-hour time point, cyanide levels in the blood were not significantly different from the control value (Table E2).

Plasma thiocyanate levels also showed substantial increases over the determined control value (2.05 µg/mL) following an oral dose of [^{14}C]-methylene bis(thiocyanate). The maximum plasma thiocyanate concentration was observed 2 hours after dosing, considerably later than when the maximum blood cyanide level was noted. Thiocyanate levels decreased at subsequent time points but had not returned to the control value 24 hours after dosing.

Discussion

In the dose-response study, the radioactivity from orally administered [^{14}C]-methylene bis(thiocyanate) was quickly eliminated; greater than 90% was excreted in 48 hours. Increasing percentages of the radioactivity remained in the tissues as the dose increased; this effect was especially striking in the stomach, where the percent remaining after administration of 10 mg/kg was increased 10-fold over that remaining after administration of 1 mg/kg. Other than the fact that it was derived from the methylene carbon of methylene bis(thiocyanate), the nature of the remaining radioactivity is not known. It could have resulted from covalent binding, *i.e.*, by displacement of thiocyanate by tissue nucleophiles or possibly from biosynthetic incorporation via the one-carbon pool. Although some biosynthetic incorporation is possible, it seems an unlikely explanation for all the remaining radioactivity, considering the uneven distribution of the label.

The results of the cyanide metabolite study indicate that the blood cyanide levels of male rats increase at least threefold shortly after administration of a single oral dose of 10 mg/kg methylene bis(thiocyanate). This initial increase in blood cyanide is followed by a marked increase in plasma thiocyanate, which may be explained in part by the rapid conversion of cyanide to thiocyanate by means of the rhodanese enzymes. Additional thiocyanate may be produced through the cleavage of thiocyanate from the parent molecule, $CH_2(SCN)_2$; the fact that concentrations of plasma thiocyanate exceed those of blood cyanide (*i.e.*, µg/mL versus ng/mL) suggests that thiocyanate may be produced by more than one pathway.

TABLE E1 Percentage of Dose in Tissues and Excreta of Male F344 Rats 48 Hours after Administration of a Single Oral Dose of [^{14}C]-Methylene Bis(thiocyanate)[1]

Parameter	Single Dose (mg/kg)		
	0.2	1	10
n	3	3	3
Tissue			
Blood	0.24 ± 0.03	0.29 ± 0.006	0.64 ± 0.28
Liver	0.50 ± 0.14	0.69 ± 0.08	1.09 ± 0.29
Kidney	0.11 ± 0.012	0.15 ± 0.03	0.24 ± 0.10
Lung	0.03 ± 0.006	0.03 ± 0.01	0.05 ± 0.02
Brain	0.013 ± 0.006	0.02 ± 0.006	0.02 ± 0.006
Fat	0.03 ± 0.02	0.08 ± 0.03	0.12 ± 0.05
Muscle	0.73 ± 0.10	1.15 ± 0.14	1.30 ± 0.19
Skin	0.53 ± 0.30	0.46 ± 0.13	0.56 ± 0.04
Testes	0.03 ± 0.006	0.04 ± 0.01	0.06 ± 0.03
Stomach	0.13 ± 0.04	0.16 ± 0.06	1.96 ± 1.18
Small intestine	0.17 ± 0.06	0.17 ± 0.04	0.30 ± 0.11
Large intestine	0.08 ± 0.03	0.08 ± 0.006	0.14 ± 0.05
Total	2.57 ± 0.56	3.31 ± 0.31	6.48 ± 0.91
Excreta			
Urine	58.85 ± 1.51	52.18 ± 5.28	62.72 ± 8.60
Feces	28.08 ± 2.77	26.50 ± 1.78	14.14 ± 2.44
Stomach content	0.02 ± 0.015	0.05 ± 0.0	0.05 ± 0.02
Small intestine content	0.07 ± 0.04	0.10 ± 0.03	0.19 ± 0.10
Large intestine content	0.39 ± 0.11	0.24 ± 0.09	1.27 ± 1.26
Expired CO_2	8.99 ± 0.58	9.94 ± 0.49	10.52 ± 1.39
Total	96.39 ± 2.12	88.98 ± 3.97	88.91 ± 9.30
Total Recovery	98.96 ± 2.40	92.29 ± 4.05	95.39 ± 8.87

[1] Data (mean ± standard deviation) are presented as percentage of dose (tissues) or cumulative percentage of dose (excreta).

TABLE E2 Percentage of Dose and Cyanide and Plasma Thiocyanate Levels in the Blood of Male F344 Rats after Administration of a Single Oral Dose of 10 mg/kg [^{14}C]-Methylene Bis(thiocyanate)[1]

Time after Dosing (hours)	Number Examined	Percentage of Dose in the Blood[2]	Increase in Blood Cyanide[3]	Increase in Plasma Thiocyanate[3]
0.5	3	15.50 ± 5.67	198.2 ± 26.9	1.71 ± 0.68
1.0	3	11.46 ± 1.27	64.9 ± 5.5	0.51 ± 0.27
2.0	3	10.72 ± 2.22	−8.8 ± 11.8	9.66 ± 3.14
4.0	3	8.94 ± 2.37	27.1 ± 38.8	3.03 ± 0.77
6.0	3	5.99 ± 0.51	—[4]	3.72 ± 0.53
12.0	3	4.20 ± 0.78	—	1.05 ± 0.14
24.0	3	1.17 ± 0.26	—	0.78 ± 0.01

[1] Data are presented as mean ± standard deviation.
[2] Percentage of dose in the blood = ^{14}C equivalents and includes parent compound and any metabolites bearing the methylene carbon.
[3] Cyanide and plasma levels are expressed as observed concentrations less control concentrations; control concentrations were 98 ng/mL for blood cyanide and 2.05 µg/mL for plasma thiocyanate.
[4] Not different from the control value.

NTP TECHNICAL REPORTS ON TOXICITY STUDIES PRINTED AS OF NOVEMBER 1993

Toxicity Report Number	Chemical	Route of Exposure	Publication Number
1	Hexachloro-1,3-butadiene	Dosed Feed	91-3120
2	*n*-Hexane	Inhalation	91-3121
3	Acetone	Drinking Water	91-3122
4	1,2-Dichloroethane	Drinking Water, Gavage	91-3123
5	Cobalt Sulfate Heptahydrate	Inhalation	91-3124
6	Pentachlorobenzene	Dosed Feed	91-3125
7	1,2,4,5-Tetrachlorobenzene	Dosed Feed	91-3126
8	D & C Yellow No. 11	Dosed Feed	91-3127
9	*o*-Cresol *m*-Cresol *p*-Cresol	Dosed Feed	92-3128
10	Ethylbenzene	Inhalation	92-3129
11	Antimony Potassium Tartrate	Drinking Water, I.P. Inject.	92-3130
12	Castor Oil	Dosed Feed	92-3131
13	Trinitrofluorenone	Dermal, Dosed Feed	92-3132
14	*p*-Chloro-α,α,α-Trifluorotoluene	Gavage (corn oil, a-CD)	92-3133
15	*t*-Butyl Perbenzoate	Gavage	92-3134
16	Glyphosate	Dosed Feed	92-3135
17	Black Newsprint Ink	Dermal	92-3340
18	Methyl Ethyl Ketone Peroxide	Dermal	92-3341
19	Formic Acid	Inhalation	92-3342
20	Diethanolamine	Drinking Water, Dermal	92-3343
21	2-Hydroxy-4-Methoxybenzophenone	Dosed Feed, Drinking Water	92-3344
22	N, N-Dimethylformamide	Inhalation	93-3345
23	*o*-Nitrotoluene *m*-Nitrotoluene *p*-Nitrotoluene	Dosed Feed	92-3346
24	1,6-Hexanediamine	Inhalation	93-3347
25	Glutaraldehyde	Inhalation	93-3348
26	Ethylene Glycol Ethers	Drinking Water	93-3349
28	Tetrachlorophthalic Anhydride	Gavage	93-3351
29	Cupric Sulfate	Drinking Water, Dosed Feed	93-3352

NTP TECHNICAL REPORTS ON TOXICITY STUDIES PRINTED AS OF NOVEMBER 1993 (continued)

Toxicity Report Number	Chemical	Route of Exposure	Publication Number
33	2-Chloronitrobenzene 4-Chloronitrobenzene	Inhalation	93-3382
35	Chemical Mixture of 25 Groundwater Contaminants	Drinking Water	93-3384
36	Pesticide/Fertilizer Mixtures	Drinking Water	93-3385
37	Sodium Cyanide	Drinking Water	94-3386