National Toxicology Program
Toxicity Report Series
Number 50

NTP Technical Report
on Toxicity Studies of

Cyclohexanone Oxime

(CAS No. 100-64-1)

Administered by Drinking Water
to B6C3F$_1$ Mice

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

Note to the Reader

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The studies described in this toxicity study report were performed under the direction of NIEHS and were conducted in compliance with NTP laboratory health and safety requirements. These studies met or exceeded all applicable federal, state, and local health and safety regulations. Animal care and use were in accord and compliance with the Public Health Service Policy on Humane Care and Use of Animals.

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This NTP report on the toxicity studies of cyclohexanone oxime is based primarily on 2-week studies that took place in January 1991 and 13-week studies that took place from April 1991 through July 1991.

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PEER REVIEW

The draft report on the toxicity studies of cyclohexanone oxime was evaluated in March 1995 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 the toxicity study report presents the experimental results and conclusions fully and clearly.

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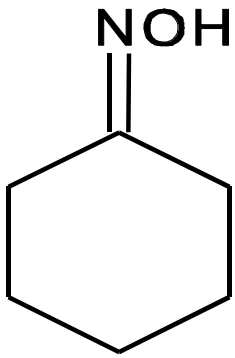
ABSTRACT

Cyclohexanone Oxime



Molecular Formula	$C_6H_{11}NO$
CAS Number	100-64-1
Molecular Weight	113.16
Synonyms	(Hydroxyimino)cyclohexane Antioxidant D

Cyclohexanone oxime is used primarily as a captive intermediate in the synthesis of caprolactam for the production of polycaprolactam (Nylon-6) fibers and plastics and also in a variety of industrial applications. Cyclohexanone oxime was selected for study because of the potential for human exposure and the interest in oximes as a chemical class. Toxicity studies of cyclohexanone oxime (approximately 99% pure) were carried out in male and female B6C3F$_1$ mice; the compound was administered in drinking water for 2 weeks or 13 weeks. In addition, the genetic toxicity of cyclohexanone oxime was evaluated by determining mutagenicity in *Salmonella typhimurium* and induction of chromosomal aberrations in cultured Chinese hamster ovary cells *in vitro*, with and without S9 activation. The frequency of micronucleated normochromatic erythrocytes in the bone marrow and peripheral blood of mice from the 13-week study was also determined.

In the 2-week study, groups of five male and five female mice were given drinking water containing 0, 106, 312, 625, 1,250, or 2,500 ppm cyclohexanone oxime. No deaths occurred, and there was no decrease in weight gain in any group. No gross lesions were observed; there were significant increases

in relative spleen weights of males and females in the 2,500 ppm group and increases in the relative liver weight of male mice exposed to 312 ppm or greater.

In the 13-week studies, groups of 10 male and 10 female mice were given drinking water containing 625, 1,250, 2,500, 5,000 or 10,000 ppm cyclohexanone oxime. Deaths occurred in the 10,000 ppm groups and weight gain was depressed in males and females given 10,000 ppm and in females given 5,000 ppm. There were significant increases in relative spleen weight at exposure levels of 5,000 and 10,000 ppm and significant increases in the relative liver weights of males and females that received 10,000 ppm. Microscopically, hematopoietic cell proliferation was observed in the spleen of males and females in the 5,000 and 10,000 ppm groups. Centrilobular cell hypertrophy was observed in the liver of males in the 2,500, 5,000, and 10,000 ppm groups and in females in the 5,000 and 10,000 ppm groups. Olfactory epithelial degeneration was observed in all exposed groups.

Cyclohexanone oxime was mutagenic in *Salmonella typhimurium* strain TA1535 with S9 activation only; results of mutagenicity testing of cyclohexanone oxime were negative in strains TA97, TA98, and TA100, with and without S9. Cyclohexanone oxime gave equivocal results in a test for induction of chromosomal aberrations in cultured Chinese hamster ovary cells without S9; with S9, results were negative. *In vivo*, no induction of micronuclei was noted in erythrocytes of mice treated with cyclohexanone oxime either for 13 weeks in drinking water or for 3 days by intraperitoneal injection.

In summary, the major targets of cyclohexanone oxime toxicity are the erythrocyte, spleen, liver, and nasal epithelium. The no-effect level for erythrotoxicity is 2,500 ppm following 13 weeks of exposure. The no-effect level for hematopoietic cell proliferation in the spleen is 2,500 ppm. The no-effect level for hepatotoxicity is 1,250 ppm for males and 2,500 ppm for females following 13 weeks of exposure. Some nasal olfactory epithelial degeneration was observed at all exposure levels; only at 625 ppm in males was the incidence of this lesion not significantly different from that in the controls. Cyclohexanone oxime was mutagenic only in *Salmonella typhimurium* strain TA1535 with S9 activation.

INTRODUCTION

Physical and Chemical Properties, Production, Use, and Exposure

Cyclohexanone oxime, a white crystalline solid, has a molecular weight of 113.16 and a melting point of 90° C. It is soluble in water and ethanol (Lide, 1992). Cyclohexanone oxime is produced by the condensation of cyclohexanone with hydroxylamine sulfate or hydroxylamine phosphate (Fisher and Cresentini, 1985). It was estimated that 100,000 to 500,000 tons of cyclohexanone oxime were produced by the United States in 1977. In 1976, 15.4 tons of cyclohexanone oxime were imported into the U.S.; more recent import or production figures are not available.

Cyclohexanone oxime is used in a wide variety of industrial applications. Primarily, it is used as a captive intermediate in the synthesis of caprolactam, which is polymerized in the production of polycaprolactam (Nylon-6) fibers and plastics (Fisher and Cresentini, 1985; NCI, 1985). The annual U.S. caprolactam production is over 500,000 tons (NCI, 1985). Approximately 90% of the monomer is used to produce fibers for clothing, carpets, home furnishings, and tire cording. The remaining 10% is used to produce nylon resins for food packaging film, extrusion compounds for bristle filaments and wire coatings, and molded plastics for automobiles and appliances (NCI, 1985). Cyclohexanone oxime is also thought to be an intermediate in the oxidative metabolism of sodium cyclamate, an artificial sweetener (Unger and McMahon, 1981).

Because of the large production volume of cyclohexanone oxime and the widespread applications of cyclohexanone oxime-derived products, a large population is potentially exposed to this chemical. No exposure limits have been set for cyclohexanone oxime (OSHA, 1983). Hematologic disorders have been reported in humans exposed to cyclohexanone oxime, and dermatitis and skin sensitization may also be potential effects of occupational exposure (Finkel, 1983). Further study of exposure to this oxime is warranted due to the potential association.

Disposition and Metabolism

A toxicokinetic study of cyclohexanone oxime in male F344 rats has been reported (Parmar and Burka, 1991; Appendix E). The chemical was found to be rapidly absorbed and cleared within 24 hours after a single oral administration of 1, 10, or 30 mg/kg of [^{14}C]-cyclohexanone oxime in aqueous solution. The majority of the cyclohexanone oxime-derived radioactivity was excreted in the urine. Three urinary metabolites were identified: cyclohexylglucuronide and the monoglucuronides of *cis*- and *trans*-cyclohexane-1,2-diol. Low levels of radioactivity (2%-3% of the dose) were retained in the tissues 24 hours after exposure. After intravenous administration of 1 mg/kg of [^{14}C]-cyclohexanone oxime, the oxime was rapidly cleared from plasma, with half lives of 1.6 minutes (alpha phase) and 18.2 minutes (beta phase). When cyclohexanone oxime was applied dermally (30 mg/kg), only 4% to 5% of the dose was recovered in the urine, feces, and tissues. The majority of the dose volatilized from the skin surface. However, the absorbed radioactivity was readily distributed and excreted, and its metabolic fate was no different than that observed after oral administration. Cyclohexanone oxime has been reported to induce increased liver microsomal activity (aniline hydroxylase and aminopyrine demethylase) in rats treated with 100 mg cyclohexanone oxime per kilogram body weight for 14 days by gavage (Komsta *et al*., 1989). In addition, cyclohexanone oxime has been reported to inhibit the oxidative metabolism of ethanol in rats and mice, an effect similar to that produced in humans as a result of disulfiram administration (Lewis and Schwartz, 1956; Koe and Tenen, 1970; Cattanach, 1976).

Toxicity

ANIMAL TOXICITY

An LD_{50} of 250 mg per kilogram body weight has been reported with intraperitoneally administered cyclohexanone oxime in male mice (Plzak and Doull, 1969). An LD_{50} of 710 mg/kg (by an unspecified route) has also been reported in male mice administered cyclohexanone oxime (Fridman *et al*., 1978).

Cyclohexanone oxime has also been observed to have acute toxic effects in rats and New Zealand White rabbits. Effects of cyclohexanone oxime in rats included neurobehavioral changes characterized by increased aggression and central nervous system depression (Rublack and Henkel, 1975). Conjunctivitis was also noted. Dermal exposure of male and female New Zealand White rabbits to 0, 0.8, 2, or 5 g cyclohexanone oxime per kilogram body weight for 24 hours resulted in reticulocytosis 1 day after the first exposure. Females had a lower hemoglobin concentration

after 7 days exposure to 5 g/kg (Gad *et al*., 1985). Oral administration of 0.1 or 1.0 g/kg to rabbits resulted in lower erythrocyte counts and elevated methemoglobin levels (Rublack and Henkel, 1975).

Two-week gavage studies in male and female Sprague-Dawley rats (Komsta *et al*., 1989) and F344 rats (Derelanko *et al*., 1985) revealed dose-related erythroid hyperplasia in the spleen and bone marrow. Sprague-Dawley rats that received 1, 10, or 100 mg cyclohexanone oxime per kg body weight for 2 weeks had hematologic differences including lower erythrocyte counts, higher platelet counts, lower hemoglobin concentrations and hematocrit levels, and greater mean red cell hemoglobin and mean red cell volume values than the control values. Bone marrow smears indicated lower myeloid, lymphocyte, and monocyte counts concomitant with elevated erythroid counts. There was also general splenic enlargement with hematopoietic cell proliferation.

Male and female F344 rats that received 10, 25, 75, 150, or 300 mg cyclohexanone oxime per kilogram body weight by gavage for 2 weeks had hematologic changes similar to those of the Sprague-Dawley rats (Derelanko *et al*., 1985). Observations included a dose-related decrease in erythrocyte counts with concomitant increases in the numbers of circulating nucleated erythrocytes and reticulocytes and reduced hematocrit levels and hemoglobin concentrations. Methemoglobin concentrations, measured at the highest dose, were significantly elevated. The rats were observed for another 2 weeks without dose administration. By Day 28, hematologic values in females had returned to normal and males displayed only slightly depressed erythrocyte counts and mildly elevated reticulocyte counts. No significant effects on body weights and no clinical signs of toxicity were noted in males or females. Splenomegaly and hepatomegaly were observed in male and female mice on Day 14 and Day 28. The hematology results suggested that the hematotoxic effects of cyclohexanone oxime administration were reversible following cessation of exposure. The authors theorized that cyclohexanone oxime induces oxidative damage to the erythrocyte resulting in hemolytic anemia compensated by increased erythropoiesis.

Results of a 13-week gavage study with male and fem?ale F344 rats were similar to those of the 2-week studies, with evidence of splenomegaly and erythroid hyperplasia in the spleen and bone marrow (Gad *et al*., 1985). Rats received doses of 0, 0.25, 2.5, and 25 mg cyclohexanone oxime per kilogram body weight five times a week for 13 weeks. All males survived to the end of the study; three of 20 females in the 25 mg/kg group died before the end of the study. Males were observed with clinical signs of toxicity that included persistent red nasal discharge (at 25 mg/kg

only), chromodacryorrhea and swollen conjunctiva (at 2.5 and 25 mg/kg), and corneal opacity (at all dose levels). No significant effects on body weight or feed consumption were observed in males or females. Hematologic differences similar to those seen in the 2-week study were noted. Dose-related anisocytosis, poikilocytosis, elevated osmotic red blood cell fragility, and a greater incidence of Howell-Jolly bodies were observed. Splenomegaly was noted at necropsy, and histopathologic examination showed erythroid hyperplasia in the bone marrow and spleen and increased hemosiderin pigment deposition in the spleen (Gad *et al*., 1985).

In an inhalation study, rats were administered 0.03, 0.1, or 1 mg cyclohexanone oxime per m^3 for 6 to 10 weeks (Tsulaya *et al*., 1975). Reduced erythrocyte count and blood cholinesterase activity were observed at exposure levels of 0.1 and 1 mg/m^3. At necropsy the rats in the 0.1 mg/m^3 group displayed degenerative differences in the parenchymatous organs and desquamation of bronchial epithelium. No adverse effects were noted in study animals at the 0.03 mg/m^3 exposure level.

GENETIC TOXICITY

Negative results were obtained in mutagenicity tests with several strains of *Salmonella typhimurium*, with and without metabolic activation (Araki *et al*., 1986; Rogers-Back *et al*., 1988) and with *Escherichia coli* strain WP2 (Araki *et al*., 1986). In addition, no increase in the frequency of sex-linked recessive lethal mutations was observed in germ cells of male *Drosophila melanogaster* administered cyclohexanone oxime (8.8 mM) by feeding (Vogel and Chandler, 1974). The only mutagenic activity reported for cyclohexanone oxime was noted in L5178Y mouse lymphoma cells treated in the absence of S9 activation; the addition of rat liver S9 eliminated the mutagenic effect (Rogers-Back *et al*., 1988).

Study Rationale and Design

The oximes, as a chemical class, are produced in relatively large quantities and are currently used in a wide variety of industrial applications. There is growing interest in using oximes as magnetic tape binders, photochemical additives, biocides for water and waste treatment, ingredients in cosmetics, and sweeteners. Despite the potential for wide-ranging occupational exposure, little is known regarding the potential toxicity of oximes. Carcinogenic effects associated with the cyclic oxime *p*-quinone dioxime (NCI, 1979) and with the aliphatic oxime, acetoxime (Mirvish *et al*., 1982), have made oximes high-priority chemicals for toxicity testing. Cyclohexanone oxime was selected by the NTP as a representative alicyclic oxime for toxicity testing in a 2-week range-finding study and a 13-week subchronic study in B6C3F_1 mice. The subacute (Derelanko *et al*., 1985) and subchronic (Gad *et al*., 1985) oral toxicity of cyclohexanone oxime in F344 rats has been reported, and studies in this species were not repeated.

Cyclohexanone oxime was administered by dosed water to male and female B6C3F_1 mice for 2 weeks at concentrations of 0,106, 312, 625, 1,250, and 2,500 ppm and for 13 weeks at concentrations of 0, 625, 1,250, 2,500, 5,000, and 10,000 ppm. Information gained from these studies may be used to set exposure levels for 2-year chronic toxicity and carcinogenicity studies in B6C3F_1 mice.

MATERIALS AND METHODS

Procurement and Characterization of Cyclohexanone Oxime

Two lots of cyclohexanone oxime, Lots 02616LT and 08812MX, were obtained from Aldrich Chemical Company (Milwaukee, WI). Lot 02616LT was used throughout the 2-week and 13-week studies. Additional cyclohexanone oxime (Lot 08812MX) was obtained to complete the 13-week study.

Chemical analyses identified the chemical, a white crystalline solid, as cyclohexanone oxime. Proton nuclear magnetic resonance and low-resolution mass spectra for Lot 02616LT were consistent with the structure of cyclohexanone oxime and literature references (*Sadtler Standard Spectra*, NIST). Gas chromatography by a single system indicated no impurities with areas greater than 0.1% relative to the major peak. The cumulative data indicated a purity greater than 99% for Lot 02616LT.

Lot 08812MX was analyzed relative to Lot 02616LT with infrared spectroscopy and gas chromatography. The infrared spectrum of Lot 08812MX was consistent with that of Lot 02616LT and a reference spectrum (Aldrich Chemical Company, 1981), confirming its identity as cyclohexanone oxime. Gas chromatography indicated no impurity peaks. The cumulative analytical data indicated a purity greater than 99% for Lot 08812MX.

Stability studies using gas chromatography indicated that cyclohexanone oxime was stable in aqueous solution at a concentration of 106 ppm for 4 weeks at 5° C when stored in a sealed container, protected from light. Solutions stored exposed to light in drinking water bottles were stable for 5 days. At the study laboratory, the bulk chemical was stored in a sealed container at 5° C or less, protected from light. The study laboratory monitored stability of the chemical throughout the study with gas chromatography; no degradation of cyclohexanone oxime was observed.

Dose Formulations

Drinking water solutions of cyclohexanone oxime were prepared in deionized water. The bulk chemical was ground to a powder, dissolved in deionized water, sonicated, and additional deionized water was added to produce the desired volume and concentration.

Dose formulations of cyclohexanone oxime were stored at 4° ± 2° C in glass vessels, protected from light, for no longer than 4 weeks. Dose formulations and animal room samples were analyzed by gas chromatography. The analytical results from all dose formulations were within 10% of the theoretical concentration. Three animal-room samples from the 2-week study were not within 10% of the theoretical concentration; this may have been due to an observable tailing of the cyclohexanone oxime peak resulting from injection temperatures that were lower than the boiling point of cyclohexanone oxime. One animal-room sample from the 13-week study was not within 10% of the theoretical concentration. This was thought to be caused by animal manipulation of the water bottle and contamination of the dosed water with fecal or bedding material that caused cyclohexanone oxime degradation.

Toxicity Study Designs

BASE STUDIES

Male and female B6C3F$_1$ mice were obtained from Taconic Laboratory Animals and Services (Germantown, NY) for the 2-week and 13-week drinking water studies. Mice were shipped to the study laboratory at approximately 4 weeks of age and quarantined for 11 or 12 days; the mice were approximately 6 weeks of age at the beginning of the studies (Table 1). Blood samples were collected from two male and two female mice at the beginning of the 2-week study and from five male and five female mice at the beginning of the 13-week study. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al*., 1986; Rao *et al*., 1989a,b). All results were negative.

During the 2-week study, groups of five male and five female mice were given 0, 106, 312, 625, 1,250, or 2,500 ppm of cyclohexanone oxime in drinking water *ad libitum* for 7 days a week. In the 13-week study, groups of 10 male and 10 female mice were given 0, 625, 1,250, 2,500, 5,000, or 10,000 ppm cyclohexanone oxime in drinking water *ad libitum* for 7 days a week.

For all studies, mice were housed in individual cages. NIH-07 Open Formula Diet (Zeigler Brothers, Inc., Gardners, PA) in pellet form was available *ad libitum*. Animal rooms were maintained with 12 hours of fluorescent light per day. The temperature was maintained at 72° ± 3° F and relative humidity at 50% ± 15%, with at least 10 room air changes per hour.

Necropsies were performed on all animals in the 2-week and 13-week studies. In the 2-week study, the liver and spleen were weighed. In the 13-week study, the heart, right kidney, liver, lungs, spleen, right testis, and thymus of each animal were 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. In the 2-week study only gross lesions were examined. In the 13-week study, complete histopathologic examinations were performed on all animals. For all paired organs (*i.e.*, kidney, ovary, adrenal gland), samples from each organ were examined. Tissues examined microscopically are listed in Table 1.

Upon completion of the laboratory pathologist's histopathologic 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

Sperm Motility and Vaginal Cytology in Mice

Sperm motility and vaginal cytology evaluations were performed on 13-week study mice (10 males and 10 females) from the 0, 1,250, 2,500, and 5,000 ppm groups at the end of the study. The parameters that were evaluated are listed in Table 1. Methods were those described in the NTP statement of work (1987). Briefly, for 12 consecutive days prior to the end of the study, the vaginal vaults were moistened with saline, if necessary, and vaginal lavage samples were taken from 10 females per group and spread on a glass slide, dried, and stained. Relative number of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (*i.e.*, diestrus, proestrus, estrus, and metestrus).

Sperm motility was evaluated at necropsy in the following manner. The left epididymis was isolated and weighed. The cauda epididymis was removed from the epididymis body (corpus epididymis) and weighed. Modified Tyrode's buffer 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 the slides. 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. Caudae were finely minced and the tissue was incubated in a saline solution and then heat fixed at 65° C. 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 phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

TABLE 1 Experimental Design and Materials and Methods in the 2-Week and 13-Week Drinking Water Studies of Cyclohexanone Oxime

2-Week Study	13-Week Study
EXPERIMENTAL DESIGN	
Study Laboratory Microbiological Associates, Inc. (Bethesda, MD)	Same as 2-week study
Strain and Species $B6C3F_1$ mice	Same as 2-week study
Animal Source Taconic Farms (Germantown, NY)	Same as 2-week study
Size of Study Groups Five males and five females	10 males and 10 females
Exposure Concentrations 0, 106, 312, 625, 1,250, or 2,500 ppm in drinking water *ad libitum* for 2 weeks	0, 625, 1,250, 2,500, 5,000, or 10,000 ppm in drinking water *ad libitum* for 13 weeks
Date of First Exposure 8 January 1991	29 April 1991
Date of Last Exposure 22 January 1991	29-30 July 1991
Date of Necropsy 22 January 1991	29-30 July 1991
Type and Frequency of Observation Observed twice daily for mortality/moribundity and clinical signs of toxicity. Clinical observations and individual body weights were recorded on Days 1 and 8 and at the end of the study. Water consumption was recorded once weekly.	Observed twice daily for mortality/moribundity and clinical signs of toxicity. Clinical observations were recorded weekly. Individual body weights were recorded at the start of the study, weekly thereafter, and at the end of the study. Water consumption was recorded twice weekly.
Necropsy Necropsies were performed on all animals. The liver and spleen were weighed at necropsy.	Necropsies were performed on all animals in the base study. The heart, right kidney, liver, lungs, spleen, right testis, and thymus were weighed at necropsy.

TABLE 1 Experimental Design and Materials and Methods in the 2-Week and 13-Week Drinking Water Studies of Cyclohexanone Oxime (continued)

2-Week Study	13-Week Study
Histopathologic Examinations	
Histopathologic examinations were performed on all tissues showing gross lesions.	Histopathologic examinations were performed on all control animals, all animals in the highest exposure groups with at least 60% survivors, all animals in the highest exposure groups, and all animals that died early. The following tissues were examined: adrenal glands, brain (three sections), esophagus, eyes (if grossly abnormal), femur with marrow, gallbladder, gross lesions, heart, intestines (large: cecum, colon, rectum; small: duodenum, jejunum, ileum), kidneys, liver, lungs, lymph nodes (mandibular and mesenteric), mammary glands, nasal cavity and turbinates (three sections), ovaries, pancreas, parathyroid glands, pituitary gland, preputial or clitoral glands, prostate gland, salivary glands, seminal vesicle, spleen, spinal cord and sciatic nerve (if neurological signs present), stomach (forestomach and glandular stomach), testis (with epididymis), thigh muscle (if neurological signs present), thymus, thyroid glands, trachea, urinary bladder, uterus, and vagina (females in vaginal cytology study only). Gross lesions and the bone marrow, liver, nasal cavity and turbinates (three sections), and spleen were examined in the lower exposure groups.
Sperm Motility and Vaginal Cytology Evaluations	
None	Sperm motility and vaginal cytology evaluations were performed on mice in the 0, 1,250, 2,500, and 5,000 ppm groups. Males were evaluated for necropsy body and reproductive tissue weights and spermatid and epididymal spermatozoal data. 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	
12 days	11 days
Age When Study Began	
6 weeks	6 weeks
Age at Necropsy	
8 weeks	19 to 20 weeks
Method of Animal Distribution	
Animals were distributed randomly into groups of approximately equal initial mean body weight.	Same as 2-week study
Diet	
NIH-07 Open Formula Diet (Zeigler Brothers, Inc., Gardners, PA) in pellet form and drinking water containing the appropriate doses, *ad libitum*.	Same as 2-week study
Animal Room Environment	
Mice were housed individually. Temperature was maintained at 72° ± 3° F and relative humidity at 50% ± 15%, with at least 10 air changes per hour. Fluorescent light was provided for 12 hours per day.	Same as 2-week study

Genetic Toxicity

SALMONELLA TYPHIMURIUM MUTAGENICITY TEST PROTOCOL

Testing was performed as reported by Zeiger *et al*. (1992). Cyclohexanone oxime was sent to the laboratory as a coded aliquot and was incubated with the *Salmonella typhimurium* tester strains (TA97, TA98, TA100, and TA1535) 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 cyclohexanone oxime. The high dose was limited by toxicity to 6,666 µg/plate. All positive assays were repeated under the conditions that elicited a positive response, and all negative assays were repeated.

CHINESE HAMSTER OVARY CELL CHROMOSOMAL ABERRATION TEST PROTOCOL

Testing was performed as reported by Galloway *et al*. (1987). Cyclohexanone oxime was sent to the laboratory as a coded aliquot. It was tested in cultured Chinese hamster ovary (CHO) cells for induction of chromosomal aberrations (Abs) both in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 and cofactor mix. Each test consisted of concurrent solvent and positive controls and of at least three doses of cyclohexanone oxime. In the absence of toxicity, 5 mg/mL was selected as the high dose. Doses in the second trial without S9 were selected to bracket the dose that gave a positive response in the first trial without S9. A single flask per dose was used, and tests yielding equivocal or positive results were repeated.

In the Abs test without S9, cells were incubated in McCoy's 5A medium with cyclohexanone oxime for 10 hours; Colcemid was added and incubation continued for 2 hours. The cells were then harvested by mitotic shake-off, fixed, and stained with Giemsa. For the Abs test with S9, cells were treated with cyclohexanone oxime and S9 for 2 hours, after which the treatment medium was removed and the cells incubated for 10 hours in fresh medium, with Colcemid present for the final 2 hours. Cells were harvested in the same manner as for the treatment without S9.

Cells were selected for scoring on the basis of good morphology and completeness of karyotype (21 ± 2 chromosomes). All slides were scored blind and those from a single test were read by the same person. Two hundred first-division metaphase cells were scored at each dose level. Classes of aberrations included simple (breaks and terminal deletions), complex (rearrangements and translocations), and other (pulverized cells, despiralized chromosomes, and cells containing 10 or more aberrations).

BONE MARROW MICRONUCLEUS TEST PROTOCOL

Preliminary range finding studies were performed. Factors affecting dose selection included chemical solubility, toxicity, and the extent of cell cycle delay induced by the chemical exposure. Based on these studies, male mice to be tested for bone marrow micronuclei were injected intraperitoneally three times at 24-hour intervals with cyclohexanone oxime dissolved in corn oil. The total dosing volume was 0.4 mL. Solvent control animals were injected with 0.4 mL corn oil only. The positive control mice received injections of cyclophosphamide. Twenty-four hours after the third injection, the mice were killed and smears of the bone marrow cells obtained from the femurs were prepared. Air-dried smears were fixed and stained; 2,000 polychromatic erythrocytes were scored for frequency of micronucleated cells in each of five animals per dose group.

PERIPHERAL BLOOD MICRONUCLEUS TEST PROTOCOL

A detailed discussion of this assay is presented in MacGregor *et al*. (1990). At the end of the 13-week toxicity study, peripheral blood samples were obtained from male and female mice, and smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were later stained with a chromatin-specific fluorescent dye (acridine orange) and coded. Two thousand normochromatic erythrocytes were scanned in each of five mice per exposure group. The criteria of Schmid (1976) were used in defining micronuclei. The results were tabulated as the mean of the pooled results from all animals within a treatment group plus or minus the standard error of the mean.

Statistical Methods

ANALYSIS AND CALCULATION OF NONNEOPLASTIC LESION INCIDENCES

The incidences of nonneoplastic lesions presented in Tables A1 and A2 are given as the number of animals bearing such lesions at a specific site and the number of animals with that site examined microscopically. For lesions detected in the 13-week study, the Fisher exact test, a procedure based on the overall proportion of affected animals, was used (Gart *et al*., 1979).

ANALYSIS OF CONTINUOUS VARIABLES

Two approaches were employed to assess the significance of pairwise comparisons between exposed and control groups in the analysis of continuous variables. Organ and body weight data, which have approximately normal distributions, were analyzed with the parametric multiple comparisons procedures of Williams (1971, 1972) and Dunnett (1955). Spermatid and epididymal spermatozoal data, which typically have skewed distributions, were analyzed with the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of exposure-response trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume monotonic exposure response (Dunnett's or Dunn's test). Trend-sensitive tests were used when Jonckheere's test was significant at a P-value less than 0.01. Prior to analysis, extreme values identified by the outlier test of Dixon and Massey (1951) were examined by NTP personnel and implausible values 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 simultaneous equality of measurements across exposure levels.

ANALYSIS OF MUTAGENICITY IN *SALMONELLA TYPHIMURIUM*

A positive response in the *Salmonella typhimurium* assay is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, not reproducible, or not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is 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 CHINESE HAMSTER OVARY CELL CHROMOSOMAL ABERRATION DATA

Chromosomal aberration data are presented as percentages of cells with aberrations. To arrive at a statistical call for a trial, analyses were conducted on both the dose-response curve and individual dose points (Galloway *et al*., 1987). For a single trial, a statistically significant ($P<0.05$) difference for one dose point and a significant trend ($P<0.015$) was considered weak evidence for a positive response; significant differences for two or more doses indicated the trial was positive. A positive trend in the absence of a statistically significant increase at any one dose point or a significant increase at a single dose point in the absence of a positive trend led to an equivocal call. Ultimately, the trial calls were based on a consideration of the statistical analyses as well as the biological information available to the reviewers.

ANALYSIS OF PERIPHERAL BLOOD AND BONE MARROW MICRONUCLEUS DATA

The results were tabulated as the mean of the pooled results from all animals within a treatment group, plus or minus the standard error of the mean. The frequency of micronucleated cells among normochromatic erythrocytes was analyzed by a statistical software package that tested for increasing trend over exposure groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each exposure group and the control group (Margolin *et al*., 1990). In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial was considered positive if the trend test P-value was less than or equal to 0.025 or the P-value for any single exposure group was less than or equal to 0.025 divided by the number of exposure groups. A final call of positive for micronucleus induction is preferably based on reproducible positive trials (as noted above). Ultimately, the final

call was determined by the scientific staff after considering the results of statistical analyses, reproducibility of any effects observed, and the magnitudes of those effects.

Quality Assurance

The animal studies of cyclohexanone oxime were performed in compliance with U.S. Food and Drug Administration Good Laboratory Practices regulations (21 CFR, Part 58). The Quality Assurance Unit of Microbiological Associates, Inc. performed audits and inspections of protocols, procedures, data, and reports throughout the course of the studies.

RESULTS

2-Week Drinking Water Study in B6C3F$_1$ Mice

All mice survived until the end of the study (Table 2). Males in the 2,500 ppm group had lower final mean body weights than the controls. The final mean body weights for females in the 625 ppm group were greater than the control value. No clinical signs of toxicity were noted in male mice. One female in the control group and one in the 106 ppm group were observed to be thin. Average daily water consumption was greater than or equal to that of the controls for both males and females at all exposure levels except 2,500 ppm; at this concentration, males and females had lower average water consumption than the controls.

TABLE 2 Survival, Weight Gain, Water Consumption, and Compound Consumption of B6C3F$_1$ Mice in the 2-Week Drinking Water Study of Cyclohexanone Oxime

Concentration (ppm)	Survival[1]	Mean Body Weight (grams) Initial	Final	Change	Final Weight Relative to Controls[2] (%)	Water Consumption (g/day)	Compound Consumption (mg/kg/day)
MALE							
0	5/5	25.5	29.6	4.1		5.3	
106	5/5	25.2	28.6	3.4	97	6.8	27
312	5/5	25.0	28.5	3.4	96	5.7	69
625	5/5	25.4	28.9	3.5	98	6.0	141
1,250	5/5	25.4	28.5	3.1	96	6.6	316
2,500	5/5	25.8	28.1	2.4	95	4.6	439
FEMALE							
0	5/5	19.4	23.1	3.7		6.5	
106	5/5	19.4	22.9	3.5	99	7.3	38
312	5/5	19.8	23.6	3.8	102	6.5	98
625	5/5	19.5	24.3	4.8	105	6.7	203
1,250	5/5	19.8	23.3	3.6	101	7.0	416
2,500	5/5	19.7	22.5	2.8	97	3.7	441

[1] Number surviving at 15 days/number of animals per group.
[2] (Exposure group mean/control group mean) × 100.

Absolute and relative liver and spleen weights for exposed mice in the 2-week study were generally slightly greater than the control values (Table 3). Male mice given 312 ppm or greater had significantly greater relative liver weights than the controls. Relative spleen weights of male mice in the 1,250 and 2,500 ppm were greater than those of the controls. The absolute and relative spleen weights of female mice given 2,500 ppm were significantly greater than those of the controls.

No gross lesions were observed in the 2-week study of cyclohexanone oxime, and no tissues were examined microscopically. Because no mortality, significant decreases in weight gain, or gross lesions were observed in the 2-week study, concentrations for the 13-week study were increased to 5,000 and 10,000 ppm.

TABLE 3 Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 2-Week Drinking Water Study of Cyclohexanone Oxime[1]

	Concentration (ppm)					
	0	106	312	625	1,250	2,500
MALE						
n	5	5	5	5	5	5
Necropsy body wt	29.6 ± 0.8	28.6 ± 0.6	28.5 ± 0.5	28.9 ± 0.9	28.5 ± 0.6	28.1 ± 0.5
Liver						
Absolute	1.565 ± 0.054	1.587 ± 0.042	1.626 ± 0.045	1.704 ± 0.070	1.662 ± 0.054	1.671 ± 0.038
Relative	52.90 ± 0.80	55.60 ± 1.85	57.08 ± 0.91*	58.84 ± 0.86**	58.37 ± 1.02**	59.44 ± 0.97**
Spleen						
Absolute	0.076 ± 0.003	0.080 ± 0.003	0.076 ± 0.002	0.078 ± 0.004	0.082 ± 0.004	0.084 ± 0.002
Relative	2.57 ± 0.04	2.80 ± 0.07	2.66 ± 0.05	2.70 ± 0.07	2.88 ± 0.16*	2.98 ± 0.09**
FEMALE						
n	5	5	5	5	5	5
Necropsy body wt	23.1 ± 0.3	22.9 ± 0.6	23.6 ± 0.9	24.3 ± 0.4	23.3 ± 0.4	22.5 ± 0.4
Liver						
Absolute	1.333 ± 0.049	1.392 ± 0.066	1.394 ± 0.065	1.527 ± 0.061	1.410 ± 0.030	1.353 ± 0.038
Relative	57.73 ± 1.75	61.09 ± 3.78	59.02 ± 1.18	62.93 ± 2.35	60.39 ± 0.28	60.21 ± 0.89
Spleen						
Absolute	0.093 ± 0.002	0.095 ± 0.006	0.098 ± 0.001	0.100 ± 0.002	0.094 ± 0.004	0.115 ± 0.006**
Relative	4.03 ± 0.08	4.14 ± 0.17	4.16 ± 0.13	4.12 ± 0.08	4.03 ± 0.17	5.11 ± 0.26**

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

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test.

** Significantly different ($P \leq 0.01$) from the control group by Williams' or Dunnett's test.

13-Week Drinking Water Study in B6C3F$_1$ Mice

Five male and five female 10,000 ppm mice were killed moribund in the third week of the study (Table 4). All other mice survived until the end of the study. The final mean body weights of males in the 5,000 and 10,000 ppm groups were less than those of the controls (Figure 1). Females in the 2,500, 5,000, and 10,000 ppm groups also had lower final mean body weights than the controls. Clinical signs of toxicity were observed in males from the higher exposure groups. At exposure levels of 2,500 and 5,000 ppm, one male in each group was observed to be hyperactive. All males in the 10,000 ppm group appeared thin, one male was observed to have abnormal posture, and four males had ruffled fur. Clinical signs of toxicity in females were restricted to the 10,000 ppm group; 9 of 10 females were observed to be thin, and two females had abnormal posture and ruffled fur. One female in the 10,000 ppm group was observed to be hyperactive. Lethargy, abnormal breathing, and ataxia were also observed. Average water consumption by males and females decreased with increasing exposure.

TABLE 4 Survival, Weight Gain, Water Consumption, and Compound Consumption of B6C3F$_1$ Mice in the 13-Week Drinking Water Study of Cyclohexanone Oxime

Concentration (ppm)	Survival[1]	Mean Body Weight (grams)			Final Weight Relative to Controls[2] (%)	Water Consumption (g/day)	Compound Consumption (mg/kg/day)
		Initial	Final	Change			
MALE							
0	10/10	26.0	41.5	15.5		5.2	
625	10/10	25.2	42.3	17.1	102	5.0	96
1,250	10/10	25.5	41.5	15.9	100	4.8	181
2,500	10/10	25.8	41.7	15.9	101	3.8	287
5,000	10/10	26.1	39.0	12.9	94	3.6	573
10,000	5/10[3]	25.6	30.6	4.3	74	3.0	1,152
FEMALE							
0	10/10	20.4	35.1	14.7		5.8	
625	10/10	20.3	35.5	15.2	101	5.6	132
1,250	10/10	20.3	35.3	15.0	100	5.2	239
2,500	10/10	20.0	33.2	13.2	95	4.2	403
5,000	10/10	20.4	30.7	10.3	87	3.4	681
10,000	5/10[3]	20.5	25.0	3.9	71	2.9	1,350

[1] Number surviving at 13 weeks/number of animals per group.
[2] (Exposure group mean/control group mean) × 100.
[3] Week of death: 3.

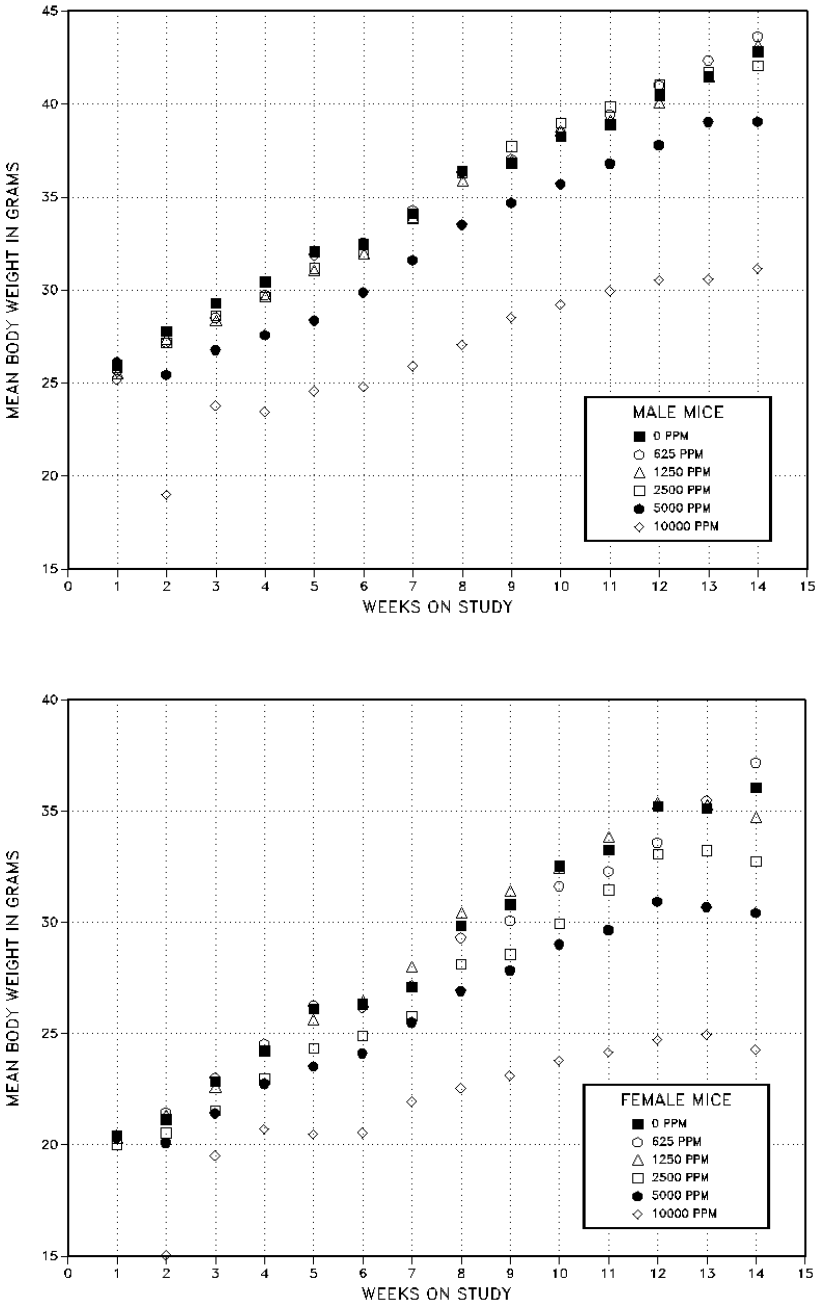


FIGURE 1 Body Weights of B6C3F$_1$ Mice Administered Cyclohexanone Oxime in Drinking Water for 13 Weeks

Spleen weights of male and female mice given 5,000 or 10,000 ppm cyclohexanone oxime were significantly greater than those of the controls (Table 5 and Appendix B). Absolute liver weights of males and females given 10,000 ppm were lower than those of the controls. Relative liver weights of males given 10,000 ppm and females given 5,000 or 10,000 ppm were significantly greater than the controls. Other differences in organ weights were considered secondary to body weight differences. Kidney weight increases may have been due to decreased water consumption.

TABLE 5 Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 13-Week Drinking Water Study of Cyclohexanone Oxime[1]

	Concentration (ppm)					
	0	625	1,250	2,500	5,000	10,000
MALE						
n	10	10	10	10	10	5
Necropsy body wt	43.5 ± 1.1	43.6 ± 0.8	43.2 ± 0.9	42.1 ± 1.0	39.1 ± 0.9**	31.1 ± 1.0**
Right kidney						
Absolute	0.310 ± 0.009	0.304 ± 0.006	0.311 ± 0.009	0.322 ± 0.011	0.322 ± 0.004	0.297 ± 0.010
Relative	7.13 ± 0.16	6.98 ± 0.22	7.20 ± 0.13	7.64 ± 0.10*	8.27 ± 0.17**	9.56 ± 0.26**
Liver						
Absolute	2.010 ± 0.129	2.088 ± 0.046	1.970 ± 0.086	2.046 ± 0.101	1.905 ± 0.056	1.594 ± 0.060*
Relative	45.97 ± 1.96	47.87 ± 0.74	45.48 ± 1.08	48.38 ± 1.27	48.76 ± 0.87	51.18 ± 1.01*
Spleen						
Absolute	0.079 ± 0.003	0.081 ± 0.003	0.077 ± 0.003	0.077 ± 0.003	0.106 ± 0.003**	0.145 ± 0.008**
Relative	1.82 ± 0.06	1.86 ± 0.06	1.79 ± 0.04	1.82 ± 0.06	2.73 ± 0.09**	4.64 ± 0.15**
FEMALE						
n	10	10	10	10	10	5
Necropsy body wt	35.6 ± 1.2	36.8 ± 0.9	34.3 ± 1.4	32.5 ± 1.0	30.4 ± 1.1**	24.2 ± 1.2**
Right kidney						
Absolute	0.209 ± 0.006	0.214 ± 0.004	0.211 ± 0.004	0.217 ± 0.005	0.225 ± 0.005	0.205 ± 0.008
Relative	5.90 ± 0.18	5.84 ± 0.13	6.21 ± 0.21	6.70 ± 0.20**	7.44 ± 0.17**	8.52 ± 0.22**
Liver						
Absolute	1.510 ± 0.052	1.623 ± 0.040	1.446 ± 0.032	1.380 ± 0.033	1.396 ± 0.053	1.172 ± 0.048**
Relative	42.64 ± 1.28	44.13 ± 0.56	42.52 ± 1.14	42.61 ± 0.94	45.92 ± 0.79*	48.72 ± 1.48**
Spleen						
Absolute	0.092 ± 0.002	0.099 ± 0.003	0.098 ± 0.005	0.097 ± 0.004	0.151 ± 0.008**	0.156 ± 0.016**
Relative	2.60 ± 0.09	2.69 ± 0.11	2.87 ± 0.14	3.00 ± 0.12	4.97 ± 0.21**	6.41 ± 0.48**

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

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test.

** Significantly different ($P \leq 0.01$) from the control group by Williams' or Dunnett's test.

There were no significant differences in sperm motility or vaginal cytology parameters between exposed and control males and females (Tables C1 and C2).

At necropsy, no gross findings other than the thin carcasses of mice from the highest exposure (10,000 ppm) groups were attributed to compound exposure. Microscopically, treatment-related effects were present in the spleen, bone marrow, liver, and nasal cavity.

In the spleen, increased hematopoietic cell proliferation was associated with exposure to cyclohexanone oxime in both male and female mice and correlated with increased spleen weights. This change, minimal to moderate in severity, was characterized by an increased amount of hematopoietic cells, primarily of the erythroid series, in the splenic red pulp of treated mice relative to that seen in the controls (Table 6). In cases of mild to moderate severity, an increase in the amount of hemosiderin pigment accompanied the increased hematopoiesis. In females, the incidence and severity of this effect was dose dependent at concentrations of 1,250 ppm and above; in males the lesion was present only at the two highest exposure levels (5,000 and 10,000 ppm). At 10,000 ppm, splenic hematopoietic cell proliferation was present only in those mice which survived to study termination.

A treatment-related increase in hematopoietic proliferation in the bone marrow, diagnosed as "hyperplasia," occurred much less frequently than in the spleen; this change was present in only three of the surviving males from the 10,000 ppm group (Table 6). Depletion of bone marrow cells attributable to moribund condition was seen in a single early death female from the 10,000 ppm group.

In the liver, exposure to cyclohexanone oxime was associated with hypertrophy of centrilobular hepatocytes, which correlated with increased relative liver weights (Table 6). Affected cells had increased amounts of cytoplasm which frequently had an eosinophilic "ground glass" appearance. Nuclei of affected centrilobular hepatocytes also tended to be larger than those of the periportal cells. In males, the incidence and severity of this change was dose dependent at concentrations of 1,250 ppm and greater; in females, the lesion was present only at the two highest exposure levels (5,000 and 10,000 ppm). At the highest exposure level (10,000 ppm), centrilobular hypertrophy was present in only those mice that survived to study termination; in those that did not survive, shrinkage of hepatic cords and loss of cytoplasmic volume consistent with glycogen depletion was observed.

In the nasal cavity, degeneration of the olfactory epithelium was present in most surviving mice from all treatment groups (Table 6). This change was characterized by decreased height and cell number and loss of cellular organization within the neuroepithelium. Occasionally, regenerative basal cell proliferation was also present in involved areas. Degeneration was most common in the dorsal meatus of the posterior nasal section.

TABLE 6 Incidence and Severity of Selected Lesions in B6C3F$_1$ Mice in the 13-Week Drinking Water Study of Cyclohexanone Oxime[1]

	Concentration (ppm)					
	0	625	1,250	2,500	5,000	10,000
MALE						
n	10	10	10	10	10	10
Spleen						
Hematopoietic cell proliferation	0	0	0	0	10** (1.6)	5* (2.2)
Bone marrow						
Hyperplasia	0	0	0	0	0	3 (1.7)
Liver						
Depletion, glycogen	0	0	0	0	0	5* (2.8)
Centrilobular, hypertrophy	0	0	1 (1.0)	7** (1.0)	10** (1.0)	5* (1.4)
Nasal cavity						
Olfactory epithelium, degeneration	0	2 (1.0)	10** (1.0)	10** (1.3)	10** (1.2)	4* (1.3)
FEMALE						
n	10	10	10	10	10	10
Spleen						
Hematopoietic cell proliferation	0	0	2 (1.5)	3 (1.0)	10** (2.4)	5* (2.4)
Bone marrow						
Depletion, cellular	0	0	0	0	0	1 (2.0)
Liver						
Depletion, glycogen	0	0	0	0	0	5* (3.4)
Centrilobular, hypertrophy	0	0	0	0	9** (1.0)	4* (1.5)
Nasal Cavity						
Olfactory epithelium, degeneration	0	10** (1.1)	10** (1.5)	10** (1.4)	9** (1.8)	5* (1.4)

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

* Significantly different ($P \leq 0.05$) from the control group by the Fisher exact test.

** Significantly different ($P \leq 0.01$) from the control group by the Fisher exact test.

Other than glycogen depletion in the liver, no microscopic changes were consistently observed in the mice that died prior to study termination. The specific cause of death could not be determined from these studies, but most were attributed to systemic toxicity and generalized debilitation.

Genetic Toxicity

Cyclohexanone oxime was tested for mutagenicity in four strains of *Salmonella typhimurium* in a preincubation protocol with and without induced rat or hamster liver S9 (Table D1). Positive results were obtained only in strain TA1535 in the presence of 5%, 10%, or 30% hamster S9; negative results were obtained in strain TA1535 with rat liver S9 and in the absence of S9. Results of mutagenicity testing of cyclohexanone oxime were negative in strains TA97, TA98, and TA100, with or without S9.

No clear indication of mutagenic activity was observed in mammalian cell cytogenicity tests with cyclohexanone oxime. Equivocal results were obtained in a test for induction of chromosomal aberrations in cultured Chinese hamster ovary (CHO) cells in the absence of S9 (Table D2). In each of two trials conducted without S9, a significant increase in aberrations was observed in one of three dose groups tested, but the responses were not related to exposure and results of the trend tests were negative. No induction of aberrations was noted in CHO cells treated with cyclohexanone oxime in the presence of S9. Two *in vivo* micronucleus tests were performed with cyclohexanone oxime in mice. Both tests had negative results. In the first test, cyclohexanone oxime was administered by intraperitoneal injection (400 to 1,000 mg/kg) to male mice three times at 24-hour intervals; no increase in the frequency of micronucleated polychromatic erythrocytes was observed in bone marrow preparations obtained 24 hours after the final injection (Table D3). In the second test, male and female mice were administered cyclohexanone oxime (625 to 10,000 ppm) in drinking water for 13 weeks; no increase in the frequency of micronucleated normochromatic erythrocytes was observed in peripheral blood smears of treated mice (Table D4).

DISCUSSION

Oximes are a class of chemicals that are produced in relatively large quantities and are used in a variety of industrial applications. Despite the potential for widespread exposure, little is known regarding the potential toxicity of oximes. Cyclohexanone oxime, an important intermediate in the production of Nylon-6, was chosen by the NTP as a representative alicyclic oxime for toxicity testing. The NTP has conducted a 2-week range-finding study and a 13-week subchronic study in B6C3F_1 mice, as well as genetic toxicology and chemical disposition studies.

All mice survived to the end of the 2-week study. Final mean body weights were between 95% and 105% of those of the controls. No clinical signs of toxicity were observed in male mice; two female mice, one of which was a control, appeared thin. Decreased water consumption was observed for males and females in the 2,500 ppm groups (the highest exposure groups). Slightly increased relative liver weights were observed in male mice given 312 ppm or greater; no significant organ weight effect was observed in female mice at any exposure level. Increased relative spleen weights were observed in male mice in the 1,250 and 2,500 ppm groups and in female mice in the 2,500 ppm group. This is in contrast to the F344 rat study of Derelanko *et al*. (1985), where splenomegaly occurred at a lower exposure than hepatomegaly. Hepatomegaly in male rats was observed at a lower exposure concentration than in female rats; this was similar to the response observed in the 2-week study in mice.

Exposure levels were increased in the 13-week study because only relatively minor effects were observed in the 2-week study. In the 13-week study, five males and five females were killed moribund in the 10,000 ppm group; final mean body weights were reduced in males in the 5,000 and 10,000 ppm groups and in females in the 2,500, 5,000, and 10,000 ppm groups. Spleen weights of males and females were elevated in the 5,000 and 10,000 ppm groups; however, in contrast to the 2-week study, spleen weights of males and females in the 1,250 and 2,500 ppm groups were similar to those of the controls. In general, relatively more cyclohexanone oxime was consumed at a given exposure level in the 2-week study compared to the 13-week study. For example, males that received 1,250 ppm in the 2-week study consumed an average of approximately 316 mg/kg daily; in contrast, in the 13-week study, males that received 1,250 ppm consumed an average of only 181 mg/kg daily. This difference may be due in part to the normal pattern of water and feed consumption as a function of body weight as young animals grow. The

spleen effect in the 2-week study may also be a consequence of the destruction of susceptible erythrocytes; as these cells are replaced with less susceptible cells, the spleen is able to recover. Gad *et al*. (1985) observed a decrease in erythrocyte fragility following 13 weeks of treatment in the rat study. This was considered to be due to the cell population being selected for younger cells that were more resistant to toxic effects.

Increased spleen weights were correlated microscopically with increased hematopoietic cell proliferation in the spleen. Hematopoietic cell proliferation (hyperplasia) was also observed in the bone marrow. Similar observations were made in the F344 rat studies (Derelanko *et al*., 1985; Gad *et al*., 1985). These observations are consistent with a cyclohexanone oxime-mediated destruction of erythrocytes. It has been proposed that erythrocyte destruction results from reaction of the hemolytic agent with hemoglobin, generating radical species which result in peroxidation of the cellular membrane which in turn damages the erythrocytes and leads to their eventual destruction and removal (Gad *et al*., 1985).

In the metabolism study of cyclohexanone oxime, it was observed that the oxime rapidly hydrolyzes to cyclohexanone and, presumably, hydroxylamine. Hydroxylamine (2,600 ppm hydroxylamine sulfate in the drinking water for 52 weeks) causes hematologic effects, such as methemoglobinemia and splenomegaly in mice (Yamamoto *et al*., 1967; Gross, 1985), similar to those observed after exposure to cyclohexanone oxime. Thus, it is not clear whether the erythrotoxicity of cyclohexanone oxime results from the parent compound or hydroxylamine, the hydrolysis product.

In a 2-year study, male and female rats were given 3,300 or 6,500 ppm, male mice were given 6,500 or 13,000 ppm, and female mice were given 6,500, 13,000, or 25,000 ppm cyclohexanone in drinking water (Lijinsky and Kovatch, 1986). No mention is made of splenomegaly or hematotoxicity in that study. There is, however, a report (Koeferl *et al*., 1981) of hemosiderin deposits and extramedullary hematopoiesis in the spleen of beagle dogs repeatedly administered cyclohexanone intravenously (284 mg/kg per day administered as a 6% solution, either 5 or 75 mL/min). There was no increase in absolute or relative spleen weight in this study. Because cyclohexanone has not been reported to be hematotoxic to other species or by other routes of administration, it is possible that the effect in dogs was due to a relatively high concentration of good lipid solvent disrupting the erythrocyte membrane. It seems far more likely that the

erythrotoxicity of cyclohexanone oxime is due to the parent or hydroxylamine rather than due to cyclohexanone or a cyclohexanone-derived metabolite.

Cyclohexanone oxime's effect on liver weight was also observed at much lower exposures in the 2-week study compared to the 13-week study. Relative liver weights of males given 10,000 ppm and females given 5,000 and 10,000 ppm were greater than those of the controls. In the 2-week study, increased liver weights were observed in males given 312 ppm or greater. Again, this may be due in part to lower actual consumption of cyclohexanone oxime at the same exposure level and also to compensation by the liver to the longer exposure.

Microscopic examination of the liver revealed hypertrophy of centrilobular hepatocytes which correlated with increased liver weights. While hepatomegaly was observed in the F344 rat studies, and the liver was examined histopathologically, no pathologic findings were reported (Derelanko *et al*., 1985; Gad *et al*., 1985). Chemical-related centrilobular hypertrophy is presumed to be due to induction of xenobiotic metabolizing enzymes and proliferation of smooth endoplasmic reticulum. The centrilobular region is where certain xenobiotic metabolizing enzymes are concentrated, and inducers sometimes result in organelle and cell proliferation in this region (Baron and Kawabata, 1983). There are apparently no studies of enzyme induction by cyclohexanone oxime in mice. However, some cyclohexanone oxime metabolites in rats appear to require cytochrome P_{450}-mediated oxidation (Parmar and Burka, 1991), and cyclohexanone oxime has been reported to induce liver microsomal activity in the rat (Komsta *et al*., 1989). In contrast, cyclohexanone apparently does not induce cytochrome P_{450} (Gupta *et al*., 1979).

The only other microscopically observed, treatment-related effect was in the nasal cavity, where degeneration of the olfactory epithelium was present in most surviving mice in all treatment groups. This lesion was not noted in the rat studies, although it may not have been looked for (Derelanko *et al*., 1985; Gad *et al*., 1985). Likewise, this lesion was not noted in the toxicity studies of cyclohexanone in mice, even when exposure was by inhalation (Gupta *et al*., 1979). Olfactory toxicity following systemic exposure has been observed with numerous chemicals and has been related to site-specific metabolism in this tissue for some chemicals (Gaskell, 1990). Studies in rabbits have demonstrated that cytochrome P_{450} is present in nasal tissue and can be induced by exposure to xenobiotics (Ding and Coon, 1990).

In parallel to the studies on cyclohexanone oxime, 2-week and 13-week studies were also performed with both F344/N rats and B6C3F$_1$ mice on an acyclic oxime analogue, methyl ethyl ketoxime. In mice, the two chemicals exhibited very similar effects. Exposure to similar concentrations of either chemical resulted in similarly increased liver and spleen weights. The only marked difference in this parameter occurred at the 10,000 ppm concentration, where the relative spleen weight following exposure to methyl ethyl ketoxime was approximately 14 mg per gram body weight, compared to 4 to 6 mg per gram body weight for the same cyclohexanone oxime exposure. Lesions were observed in the liver, spleen, and nasal epithelium for both chemicals. Hyperplasia of the urinary bladder transitional epithelium was observed in the methyl ethyl ketoxime study but not in the cyclohexanone oxime study.

In summary, the major targets of cyclohexanone oxime toxicity are the erythrocyte, the liver, and nasal epithelium. The no-effect level for erythrotoxicity is 2,500 ppm following 13 weeks of exposure. The no-effect level for hematopoietic cell proliferation in the spleen is 2,500 ppm. The no-effect level for hepatotoxicity is 1,250 ppm for males and 2,500 ppm for females following 13 weeks of exposure. Some nasal olfactory epithelial degeneration was observed at all exposure levels; only at 625 ppm in males was the incidence of this lesion not significantly different from that in the controls. Cyclohexanone oxime was mutagenic only with *Salmonella typhimurium* strain TA1535 with S9 activation.

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APPENDIX A

Summary of Nonneoplastic Lesions in Mice

TABLE A1 Summary of the Incidence of Nonneoplastic Lesions in Male B6C3F$_1$ Mice in the 13-Week Drinking Water Study of Cyclohexanone Oxime[1]

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Moribund						5
Survivors						
Terminal sacrifice	10	10	10	10	10	5
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Depletion glycogen						5 (50%)
Hemorrhage		1 (10%)				
Inflammation		1 (10%)				
Centrilobular, hypertrophy			1 (10%)	7 (70%)	10 (100%)	5 (50%)
Centrilobular, cytoplasmic vacuolization	1 (10%)	3 (30%)	4 (40%)	2 (20%)		
Pancreas	(10)				(10)	(10)
Inflammation	1 (10%)					
Cardiovascular System						
None						
Endocrine System						
None						
General Body System						
None						
Genital System						
None						
Hematopoietic System						
Bone marrow	(10)	(10)	(10)	(10)	(10)	(10)
Hyperplasia						3 (30%)
Lymph node, mandibular	(10)				(10)	(10)
Hemorrhage	3 (30%)				2 (20%)	1 (10%)
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Hematopoietic cell proliferation					10 (100%)	5 (50%)
Red pulp, cellular depletion						4 (40%)
Thymus	(10)				(10)	(9)
Atrophy						4 (44%)
Hemorrhage					2 (20%)	

TABLE A1 Summary of the Incidence of Nonneoplastic Lesions in Male B6C3F$_1$ Mice in the 13-Week Drinking Water Study of Cyclohexanone Oxime (continued)

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Integumentary System						
Skin	(10)			(1)	(10)	(10)
Hair follicle, atrophy				1 (100%)		
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(10)				(10)	(10)
Hemorrhage	1 (10%)					
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Olfactory epithelium, degeneration		2 (20%)	10 (100%)	10 (100%)	10 (100%)	4 (40%)
Olfactory epithelium, hemorrhage		1 (10%)				
Special Senses System						
None						
Urinary System						
None						

[1] Number of animals examined microscopically at site and number of animals with lesion.

TABLE A2 Summary of the Incidence of Nonneoplastic Lesions in Female B6C3F$_1$ Mice in the 13-Week Drinking Water Study of Cyclohexanone Oxime[1]

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Early deaths						
Moribund						4
Natural death						1
Survivors						
Terminal sacrifice	10	10	10	10	10	5
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Depletion glycogen						5 (50%)
Inflammation	1 (10%)					
Centrilobular, hypertrophy					9 (90%)	4 (40%)
Hematopoietic cell proliferation				1 (10%)		
Pancreas	(10)	(1)			(10)	(10)
Duct, cyst		1 (100%)				
Salivary glands	(10)				(10)	(10)
Infiltration cellular, lymphocyte	1 (10%)				1 (10%)	
Cardiovascular System						
None						
Endocrine System						
Parathyroid gland	(8)				(8)	(8)
Infiltration cellular, lymphocyte	1 (13%)					
General Body System						
None						
Genital System						
Uterus	(10)				(10)	(10)
Atrophy						4 (40%)
Hematopoietic System						
Bone marrow	(10)	(10)	(10)	(10)	(10)	(10)
Depletion cellular						1 (10%)
Hemorrhage						1 (10%)
Lymph node, mandibular	(10)				(10)	(9)
Hemorrhage	2 (20%)				1 (10%)	1 (11%)
Infiltration cellular, lymphocyte						1 (11%)
Lymph node, mesenteric	(10)				(10)	(8)
Atrophy						1 (13%)
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Hematopoietic cell proliferation			2 (20%)	3 (30%)	10 (100%)	5 (50%)

Lymphoid follicle, depletion	2 (2 0 %)
Red pulp, cellular depletion	1 (10%)

TABLE A2 Summary of the Incidence of Nonneoplastic Lesions in Female B6C3F$_1$ Mice in the 13-Week Drinking Water Study of Cyclohexanone Oxime (continued)

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Hematopoietic System (continued)						
Thymus	(10)				(10)	(8)
Atrophy						4 (50%)
Hemorrhage						1 (13%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(10)				(10)	(10)
Hemorrhage						1 (10%)
Nose	(10)	(10)	(10)	(10)	(10)	(10)
Olfactory epithelium, degeneration		10 (100%)	10 (100%)	10 (100%)	9 (90%)	5 (50%)
Special Senses System						
None						
Urinary System						
Urinary bladder	(10)				(10)	(10)
Infiltration cellular, lymphocyte	1 (10%)				1 (10%)	1 (10%)
Inflammation					1 (10%)	

[1] Number of animals examined microscopically at site and number of animals with lesion.

APPENDIX B

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

TABLE B Organ Weights and Organ-Weight-to-Body-Weight Ratios for B6C3F$_1$ Mice in the 13-Week Drinking Water Study of Cyclohexanone Oxime[1]

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
n	10	10	10	10	10	5
MALE						
Necropsy body wt	43.5 ± 1.1	43.6 ± 0.8	43.2 ± 0.9	42.1 ± 1.0	39.1 ± 0.9**	31.1 ± 1.0**
Heart						
Absolute	0.160 ± 0.005	0.173 ± 0.006	0.166 ± 0.005	0.177 ± 0.007	0.171 ± 0.005	0.156 ± 0.005
Relative	3.69 ± 0.07	3.96 ± 0.11	3.84 ± 0.07	4.21 ± 0.12**	4.39 ± 0.10**	5.01 ± 0.09**
Right kidney						
Absolute	0.310 ± 0.009	0.304 ± 0.006	0.311 ± 0.009	0.322 ± 0.011	0.322 ± 0.004	0.297 ± 0.010
Relative	7.13 ± 0.16	6.98 ± 0.22	7.20 ± 0.13	7.64 ± 0.10*	8.27 ± 0.17**	9.56 ± 0.26**
Liver						
Absolute	2.010 ± 0.129	2.088 ± 0.046	1.970 ± 0.086	2.046 ± 0.101	1.905 ± 0.056	1.594 ± 0.060*
Relative	45.97 ± 1.96	47.87 ± 0.74	45.48 ± 1.08	48.38 ± 1.27	48.76 ± 0.87	51.18 ± 1.01*
Lungs						
Absolute	0.298 ± 0.016	0.308 ± 0.018	0.304 ± 0.011	0.303 ± 0.007	0.320 ± 0.007	0.314 ± 0.006
Relative	6.88 ± 0.37	7.03 ± 0.37	7.06 ± 0.22	7.21 ± 0.15	8.22 ± 0.18**	10.09 ± 0.22**
Spleen						
Absolute	0.079 ± 0.003	0.081 ± 0.003	0.077 ± 0.003	0.077 ± 0.003	0.106 ± 0.003**	0.145 ± 0.008**
Relative	1.82 ± 0.06	1.86 ± 0.06	1.79 ± 0.04	1.82 ± 0.06	2.73 ± 0.09**	4.64 ± 0.15**
Right testis						
Absolute	0.120 ± 0.004	0.128 ± 0.004	0.127 ± 0.002	0.128 ± 0.003	0.128 ± 0.002	0.120 ± 0.001
Relative	2.78 ± 0.10	2.94 ± 0.06	2.95 ± 0.07	3.05 ± 0.08*	3.29 ± 0.10**	3.86 ± 0.11**
Thymus						
Absolute	0.070 ± 0.009	0.069 ± 0.007	0.074 ± 0.006	0.066 ± 0.005	0.056 ± 0.006	0.047 ± 0.009
Relative	1.60 ± 0.19	1.58 ± 0.15	1.71 ± 0.14	1.59 ± 0.14	1.43 ± 0.15	1.49 ± 0.23
FEMALE						
Necropsy body wt	35.6 ± 1.2	36.8 ± 0.9	34.3 ± 1.4	32.5 ± 1.0	30.4 ± 1.1**	24.2 ± 1.2**
Heart						
Absolute	0.137 ± 0.003	0.134 ± 0.002	0.144 ± 0.004	0.133 ± 0.003	0.137 ± 0.007	0.122 ± 0.008
Relative	3.87 ± 0.12	3.64 ± 0.07	4.26 ± 0.18	4.14 ± 0.16	4.52 ± 0.17**	5.04 ± 0.14**
Right kidney						
Absolute	0.209 ± 0.006	0.214 ± 0.004	0.211 ± 0.004	0.217 ± 0.005	0.225 ± 0.005	0.205 ± 0.008
Relative	5.90 ± 0.18	5.84 ± 0.13	6.21 ± 0.21	6.70 ± 0.20**	7.44 ± 0.17**	8.52 ± 0.22**
Liver						
Absolute	1.510 ± 0.052	1.623 ± 0.040	1.446 ± 0.032	1.380 ± 0.033	1.396 ± 0.053	1.172 ± 0.048**
Relative	42.64 ± 1.28	44.13 ± 0.56	42.52 ± 1.14	42.61 ± 0.94	45.92 ± 0.79*	48.72 ± 1.48**
Lungs						
Absolute	0.283 ± 0.008	0.269 ± 0.017	0.311 ± 0.010	0.301 ± 0.012	0.288 ± 0.014	0.278 ± 0.014
Relative	8.07 ± 0.39	7.34 ± 0.46	9.17 ± 0.41	9.27 ± 0.34	9.53 ± 0.44*	11.61 ± 0.68**
Spleen						
Absolute	0.092 ± 0.002	0.099 ± 0.003	0.098 ± 0.005	0.097 ± 0.004	0.151 ± 0.008**	0.156 ± 0.016**
Relative	2.60 ± 0.09	2.69 ± 0.11	2.87 ± 0.14	3.00 ± 0.12	4.97 ± 0.21**	6.41 ± 0.48**
Thymus						
Absolute	0.071 ± 0.005	0.066 ± 0.004	0.067 ± 0.005	0.065 ± 0.003	0.059 ± 0.005	0.057 ± 0.003
Relative	2.00 ± 0.11	1.80 ± 0.10	1.93 ± 0.08	2.03 ± 0.12	1.92 ± 0.11	2.41 ± 0.22

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

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test.

** Significantly different ($P \leq 0.01$) from the control group by Williams' or Dunnett's test.

APPENDIX C

Reproductive Tissue Evaluations and Estrous Cycle Characterization

TABLE C1 Summary of Reproductive Tissue Evaluations in Male B6C3F$_1$ Mice in the 13-Week Drinking Water Study of Cyclohexanone Oxime[1]

Study Parameters	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
n	10	10	10	10
Weights (g)				
Necropsy body weight	43.5 ± 1.1	43.2 ± 0.9	42.1 ± 1.0	39.1 ± 0.9**
Left epididymis	0.054 ± 0.002	0.051 ± 0.001	0.053 ± 0.001	0.053 ± 0.001
Left cauda epididymis	0.019 ± 0.001	0.020 ± 0.001	0.021 ± 0.001	0.019 ± 0.001
Left testis	0.120 ± 0.003	0.123 ± 0.003	0.122 ± 0.003	0.125 ± 0.002
Spermatid measurements				
Spermatid heads (10^7/g testis)	20.34 ± 0.50	20.24 ± 0.40	19.40 ± 0.61	20.50 ± 0.35
Spermatid heads (10^7/testis)	2.43 ± 0.05	2.49 ± 0.05	2.35 ± 0.04	2.57 ± 0.05
Spermatid count (mean/10^{-4} mL suspension)	76.05 ± 1.56	77.80 ± 1.42	73.33 ± 1.31	80.33 ± 1.45
Epididymal spermatozoal measurements				
Motility (%)	87.89 ± 0.59	88.75 ± 0.58	87.80 ± 0.36	86.93 ± 0.50
Concentration (10^6/g cauda epididymal tissue)	755 ± 31	716 ± 67	647 ± 36	668 ± 44

[1] Data are presented as mean ± standard error. Differences from the control group for spermatid and epididymal spermatozoal measurements are not significant by Dunn's test.

** Significantly different ($P \leq 0.01$) from the control group by Shirley's test.

TABLE C2 Summary of Estrous Cycle Characterization in Female B6C3F$_1$ Mice in the 13-Week Drinking Water Study of Cyclohexanone Oxime[1]

Study Parameters	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
n	10	10	10	10
Necropsy body weight (g)	35.6 ± 1.2	34.3 ± 1.4	32.5 ± 1.0	30.4 ± 1.1**
Estrous cycle length (days)	4.90 ± 0.26	4.90 ± 0.37	4.70 ± 0.13	4.60 ± 0.15
Estrous stages (% of cycle)				
Diestrus	33.3	29.2	25.0	26.7
Proestrus	15.8	17.5	20.0	17.5
Estrus	31.7	34.2	33.3	33.3
Metestrus	19.2	19.2	21.7	22.5

[1] Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the control group for estrous cycle length are not significant by Dunn's test. By multivariate analysis of variance, exposed groups did not differ significantly from the control group in the relative length of time spent in the estrous stages.

** Significantly different ($P \leq 0.01$) from the control group by Shirley's test.

APPENDIX D

Genetic Toxicology

TABLE D1 Mutagenicity of Cyclohexanone Oxime in *Salmonella typhimurium*[1]

Strain	Dose (g/plate)	Revertants/plate[2] -S9	+30% hamster S9	+30% rat S9
TA97	0	118 ± 11.4	159 ± 5.5	195 ± 4.0
	10			35 ± 4.0
	33	135 ± 8.1	168 ± 23.4	208 ± 5.0
	100	153 ± 3.5	158 ± 4.7	175 ± 5.7
	333	130 ± 9.5	177 ± 12.0	208 ± 5.7
	1,000	128 ± 10.7	156 ± 4.0	198 ± 15.4
	3,333	69 ± 2.4	163 ± 11.4	179 ± 7.8
Trial summary		Negative	Negative	Negative
Positive control[3]		350 ± 12.8	383 ± 11.5	357 ± 17.9
TA98	0	35 ± 2.3	35 ± 7.5	49 ± 5.7
	33	40 ± 4.6	45 ± 2.2	38 ± 2.7
	100	39 ± 6.7	43 ± 2.9	37 ± 2.2
	333	35 ± 2.2	37 ± 4.8	36 ± 2.5
	1,000	31 ± 6.9	55 ± 3.3	21 ± 2.4
	3,333		34 ± 7.0	21 ± 4.3
Trial summary		Negative	Negative	Negative
Positive control		466 ± 19.4	435 ± 28.9	151 ± 19.4
TA100	0	149 ± 5.0	145 ± 4.1	159 ± 5.4
	10	145 ± 2.3		
	33	155 ± 10.9	176 ± 7.3	155 ± 20.9
	100	118 ± 3.1	135 ± 4.9	170 ± 7.7
	333	135 ± 4.0	149 ± 5.4	138 ± 20.6
	1,000	115 ± 11.4	157 ± 11.3	148 ± 5.2
	3,333		127 ± 7.5	122 ± 19.7
Trial summary		Negative	Negative	Negative
Positive control		764 ± 25.4	557 ± 16.2	406 ± 10.5

TABLE D1 Mutagenicity of Cyclohexanone Oxime in *Salmonella typhimurium* (continued)

Strain	Dose (μg/plate)	Revertants/plate -S9 Trial 1	-S9 Trial 2	+hamster S9 5%	+hamster S9 5%	+hamster S9 10%	+hamster S9 10%	+hamster S9 10%
TA1535	0	10 ± 1.7	8 ± 2.2	8 ± 1.0	11 ± 2.2	10 ± 3.2	12 ± 2.2	10 ± 2.7
	3		8 ± 1.2					
	10		7 ± 0.9					
	33	18 ± 5.8	7 ± 0.3					
	100	19 ± 1.5	6 ± 1.5			12 ± 1.2		
	333	18 ± 4.4	8 ± 1.5	11 ± 2.2	22 ± 1.7	8 ± 2.5	23 ± 2.3	14 ± 3.8
	666			9 ± 1.5	33 ± 1.7	10 ± 0.7	52 ± 1.9	27 ± 3.6
	1,000	17 ± 4.2		10 ± 2.6	33 ± 3.3	13 ± 3.7	58 ± 5.4	48 ± 2.2
	1,666			8 ± 2.1	37 ± 1.5	11 ± 0.6	59 ± 4.5	63 ± 3.5
	3,333	6 ± 2.6		3 ± 1.0	30 ± 3.4	5 ± 1.0	104 ± 4.2	56 ± 7.3
	6,666			0 ± 0.0	1 ± 1.0[4]	1 ± 0.9		0 ± 0.0[4]
Trial summary		Equivocal	Negative	Negative	Positive	Negative	Positive	Positive
Positive control		826 ± 29.9	1,186 ± 56.2	95 ± 13.9	102 ± 7.4	69 ± 3.5	63 ± 1.9	73 ± 4.0

TA1535 (continued)

Dose (μg/plate)	+hamster S9 (continued) 30%	30%	30%	+rat S9 30%
0	11 ± 0.3	10 ± 2.9	10 ± 0.7	14 ± 2.4
33	11 ± 1.5			10 ± 0.6
100	5 ± 0.3			10 ± 1.8
333	6 ± 0.3	9 ± 2.7	14 ± 1.7	12 ± 2.3
666		10 ± 3.6	25 ± 1.5	
1,000	17 ± 1.0	11 ± 1.3	37 ± 1.2	17 ± 1.3
1,666		21 ± 5.2	53 ± 2.6	
3,333	31 ± 1.2	17 ± 2.5	72 ± 5.0	16 ± 2.6
6,666		5 ± 1.2	9 ± 1.7[4]	
Trial summary	Equivocal	Equivocal	Positive	Negative
Positive control	279 ± 10.5	141 ± 14.0	75 ± 7.0	51 ± 5.6

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

TABLE D2 Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Cyclohexanone Oxime[1]

-S9					+S9				
Dose (g/mL)	Total Cells	No. of Abs	Abs/ Cell	Cells with Abs (%)	Dose (g/mL)	Total Cells	No. of Abs	Abs/ Cell	Cells with Abs (%)
Trial 1 — Harvest time: 12 hours Summary: Equivocal					**Trial 1** — Harvest time: 12 hours Summary: Negative				
Dimethylsulfoxide					Dimethylsulfoxide				
	200	2	0.01	1.0		200	1	0.01	0.5
Mitomycin-C					Cyclophosphamide				
0.4	25	10	0.40	36.0	20	25	14	0.56	44.0
Cyclohexanone oxime					Cyclohexanone oxime				
1,081	200	10	0.05	4.5*	1,081	200	1	0.01	0.5
2,325	200	4	0.02	1.5	2,325	200	0	0.00	0.0
5,000[2]	200	1	0.01	0.5	5,000[2]	200	3	0.02	1.5
				P=0.853[3]					P=0.158
Trial 2 — Harvest time: 12 hours Summary: Equivocal									
Dimethylsulfoxide									
	200	2	0.01	0.5					
Mitomycin-C									
0.4	25	16	0.64	32.0					
Cyclohexanone oxime									
500	200	3	0.02	1.5					
1,000	200	7	0.04	3.5*					
2,000	200	3	0.02	0.5					
				P=0.301					

[1] Study performed at Sitek Research Laboratories. A detailed description of the protocol is presented in Galloway *et al.* (1987). Abs = aberrations.
[2] Precipitate formed at this dose level.
[3] Significance of percent cells with aberrations tested by the linear regression trend test versus log of the dose.
* Positive (P<0.05).

TABLE D3 Induction of Micronuclei in Bone Marrow Erythrocytes of Male B6C3F$_1$ Mice by Cyclohexanone Oxime[1]

	Dose (mg/kg)	Micronucleated PCEs/1,000 PCEs[2]	Number examined
Corn oil		1.1 ± 0.29	5
Cyclophosphamide	25	8.4 ± 0.24	5
Cyclohexanone oxime	400	1.2 ± 0.41	5
	600	1.2 ± 0.56	5
	800	0.5 ± 0.22	5
	1,000	1.5 ± 0.41	4
		P=0.527[3]	

[1] PCEs = polychromatic erythrocytes. Data are presented as mean ± standard error. Differences from the control group were not significant by a *t*-test.
[2] Two thousand polychromatic erythrocytes were scored per animal.
[3] Significance of micronucleated PCEs/1,000 PCEs tested by a one-tailed trend test.

TABLE D4 Frequency of Micronuclei in Peripheral Blood of Male and Female B6C3F$_1$ Mice Exposed to Cyclohexanone Oxime in Drinking Water[1]

	Concentration (ppm)	Micronucleated NCEs/1,000 NCEs[2]	Number examined
MALE	0	4.3 ± 0.3	5
	625	3.9 ± 0.9	5
	1,250	3.6 ± 0.2	5
	2,500	3.6 ± 0.7	5
	5,000	1.1 ± 0.3	5
	10,000	1.2 ± 0.5	5
		P=1.000[3]	
FEMALE	0	2.9 ± 0.6	5
	625	3.2 ± 0.7	5
	1,250	1.8 ± 0.5	5
	2,500	2.4 ± 0.7	5
	5,000	0.7 ± 0.3	5
	10,000	0.9 ± 0.2	5
		P=1.000	

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

APPENDIX E

Metabolism and Disposition Study

Metabolism and Disposition of Cyclohexanone Oxime in Male F-344 Rats

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Drug Metabolism and Disposition **19, 1101-1107 (1991)**

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METABOLISM AND DISPOSITION OF CYCLOHEXANONE OXIME IN MALE F-344 RATS

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ABSTRACT:

Cyclohexanone oxime (CHOX), an intermediate used in the synthesis of Polycaprolactam/Nylon, was found to be rapidly absorbed and cleared from the body within 24 hours after a single oral administration of 1, 10 and 30 mg/kg of [^{14}C]-CHOX to the adult male Fischer rats. The majority of the CHOX derived radioactivity (65–90% of the dose) was excreted in the urine. Elimination in the feces accounted for 5–10% of the dose and very low levels of radioactivity (2–3%) were retained in the tissues 24 hours after exposure. After iv administration of 1 mg/kg of [^{14}C]-CHOX, the oxime was rapidly cleared from plasma with half-lives of 1.6 (alpha phase) and 18.2 min (beta phase). However, when CHOX was applied dermally (30 mg/kg), only about 4–5% of the dose was recovered in urine, feces and the tissues. The majority of the dose volatilized from the skin surface. However, the absorbed oxime was readily distributed and excreted, and its metabolic fate was no different than observed after oral administrations. HPLC analysis of urine showed that the majority of the radioactivity excreted was in the form of three metabolites, cyclohexylglucuronide and the monoglucuronides of *cis*- and *trans*-cyclohexane-1,2-diol. *In vitro* studies showed that these metabolites arise primarily by hydrolysis of the oxime to cyclohexanone which is then reduced to cyclohexanol and eliminated as the glucuronide conjugate. The cyclohexanol, in turn could be metabolized to *cis*- and *trans*-cyclohexane-1,2-diols, which are excreted as their monoglucuronides.

Oximes have a wide variety of applications, as chemical and pesticide intermediates; blocking agents in the polymer industry; chelators in the metal industry; drugs; antioxidants; anti-knock agents in fuels; and in dye synthesis. CHOX[1] is almost exclusively used as a captive intermediate for the production of caprolactam, which, in turn, is used to form polycaprolactam or Nylon 6, a fiber used in clothing, carpeting, home furnishings, and similar products.

Although there are no reports of occupational exposure to CHOX in humans, the large volume of CHOX produced yearly necessitates a thorough understanding of the toxicity of this compound. Studies in laboratory animals have shown that CHOX produces toxic lesions in the hematopoietic system (1–3).[2] Systemically absorbed CHOX is reported to induce oxidative damage to the erythrocytic population in animals resulting in hemolytic anemia accompanied by increased erythropoiesis. Oral administration of CHOX for 2 weeks produced a dose-related decrease in the erythrocyte number, Hb, and hematocrit with an accompanying increase in reticulocytes and circulating nucleated erythrocytes in male and female rats. Methemoglobin levels were also significantly elevated in both sexes. Splenomegaly and hepatomegaly were also observed and the effects persisted for up to 2 weeks after exposure. Histopathological examination revealed compound-related effects in the spleen and bone marrow of male and female rats (1). Similar hematotoxic effects were reported when animals were treated subchronically for 13 weeks by gavage (2). Gad *et al.* (2) suggested that significant amounts of CHOX, sufficient to elicit toxic manifestations, may be absorbed through the skin. They reported that dermal application of 0.8 to 5 g/kg of CHOX increased reticulocyte counts 24 hr after exposure in male rabbits, while the Hb levels in females were depressed (2). Rats exposed by inhalation to 0.03, 0.1, and 1 mg/m^3 of CHOX for 6 and 10 weeks also exhibited decreased erythrocyte counts and increased levels of methemoglobin. Degenerative changes in the parenchymatous organs and desquamation of bronchial epithelium were observed in the high dose group (3).

CHOX was reported to be mutagenic in mouse lymphoma cells without exogenous metabolic activation. However, no mutagenic activity was observed in the presence of S-9 activation, suggesting that the chemical may be deactivated by the enzymes in S-9 (4). CHOX was non-mutagenic in *Salmonella typhimurium* and in *Escherichia coli* with or without metabolic activation (4, 5). CHOX also failed to induce sex-linked recessive mutations in *Drosophila* (6, 7).

At present, little information is available on the disposition and metabolism of CHOX, except that some oximes, including CHOX, are reported to inhibit the oxidative metabolism of ethanol (8, 9). Additional interest has been generated in the toxicity of CHOX since there are reports suggesting that CHOX is formed by the oxidative metabolism of sodium cyclamate, an artificial sweetener (10). The present study was designed to provide greater insight into the fate of CHOX by studying its absorption, tissue distribution, metabolism, and excretion in adult male Fischer rats.

Materials and Methods

Chemicals. Cyclohexanone oxime [1-^{14}C], specific activity 6.85 mCi/mmol, was synthesized by NEN Research Products (Boston, MA). Radiochemical purity was determined by HPLC to be greater than 99%. Unlabeled CHOX, cyclohexanol, cyclohexanone, and *trans*- and *cis*-cyclohexane-1,2-diol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Unlabeled CHOX was recrystallized from hexane before use. NADPH, NADP, nicotinamide, β-glucuronidase (type B-1), and glucose 6-phosphate were obtained from Sigma Chemical Co. (St. Louis,

[1] Abbreviations used are: CHOX, cyclohexanone oxime; b.w., body weight.
[2] Ref. 3, per citation in refs. 1 and 2.

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MO). All other chemicals were of the highest purity commercially available.

Apparatus. *HPLC.* The HPLC system used consisted of Waters Associates (Milford, MA) pumps, automated gradient controller, and tunable absorbance detector. A Flow-One radioactive flow detector (Radiomatic Instruments Co, Tampa, FL) was used for analysis and a Beckman 171 radioisotope detector (Beckman Instruments, Fullerton, CA) was used for isolation of metabolites. A 25 cm × 4.6 mm Microsorb C-18 column (Rainin Instrument Co., Worburn, MA) was used for both analysis and isolation.

Gas Chromatography. GC was performed on a Hewlett-Packard (Avondale, PA) model 5880-A gas chromatograph equipped with a flame ionization detector, a Grob-type splitless injector, and a 25-m fused silica capillary column having 0.22 mm i.d. and a 0.25-μm film thickness of BP10 (Bodman Chemicals, Doraville, GA). The oven temperature was programmed to rise from 35°C to 110°C at a rate of 7.5°/min for the analysis of cyclohexanol and cyclohexanone and from 35°C to 200°C for *cis*- and *trans*-cyclohexane-1,2-diol. The injector and detector temperatures were 250°C and 275°C, respectively.

^1H NMR Spectra. These were recorded on a General Electric (Fremont, CA) GN-500 NMR spectrometer using D_2O as solvent. The chemical shifts reported are in ppm relative to tetramethylsilane as the external standard.

Mass Spectra. Mass spectra were recorded on a Concept 1 SQ hybrid mass spectrometer (Kratos Analytical, Manchester, UK). This instrument utilizes a Hewlett-Packard model 5890 gas chromatograph. A J&W DB-5 capillary column, 0.25 mm i.d. × 60 m, with a 0.25-μm film thickness (J&W Scientific, Fulsom, CA) was used for analysis of *in vivo* metabolites; a similar 30-m column was used for analysis of the *in vitro* metabolites.

Animals. Male Fischer 344 rats, 8–10 weeks old (200–250 g), obtained from Charles River Breeding Laboratories (Raleigh, NC), were used throughout the study. All animals received rat chow and water *ad libitum*.

Disposition Studies. Rats were housed in individual glass metabolic cages allowing for separate collection of urine, feces, CO_2, and other volatile materials. For oral administration the dosing solutions were prepared in distilled water. Radiolabeled CHOX was diluted as needed with unlabeled CHOX to administer approximately 50 μCi/kg body weight (b.w.). The oral doses administered were 1, 10, and 30 mg/kg b.w. in 5 ml/kg of dosing solution, and the animals were sacrificed 24 hr later. A group of animals was also sacrificed 6 hr after oral exposure to 30 mg/kg of CHOX. A dermal dose of CHOX, 30 mg/kg, was dissolved in acetone and applied at 0.2 ml/kg to the interscapular area. Radiolabeled CHOX was diluted as needed with unlabeled CHOX to administer 50 μCi/kg to each animal. The interscapular area was clipped 24 hr before dosing. At the time of dosing each animal was anesthetized with 1 ml/kg of Ketamine. The dermal dose was applied with a microliter syringe with a blunt needle in a circular area of 1 cm^2. This area was then covered with a perforated metal tissue capsule (Lipshaw, Detroit, MI) to prevent removal or ingestion of the dermal dose by grooming. The capsule was glued directly to the skin with cyanoacrylate glue and remained in place throughout the course of the study. The animals were kept in individual glass metabolism cages and sacrificed 24 hr after the treatment except for one group which was sacrificed 3–5 min after dosing.

Three animals were used in each group and the animals were treated between 9:00 and 11:00 a.m. Following sacrifice, animals were immediately dissected and the tissues were removed, weighed, and then stored at −20°C until assayed. The radioactivity in each tissue was analyzed by combustion of triplicate 50- to 100-mg samples to $^{14}CO_2$ in a Packard Tricarb sample oxidizer (Packard Instrument Co., Downers Grove, IL). The $^{14}CO_2$ was then counted in a Beckman model LS-9800 liquid scintillation counter. Body composition estimates for blood and muscle were 8 and 50%, respectively (11), and 11 and 16% for adipose tissue and skin, respectively (12). All other tissues were determined gravimetrically. Excretion of CHOX-derived radioactivity was determined by complete collection of the urine and feces for 24 hr after exposure. Urine samples (0.02–0.05 ml) were counted directly without combustion. Fecal samples were air-dried, weighed, and ground to a fine powder. Triplicate samples were oxidized and the radioactivity was determined as described above. For the group sacrificed immediately after dermal administration, the application site was excised, washed with acetone two to three times, and cut into several pieces. Radioactivity in the washings was determined by adding 0.05-ml aliquots to scintillation fluid and counting. The radioactivity retained in the skin after acetone extraction was determined by combustion.

Radioactivity excreted in the urine was subjected to enzymatic hydrolysis by β-glucuronidase to free possible glucuronide conjugates. The incubation mixture contained 100–200 μg of CHOX-derived material and 2000 units of β-glucuronidase in 0.1 M sodium acetate buffer, pH 5.0. The reaction mixture was incubated at 37°C for 15 hr and then analyzed by GC or HPLC. Similar incubation mixtures without enzyme served as controls.

Collection of CO_2 and Volatiles. After oral or dermal administration of ^{14}C-CHOX, rats were placed in Metabowl-Mark III glass metabolism cages (Jencons, Hemel Hempstead, Herfordshire, UK) maintained at an air flow of 0.4–0.5 liter/min. Total air flow through the cage was first passed through an ethanol (200 ml) trap for the collection of volatiles and then through 200 ml of a 7:3 (v/v) mixture of 2-methoxyethanol/ethanolamine for $^{14}CO_2$ collection. The percentage of the total dose of ^{14}C-CHOX eliminated as $^{14}CO_2$ and volatiles was determined by counting triplicate 1-ml aliquots of each trapping solution.

For the dermal studies, activated charcoal filters (SKC Inc., Eighty Four, PA) were used in place of ethanol for trapping the expired volatiles. The expired volatiles absorbed by the charcoal traps were extracted with 1 ml of acetonitrile from each section of the filter (sample zone with 200 mg of activated charcoal and back-up zone with 100 mg), and radioactivity was counted in a 20-μl aliquot. Following extraction, the activated charcoal was also analyzed by combustion of triplicate 25-mg samples and the radioactivity was determined as described earlier.

Pharmacokinetic Studies. An iv dose of 1 mg/kg CHOX dissolved in 1 ml/kg normal saline was used. Radiolabeled CHOX was diluted with unlabeled CHOX to administer 50 μCi/kg to each animal. Male rats weighing 200–230 g were anesthetized with 1 ml/kg of Ketamine. A silicone tube was installed in the jugular vein and exteriorized through the skin at the back of the neck. Following a 24-hr recovery period, an iv dose was injected through the tubing. At specified times after dosing, known amounts of blood were withdrawn. The blood was immediately centrifuged and the plasma stored frozen at −20°C until analysis. The concentration of unmetabolized oxime was determined in the plasma samples by HPLC using an isocratic system of 90:10 water/acetonitrile at a flow rate of 1.5 ml/min for 20 min and a Flow-one radioactivity detector. Pharmacokinetic parameters were calculated using NONLIN (Statistical Consultants, Inc., Lexington, KY). Initial parameters were obtained graphically. The data were weighted by the reciprocal of the concentration.

Metabolism of ^{14}C-CHOX *In Vivo*. Metabolism was studied by determining the relative amounts of CHOX metabolites in urine and liver extracts. Urine samples were collected at intervals of 0–2, 2–4, 4–6, 6–8, 8–12, and 12–24 hr after treatment. These samples were acidified with 10 μl of glacial acetic acid per ml and centrifuged at low speed in a clinical centrifuge. The supernatants were then filtered through type HV 0.45-μm filter units (Millipore Corp., Bedford, CA) and analyzed by HPLC. Livers from animals sacrificed 6 hr after treatment were homogenized in acetonitrile and centrifuged, and the supernatant was filtered before HPLC analysis. HPLC analysis was carried out with a C-18 column using a linear gradient of 99.9:0.1 water/acetic acid to 74.9:25:0.1 water/acetonitrile/acetic acid in 28 min at a flow rate of 1.5 ml/min.

Isolation and Identification of the Metabolites. The three major metabolites in urine were isolated isocratically using a C-18 column with 89.9:10:0.1 water/acetonitrile/trifluoroactetic acid at a flow rate of 1.5 ml/min. The radioactive peaks, M-1, M-2, and M-3, were collected and freeze-dried. Peaks M-1 and M-2 were further purified isocratically using 98.9:1:0.1 water/acetonitrile/trifluoroacetic acid. NMR spectra of the isolated metabolites were obtained:

M-1, ^{1}H NMR, 1.1–1.4 (m, 4H, 2× CH_2), 1.65 (br s, 2H, CH_2), 1.94 (br

d with 12 Hz separation between lines, 1H, $C\underline{H}_aH_b$), 2.11 (br d with 14 Hz separation, 1H, $CH_a\underline{H}_b$), 3.34 (t, J = 8Hz, 1H, HCOH), 3.45–3.6 (m, 3H, 3× HCOH), and 3.98 ppm (d, J = 9Hz, 1H, $C\underline{H}CO_2H$); ^{13}C NMR, 23.4 (CH_2), 23.6 (CH_2), 31.6 (CH_2), 32.3 (CH_2), 71.4 (HCO—), 73.2 (HCO—), 73.5 (HCO—), 74.7 (HCO—), 75.2 (HCO—), 85.8 (O$\underline{C}$HCO2H), 103.1 (OCHO), and 172.8 ppm (CO_2H).

M-2, ^1H NMR, 1.25–1.4 (m, 2H, CH_2), 1.45–1.7 (m, 4H, 2× CH_2), 1.7–1.85 (m, 2H, CH_2), 3.36 (3 line multiplet, 1H, HCO—), 3.55 (4 line multiplet, 2H, 2× HCO—), 3.8–4.05 (m, 3H, 2× HCO— and $C\underline{H}CO_2H$) and 4.55–4.65 ppm (4 line multiplet, 1H, OCHO).

M-3, ^1H NMR, 1.1–1.4 (m, 5H, 2× CH_2 and $C\underline{H}_aH_b$), 1.52 (m, 1H, $CH_a\underline{H}_b$), 1.73 (m, 2H, CH_2), 1.95 (m, 2H, CH_2), 3.25 (t, J = 8Hz, 1H, HCO—), 3.5 (m, 2H, 2× CHO—), 3.72 (d, J = 9Hz, 1H, $C\underline{H}CO_2H$), 3.75 (m, 1H, HCO—), and 4.58 ppm (d, J = 8Hz, 1H, OCHO)

To characterize the three glucuronide conjugates further, the metabolites were hydrolysed by β-glucuronidase and analyzed by GC or GC/MS. Prior to analysis, hydrolysis products from metabolites M-1 and M-2 were treated with trimethylsilyl chloride (0.1 ml) and the mixture was kept overnight at room temperature. The silylation mixture was diluted with methylene chloride before analysis. The hydrolysis product from M-3 was extracted into methylene chloride and analyzed without derivatization.

***In Vitro* Metabolism of ^{14}C-CHOX.** The *in vitro* metabolism was studied by incubating CHOX with rat liver S-9 and microsomes in the presence and absence of an NADPH-generating system. The reaction mixture (final volume 3 ml) contained 7.4 μmol of CHOX, 0.5 μmol of NADP, 100 μmol of nicotinamide, 25 μmol of glucose 6-phosphate, 75 μmol of $MgCl_2$, and S-9 corresponding to 200–250 mg of liver. When microsomes were incubated with CHOX, 1 mM NADPH was used. Nonenzymatic metabolism was studied using boiled S-9 and microsomes. The reaction mixtures were incubated at 37°C for 0.5–2 hr and then extracted with equal amounts of methylene chloride. A portion of the extract was analyzed by GC for cyclohexanol and cyclohexanone, while the remaining extract was silylated for analysis of M-1 and M-2 by GC-MS.

Results

Absorption, Distribution, and Excretion 24 hr after Oral Administration. The effect of dose on absorption, distribution, and excretion of CHOX was studied in adult male rats by administering CHOX orally at doses of 1, 10, and 30 mg/kg b.w. The data indicate that the oxime was rapidly cleared from the body irrespective of the dose (table 1). The majority (70–90%) of the radioactivity was excreted in urine by 24 hr. The other important route of elimination was feces where about 5–10% of the label was recovered. Very little radioactivity (0.3–1.5%) was eliminated in the expired air as volatiles. About 1.5–2% of the radioactivity was eliminated as CO_2. Tissue distribution studies show that there was little retention of radioactivity in tissues 24 hr after treatment and the relative amount of radioactivity did not change appreciably with dose (table 2). CHOX-derived radioactivity was found to be mainly in blood, liver, skin, muscle, and small and large intestinal contents.

Absorption, Distribution, and Excretion 6 hr after Oral Administration. Since nearly all of the radioactivity was eliminated by 24 hr, disposition studies were also carried out in a group of animals 6 hr after oral administration of 30 mg/kg CHOX to provide a better estimate of the absorption, distribution, and metabolism of this oxime in the first few hr following exposure. About 40% of the ^{14}C was excreted in urine and feces by 6 hr (table 3). Tissue distribution studies showed widespread distribution of CHOX-derived radioactivity (table 3). Large-mass tissues such as muscle, skin, and fat accounted for almost half of the radioactivity. The gastrointestinal tract and its contents, liver, kidney, and blood also contained high levels of radioactivity, whereas brain, spleen, testes, and lungs contained low levels of radioactivity (table 3).

Absorption, Distribution, and Excretion 24 hr after Dermal Application. The excretion of CHOX-derived radioactivity in dermally treated rats was also followed for 24 hr after treatment. Only about 4–5% of the dose was recovered in urine, feces, and tissues 24 hr after dermal application. Approximately 4–5% of the radiolabel remained at the administration site and 20–25% of the radioactivity was recovered as volatiles in the charcoal traps (table 1). GC analysis of extracts of the charcoal traps showed the presence of cyclohexanol and cyclohexanone in addition to CHOX. The distribution and elimination of the absorbed dose were no different from those observed after oral administration. The major route of elimination was in urine and there was little retention of radioactivity in any tissue. Among the different tissues, skin, muscle, liver, and blood contained most of the radioactivity (table 2).

Since recovery of radioactivity was low, experiments were also conducted to study volatilization of the oxime from the skin surface. It was found that within 3–5 min of dosing (the time between dosing, gluing the metal capsule to the site of administration, and placing the animal in the metabolism cage), 20–30% of the radioactivity evaporated from the application site *i.e.* almost one-third of the dose was lost during the administration process.

Pharmacokinetic Studies. After a single iv dose, CHOX was rapidly eliminated from plasma (fig. 1). The plasma concentration of CHOX at the first sampling point (2 min) was in the range of 1.5–3.5 μg/ml. The plasma concentration declined rapidly and was below the quantitation limit of the assay by 90 min after treatment. The decrease in CHOX plasma was best described by a biexponential function of the form $Ae^{-\alpha t} + Be^{-\beta t}$. A short α phase was observed for CHOX with a $t_{1/2}$ of 1.6 min

TABLE 1

Total recovery of CHOX-derived radioactivity (percentage of dose) 24 hr after administration

	Oral			Dermal
	1 mg/kg	10 mg/kg	30 mg/kg	(30 mg/kg)
Urine	86.7 ± 5.2[a]	73.7 ± 15.5	68.0 ± 11.3	3.860 ± 0.86
Feces	4.89 ± 2.6	8.04 ± 2.10	9.40 ± 4.70	0.089 ± 0.01
Tissues	1.91 ± 0.14	2.40 ± 0.39	3.10 ± 1.09	0.408 ± 0.02
Volatiles	0.29 ± 0.29	1.54 ± 2.00	0.66 ± 0.22	22.60 ± 3.20
CO_2	1.93 ± 0.08	1.80 ± 0.06	1.67 ± 0.16	
Application site				4.52 ± 1.30
Total	95.8 ± 3.70	87.5 ± 15.8	82.9 ± 9.3	31.4 ± 3.9

[a] Values are the mean ± SD of three animals.

TABLE 2

Tissue distribution of CHOX-derived radioactivity (percentage of dose) 24 hr after administration

	Oral			Dermal (30 mg/kg)
	1 mg/kg	10 mg/kg	30 mg/kg	
Blood	0.173 ± 0.050[a]	0.220 ± 0.01	0.290 ± 0.03	0.053 ± 0.009
Liver	0.240 ± 0.040	0.320 ± 0.06	0.310 ± 0.08	0.036 ± 0.005
Kidney	0.027 ± 0.002	0.028 ± 0.01	0.039 ± 0.01	0.007 ± 0.001
Skin	0.289 ± 0.070	0.390 ± 0.14	0.580 ± 0.20	0.099 ± 0.020
Muscle	0.477 ± 0.060	0.580 ± 0.11	0.710 ± 0.20	0.099 ± 0.004
Fat	0.065 ± 0.006	0.090 ± 0.02	0.109 ± 0.02	0.036 ± 0.007
Small intestine	0.024 ± 0.005	0.032 ± 0.01	0.067 ± 0.05	0.007 ± 0.0001
Small intestine contents	0.147 ± 0.030	0.230 ± 0.03	0.270 ± 0.08	0.026 ± 0.006
Large intestine	0.025 ± 0.005	0.035 ± 0.004	0.044 ± 0.01	0.006 ± 0.001
Large intestine contents	0.382 ± 0.100	0.396 ± 0.11	0.466 ± 0.21	0.046 ± 0.020

[a] Values are the mean ± SD of three animals.

TABLE 3

Tissue distribution of CHOX-derived radioactivity 6 hr after oral administration

	% of Dose	μg eq/g Tissue
Blood	2.09 ± 0.11[a]	7.96 ± 0.43
Liver	5.98 ± 0.44	43.8 ± 3.80
Kidney	0.78 ± 0.08	30.7 ± 3.10
Skin	4.00 ± 0.22	7.59 ± 0.41
Fat	2.88 ± 0.15	6.29 ± 0.39
Muscle	12.6 ± 1.30	7.65 ± 0.83
Small intestine	1.18 ± 0.15	38.2 ± 5.20
Small intestine contents	6.40 ± 1.50	95.9 ± 28.0
Large intestine	0.44 ± 0.06	17.6 ± 4.00
Large intestine contents	4.82 ± 1.11	42.8 ± 6.80
Brain	0.20 ± 0.02	7.32 ± 0.32
Spleen	0.07 ± 0.01	9.51 ± 1.15
Testes	0.31 ± 0.03	8.72 ± 0.58
Lungs	0.13 ± 0.03	7.91 ± 0.44
Stomach	1.52 ± 0.62	24.1 ± 9.27
Stomach contents	5.27 ± 0.66	83.1 ± 28.7
Urine	38.2 ± 10.6	
Feces	1.15 ± 1.76	

[a] Values are the mean ± SD of three animals.

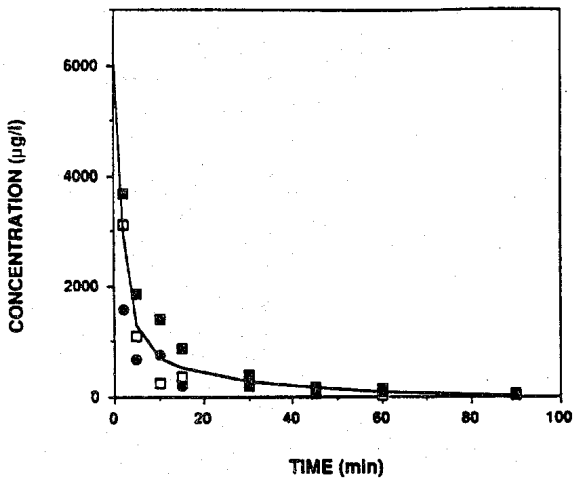

FIG. 1. *Plasma concentration of CHOX (not total radioactivity) following a 1 mg/kg iv dose.*

■, ●, and □ represent data from individual rats. The mean values for A (5330 μg/liter), B (976 μg/liter), α (0.481 min^{-1}), and β (0.0407 min^{-1}) from the three animals were used to generate the line.

TABLE 4

Pharmacokinetic parameters after iv administration of ^{14}C-CHOX (1 mg/kg)

Parameter	Value
$t_{1/2}\alpha$	1.60 ± 0.65 min[a]
$t_{1/2}\beta$	18.2 ± 5.80 min
AUC_0^∞	32,100.000 ± 15,700 min·μg/liter
Cl	0.036 ± 0.015 liter/kg/min
V_{ss}	0.649 ± 0.478 liter/kg

[a] Values are the mean ± SE of three animals.

which was followed by a β phase with a $t_{1/2}$ of 18.2 min. The AUC obtained for CHOX ranged from 20,600 to 50,000 min·μg/liter and the total clearance averaged 0.036 liter/kg/min with a range of 0.020 to 0.048 liter/kg/min. The calculated volume of distribution (V_{ss}) of CHOX ranged from 0.309 to 1.19 liter/kg (table 4).

Metabolism. *In Vivo.* HPLC analysis of urine indicated that CHOX was rapidly metabolized after oral or dermal administration. Identical metabolic profiles were observed in urine samples from animals treated by either route or when treated with different doses. Similar metabolic profiles were also observed when liver extracts from animals sacrificed after 6 hr were analyzed by HPLC. Only trace amounts of the total radioactivity injected had a retention time similar to that of CHOX, even in urine samples collected as early as 0–2 hr after exposure. The majority of the radioactivity eliminated in the first 24 hr was in the form of three metabolites, M-1, M-2, and M-3. M-3 predominated in urine collected at early hours (0–2 hr), while M-1 and M-2 predominated in the urine at later time points, 8–12 and 12–24 hr after treatment (fig. 2).

GC analysis of a methylene chloride extract of urine showed the presence of a compound which coeluted with cyclohexanone with a R_i of 12.39 min. When a urine sample hydrolysed by β-glucuronidase was analyzed, a peak coeluting with cyclohexanol (R_i = 12.13 min) was also identified, suggesting that one of the metabolites could be a glucuronide of cyclohexanol.

The three urinary metabolites, M-1, M-2, and M-3, were isolated by HPLC using a reverse phase column and characterized by NMR spectrometry.

M-1. The ^{1}H NMR spectrum of this metabolite was consistent with it being a glucuronide with several signals in the 3.3 to 3.6 ppm region corresponding to protons attached to hydroxyl-bearing carbons and a signal at 3.98 for the proton on the carbon bearing both the ether oxygen and the carboxyl group. The signal

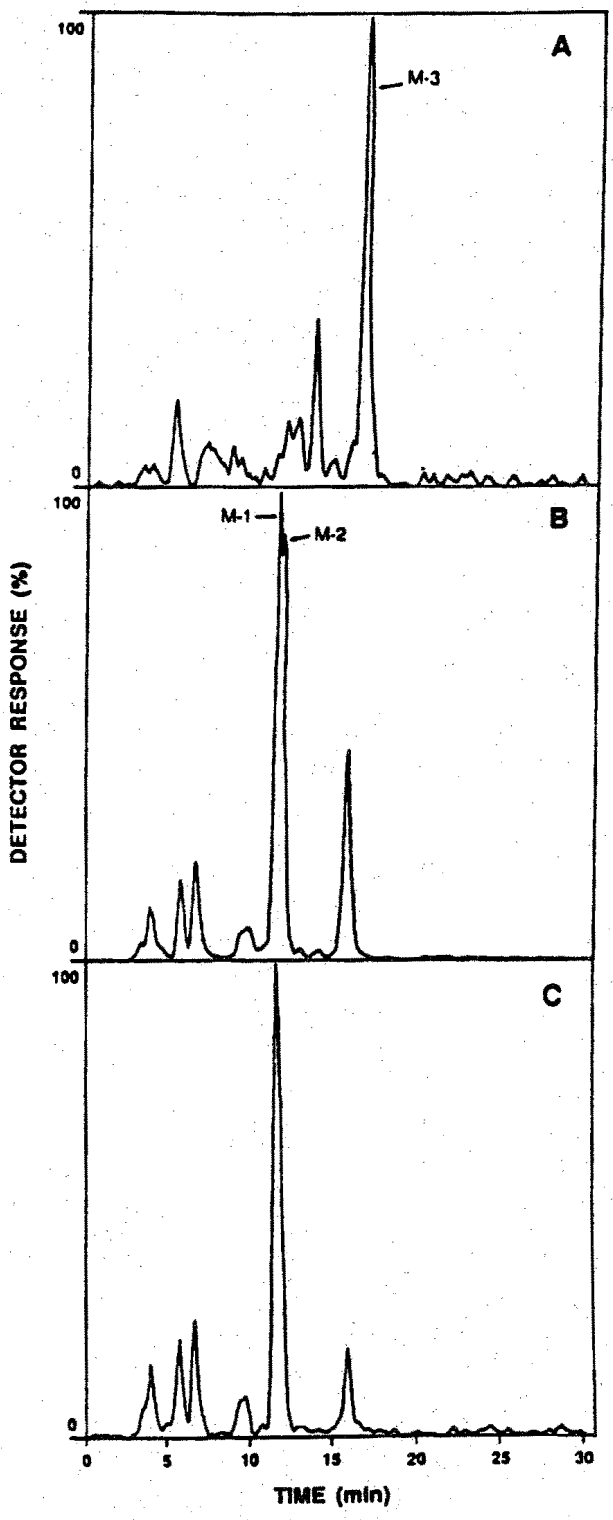

FIG. 2. *Representative HPLC chromatograms of metabolites in urine collected at 0–2 hr (A), 8–12 hr (B), and 12–24 hr (C) after oral administration of* ^{14}C-CHOX. CHOX had a R_t *of 14.7 min under the assay conditions.*

for the anomeric proton was obscured by the HOD peak in the ^{1}H NMR spectrum. The anomeric carbon could, however, be seen at 103.1 ppm in the ^{13}C NMR. The remainder of the molecule was identified as *trans*-cyclohexane-1,2-diol based in part on the similarity of the ^{1}H NMR of M-1 to that of *trans*-cyclohexane-1,2-diol. There are four signals in the 1- to 2-ppm region for M-1 at about 1.2, 1.6, 1.9, and 2.1 ppm. There are similar signals at 1.2, 1.6, and 1.9 in the ^{1}H NMR of *trans*-cyclohexane-1,2-diol. The splitting of the 1.9 signal into two signals is likely due to the fact that M-1 is asymmetric and the diol is not. The NMR signals for the protons on the hydroxyl-bearing carbon of the diol are at 3.36 ppm; corresponding signals are present in the spectrum of M-1. Integration of the ^{1}H NMR spectrum is consistant with M-1 being the monoglucuronide of *trans*-cyclohexane-1,2-diol. For further confirmation of the structure of M-1, the metabolite was hydrolyzed with β-glucuronidase and the freed diol was subjected to GC analysis after derivatization as the TMS ether. The silylated derivative showed a peak at 18.59 min which coeluted with the trimethylsilyl ether of *trans*-cyclohexane-1,2-diol. M-1 was thus identified as a monoglucuronide of *trans*-cyclohexane-1,2-diol.

M-2. The ^{1}H NMR spectrum of this metabolite was also consistent with it being a glucuronide with several signals in the 3.3- to 4.0-ppm region. The anomeric proton was not obscured by the HOD peak and was seen as two doublets at 4.5 and 4.6 ppm. For this metabolite there were only three groups of signals in the 1- to 2-ppm region, broad multiplets at about 1.3, 1.6, and 1.8 ppm. This pattern corresponds closely to that seen for *cis*-cyclohexane-1,2-diol where there are multiplets of similar appearance at 1.3, 1.55, and 1.65 ppm. The NMR signal for protons on the hydroxyl-bearing carbons are at lower field, 3.75 ppm, for the *cis*-diol compared to the *trans*-diol. There is a corresponding signal in the NMR of M-2. The integral from the NMR spectrum indicates that M-2 is a monoglucuronide. The observation of two doublets for the anomeric proton probably arises from the fact that there could be two conformations of similar energy for M-2, one in which the glucuronide substituent is axial and one in which this substituent is equitorial. However, running the spectrum at temperatures up to 70°C did not result in coalesence of the signals as would have been expected if the two conformations were easily interconvertable. M-2 was hydrolyzed with β-glucuronidase, the freed diol was silylated and subjected to GC/MS using the 60-m column. The di-TMS ether of *cis*-cyclohexane-1,2-diol had the same retention time (15.0 min) and mass spectrum (M-CH_3 at *m/z* 245) as the compound obtained from M-2. Based on this information M-2 was identified as the monoglucuronide of *cis*-cyclohexane-1,2-diol.

M-3. As with the previous metabolites, there was evidence that M-3 was a glucuronide from the presence of several signals in the 3.2- to 3.8-ppm region of the NMR spectrum. The anomeric proton could be seen as a doublet at about 4.6 ppm. The rest of the NMR spectrum consisted of four multiplets at about 1.25, 1.5, 1.7, and 1.95 ppm. Signals similar in appearance are present at 1.25, 1.5, 1.7, and 1.85 ppm in the NMR spectrum of cyclohexanol. The NMR signal for the proton on the hydroxyl-bearing carbon in cyclohexanol was at 3.6 ppm; an absorption of similar appearance was found at 3.75 in the NMR of M-3. GC analysis of the methylene chloride extract of the β-glucuronidase hydrolysate of M-3 showed the presence of a peak with a R_t of 12.13 min which cochromatographed with cyclohexanol. M-3 was therefore identified as cyclohexylglucuronide.

In Vitro. When the methylene chloride extract of the reaction