NTP REPORT ON THE

TOXICITY STUDIES OF HEXACHLORO-1,3-BUTADIENE IN B6C3F$_1$ MICE

(FEED STUDIES)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

January 1991

NTP TOX 1
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U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

FOREWORD

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control. The NTP coordinates the relevant programs, staff, and resources from these Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

The NTP develops, evaluates, and disseminates scientific information about potentially toxic and hazardous chemicals. This knowledge is used for protecting the health of the American people and for the primary prevention of disease.

The studies described in this Toxicity Study Report were performed under the direction of the NIEHS and were conducted in compliance with NTP chemical health and safety requirements and must meet or exceed all applicable Federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals.

These studies are designed and conducted to characterize and evaluate the toxicologic potential of selected chemicals in laboratory animals. Chemicals selected for NTP toxicology studies are chosen primarily on the bases of human exposure, level of production, and chemical structure.

Anyone who is aware of related ongoing or published studies not mentioned in this report, or of any errors in this report is encouraged to make this information known to the NTP. Comments and questions should be directed to Dr. J.R. Bucher, NIEHS, P.O. Box 12233, Research Triangle Park, NC 27709 (919-541-4532).

These NTP Toxicity Study Reports are available for sale from the National Technical Information Service, U.S. Department of Commerce, 5285 Port Royal Road, Springfield, VA 22161 (703-487-4650). Single copies of this Toxicity Study Report are available without charge while supplies last from the NTP Public Information Office, NIEHS, P.O. Box 12233, Research Triangle Park, NC 27709 (919-541-3991).

TOXICITY STUDIES OF HEXACHLORO-1,3-BUTADIENE IN $B6C3F_1$ MICE

(FEED STUDIES)

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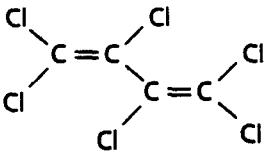

HEXACHLORO-1,3-BUTADIENE

CAS No. 87-68-3

C_4Cl_6 Molecular weight 260.7

Synonyms: HCBD; hexachlorobutadiene; 1,1,2,3,4,4-hexachloro-1,3-butadiene; perchlorobutadiene

Trade Names: C 46; Dolen-Pur

ABSTRACT

Two-week and 13-week toxicity studies of hexachloro-1,3-butadiene incorporated in the diet were conducted in B6C3F$_1$ mice. Groups of five mice of each sex received diets containing 0, 30, 100, 300, 1,000, or 3,000 ppm hexachloro-1,3-butadiene for 15 days. Toxic responses in the 2-week studies, primarily in the higher dose groups, included abnormal clinical signs (lethargy, hunched posture, rough hair coats, light sensitivity, and/or incoordination), deaths (all mice in the two highest dose groups died by day 7), body and organ weight depression, and gross and histopathologic changes. The most prevalent microscopic lesion, seen in all hexachloro-1,3-butadiene-dosed mice, was renal tubular cell necrosis and/or regeneration. Regeneration was seen in lower dose groups. In addition to kidney lesions, histopathologic changes were also seen in the liver (hepatocyte necrosis, cytoplasmic vacuolization), lymphoid tissues (lymph node necrosis, depletion), and testis (seminiferous tubule giant cells) of mice in the two highest dose groups which died during the first week of the studies.

Thirteen-week studies were conducted in which groups of 10 mice per sex received 0, 1, 3, 10, 30, or 100 ppm hexachloro-1,3-butadiene in feed (corresponding to doses of 0, 0.1, 0.4, 1.5, 4.9, or 16.8 mg/kg per day for males and 0.2, 0.5, 1.8, 4.5, or 19.2 mg/kg per day for females). No compound-related clinical signs or deaths were observed. Compared with controls, body weight gain was reduced in males receiving 30 and 100 ppm (—49% and —56%, respectively) and females receiving 100 ppm (—47%). Kidney weights were reduced in the males receiving 30 and 100 ppm and females receiving 100 ppm. A compound-related increase in tubular cell regeneration in the renal cortex occurred in male and female mice. This lesion, characterized by a diffuse increase in basophilia of the tubular epithelial cytoplasm and an increase in the number of nuclei, increased in severity with increased dose. The motility of sperm from dosed mice was lower, though not dose related, than that from controls. Female mice were more susceptible to the toxicity of hexachloro-1,3-butadiene than male mice. Based on the histopathologic evaluations, the no-observed-adverse-effect level appeared to be 10 ppm for the male mice in this 13-week study; no such level was identified for the female mice.

CONTRIBUTORS

The NTP Report on the Toxicity Studies of hexachloro-1,3-butadiene is based on the 2-week studies that began in March 1985 and on the 13-week studies that began in August 1985 at Microbiological Associates, Inc. (Bethesda, MD).

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PEER REVIEW PANEL

The members of the Peer Review Panel who evaluated the draft report on the Toxicity Studies on, hexachloro-1,3-butadiene on March 13,1989, are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, Panel members have four major responsibilities: (a) to ascertain that all relevant literature data have been adequately cited and interpreted, (b) to determine if the design and conditions of the NTP studies were appropriate, (c) to ensure that the Technical Report presents the experimental results and conclusions fully and clearly, and (d) to judge the significance of the experimental results by scientific criteria.

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SUMMARY OF PEER REVIEW COMMENTS ON THE TOXICITY STUDIES OF HEXACHLORO-1,3-BUTADIENE

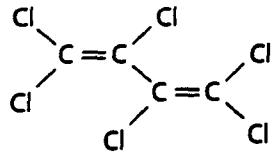
On March 13, 1989, the draft report on the toxicity studies of hexachloro-1,3-butadiene received public review by the National Toxicology Program Board of Scientific Counselors' Technical Reports Review Subcommittee and associated Panel of Experts. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. R.S.H. Yang, NIEHS, introduced the short-term toxicity studies of hexachloro-1,3-butadiene by reviewing the rationale, experimental design, and results.

Dr. Klaassen, a principal reviewer, had several editorial suggestions and commented that nearly all of the conclusions were drawn from the histopathologic data and that it would also be useful to use other measures, e.g., clinical chemistry. Dr. Yang said that no clinical pathology was done.

Dr. Scala, a second principal reviewer, stated that a clearer exposition of the kidney pathology with more extensive tabulation would have been desirable. Dr. Scala asked whether the unexplained losses of hexachloro-1,3-butadiene from the feed in the animal room might simply have resulted from volatilization. Dr. Yang replied that volatilization did appear to be responsible for the losses.

In response to a question concerning the purpose of the Toxicity Study Reports, Dr. Griesemer stated that the goal of these and the other short-term toxicity studies to be reviewed was to design and conduct stand-alone toxicologic characterization studies. Dr. Scala concluded that the Panel would accept the Report with the clarifications noted.



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Trade Names: C 46; Dolen-Pur

I. INTRODUCTION

Properties, Use, and Production

Hexachloro-1,3-butadiene is a colorless liquid with a faint, turpentine-like odor. It is practically insoluble in water (5 µg/ml at 20° C) but is soluble in ethanol and diethyl ether. It has a melting point of about - 21° C and a specific gravity of 1.675 at 15.5° C. It is somewhat volatile (22 mm mercury at 100° C) and is a nonflammable, stable compound (Condensed Chemical Dictionary, 1977).

Hexachloro-1,3-butadiene is produced in the United States as a by-product of the manufacture of chlorinated hydrocarbons such as tetrachloroethylene, trichloroethylene, and carbon tetrachloride. An estimated 3.3-6.6 million kilograms is produced per year. In 1974, approximately 0.23 million kilograms was imported into the United States (USEPA, 1975, 1980). There are no current production/import data (U.S. International Trade Commission, personal communication to NTP, 1986).

Hexachloro-1,3-butadiene is used as a solvent for many organic substances, as a fluid for gyroscopes, as a heat-transfer liquid in transformers, as hydraulic fluid, as a chemical intermediate in the production of lubricants, and as an intermediate in the manufacture of rubber compounds; it is also used for the recovery of chlorine-containing gas in chlorine plants (IARC, 1979; USEPA, 1980).

The USSR is believed to be one of the major users of hexachloro-1,3-butadiene; 0.59-0.77 million kilograms has been used annually as a fumigant against Phylloxera on grapes (USEPA, 1975; IARC, 1979). It is also used as a fumigant in vineyards in France, Italy, Greece, Spain, and Argentina.

Disposition and Metabolism

The biotransformation of hexachloro-l,3-butadiene was reviewed by Yang (1988). A summary of the metabolic pathways and bioactivation of hexachloro-1,3-butadiene is presented in Figure 1.

Nash et al. (1984) demonstrated that after oral administration of a nephrotoxic dose (200 mg/kg) to male Alderley Park rats, the principal route of excretion of hexachloro-1,3-butadiene was biliary. Based on chromatographic and mass spectrometric evidence, the major biliary metabolite was identified as a conjugate of hexachloro-1,3-butadiene and glutathione. The

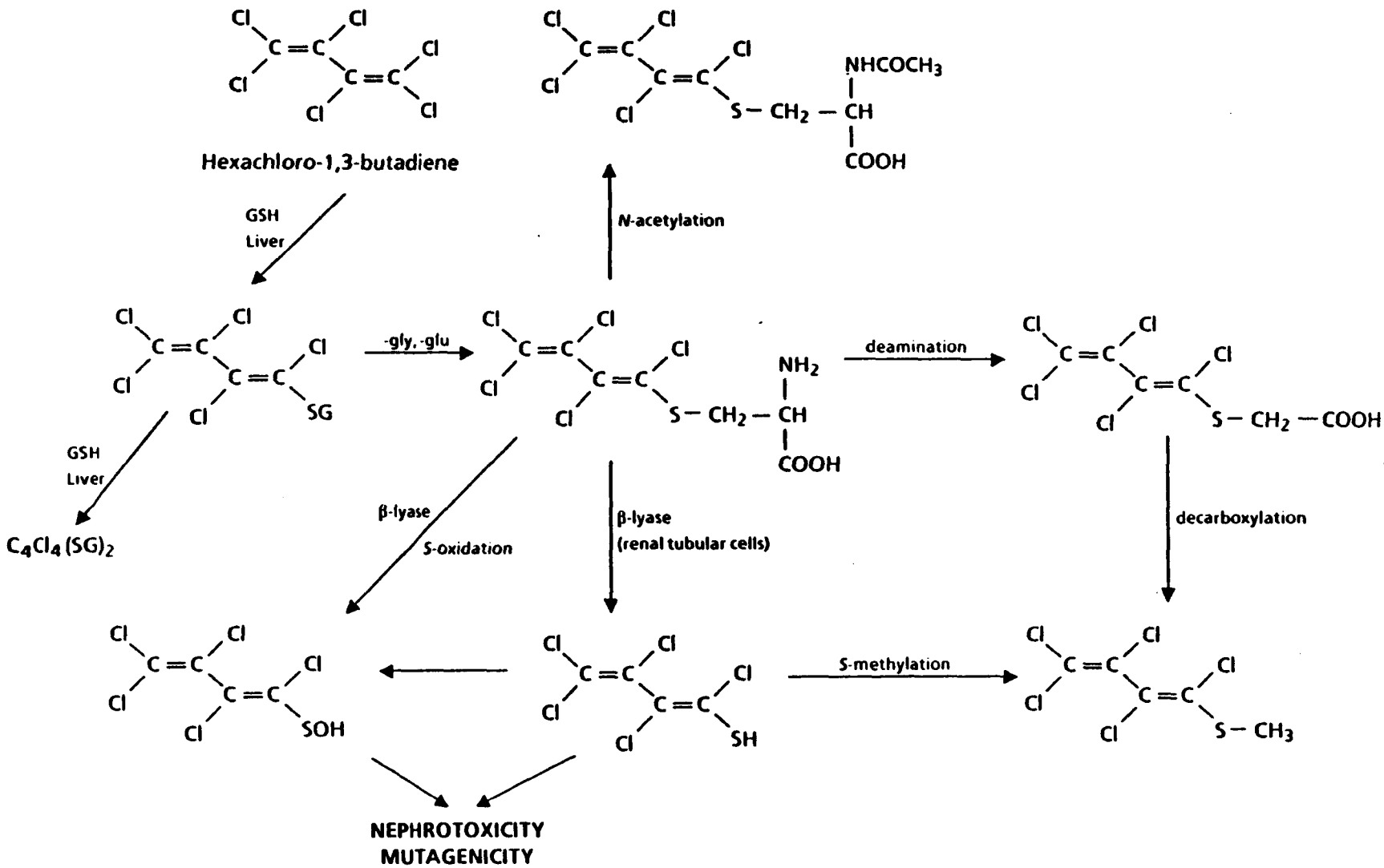

FIGURE 1. SUMMARY OF PROPOSED METABOLIC PATHWAYS OF HEXACHLORO-1,3-BUTADIENE AND ITS BIOACTIVATION

(Jaffe et al., 1983; Nash et al., 1984; Wolf et al., 1984; Jones et al., 1985; Keicherl et al., 1985; Keicherl and Schulz, 1986; Wild et al., 1986)

cysteinylglycine conjugate of hexachloro-1,3-butadiene, a metabolite of the glutathione conjugate, was also found in the bile.

When hexachloro-1,3-butadiene was administered orally to rats, its glutathione conjugate of hexachloro-1,3-butadiene, its mercapturic acid derivative, and bile-containing hexachloro-1,3-butadiene metabolites were all nephrotoxic (Nash et al., 1984). However, rats fitted with biliary cannulae were completely protected from kidney damage when dosed with hexachloro-1,3-butadiene, indicating that hepatic metabolites were solely responsible for the nephrotoxicity of hexachloro-1,3-butadiene. These investigators proposed that the hepatic glutathione conjugate of hexachloro-1,3-butadiene was degraded to its equivalent cysteine conjugate, which was then cleaved by the renal cytosolic enzyme b-lyase to give a toxic thiol that, in turn, caused localized kidney damage (the proposed metabolic pathway is presented in Figure 1). Their identification of a urinary metabolite, 1,1,2,3,4-pentachloro-l,3-butadienyl sulfenic acid, is consistent with this hypothesis. A similar mode of metabolic activation involving glutathione conjugation was suggested for a renal toxin, S-(l,2-dichlorovinyl) cysteine (Derr and Schultze, 1963; Anderson and Schultze, 1965; Gandolfi et al., 1981; Hassall et al., 1983).

Independently, Reichert et al. (1985) reported the identification of two major urinary metabolites of hexachloro-1,3-butadiene in female Wistar rats administered a single oral dose of hexachloro-1,3-butadiene: pentachloro-1-methylthio-l,3-butadiene and pentachlorocarboxymethylthio-l,3-butadiene. In addition, they indicated that approximately equal portions (5.3%) of a single 1.0 or 50 mg/kg oral dose of hexachloro-1,3-butadiene were exhaled unchanged by rats and that the gastrointestinal absorption pathway for hexachloro-l,3-butadiene appeared to be saturated at the higher dose. Covalent binding to proteins in kidney and liver was consistent with the organ-specific toxicity of hexachloro-1,3-butadiene; binding was higher in the kidney and independent of the dose.

Results of subsequent studies (Jones et al., 1985; Reichert and Schutz, 1986) strengthened the hypothesis that a biologically active intermediate is derived from the glutathione conjugation pathway (Jaffe et al., 1983; Nash et al., 1984; Wolf et al., 1984). The formation in vitro and in vivo of both a mono- and a disubstituted glutathione conjugate of hexachloro-1,3-butadiene (Jones et al., 1985) and the strong mutagenic activity of mercapturic acid derivative of hexachloro-l,3-butadiene (Reichert and Schutz, 1986) were reported.

Short-Term Toxicity

The short-term toxicity data on hexachloro-1,3-butadiene were reviewed by Gehring and MacDougall (1971). The oral LD_{50} values were reported to be 200-350 mg/kg for rats, 87-116 mg/kg for mice, and 90 mg/kg for guinea pigs (Murzakaev, 1963; Gulko et al., 1964; Gehring and MacDougall, 1971). Although dermal exposure to hexachloro-1,3-butadiene at 120 mg/kg for 4 hours or 63 mg/kg for 24. hours was not lethal, 1/2 rabbits died after exposure at 126 mg/kg for 7 hours and 4/4 died after 24 hours.

All rats survived inhalation exposure to hexachloro-l,3-butadiene at 161 ppm for 0.88 hours or 34 ppm for 3.5 hours (Gehring and MacDougall, 1971). Some or all rats died after exposure to hexachloro-1,3-butadiene at 133-500 ppm for 4-7 hours; most guinea pigs and cats died after exposure at 161 ppm for 0.88 hours or at 34 ppm for 7.5 hours.

Neonatal rats appeared to be more sensitive to the acute lethal effect of hexachloro-l,3-butadiene than were adults. This was first suggested by Poteryaeva (1966) after the deaths for 3 months of newborn rats of mothers given a single subcutaneous injection of hexachloro-1,3-butadiene at 20 mg/kg before mating. A subsequent study by Kociba et al. (1977) confirmed the greater susceptibility of weanling rats (Table 1).

Short-Term Repeated Exposure and Toxicity

Harleman and Seinen (1979) studied hexachloro-l,3-butadiene toxicity in Wistar-derived (CpbWU/WI) rats in a 14-day range-finding study, a 13-week gavage study, and a dietary reproduction study. Histologic changes were

TABLE 1. RESULTS OF ORAL LD_{50} STUDIES OF HEXACHLORO-1,3-BUTADIENE IN WEANLING AND ADULT RATS (a)

Age	LD_{50} (mg/kg) (b) Male	Female
Adult	580 (504-667)	200-400
21-22 days old	65 (46-91)	46 (26-81)

(a) Kociba et al., 1977
(b) 95% confidence limits

found in the kidney of all rats after dietary exposure to hexachloro-1,3-butadiene at 0, 50, 150, or 450 ppm (approximately 0, 3, 9, or 27 mg/kg per day) for 14 days. The lesion was characterized by degeneration of tubular epithelial cells, especially in the straight limbs of the proximal tubules located in the outer zone of the medulla. No changes were found in other organs. Other toxic responses included dose-related depression of weight gain and feed consumption, as well as increases in relative kidney weights in the males and females at the two highest doses.

Kociba et al. (1977) noted renal toxicity, as shown by an increase in relative kidney weight and renal tubular degeneration, in rats receiving hexachloro-1,3-butadiene in feed at 30, 65, or 100 mg/kg per day for 30 days. The kidney was the organ most sensitive to the effects of hexachloro-l,3-butadiene. In the 13-week toxicity studies conducted by Harleman and Seinen (1979), male and female rats (10 rats per dose per sex) were given 0, 0.4, 1, 2.5, 6.3, or 15.6 mg/kg per day by gavage in arachid oil. The no-effect levels for females and males were suggested to be 1 and 2.5 mg/kg per day, respectively. Lower body weight gain was observed in each sex at the two highest doses, and degeneration of proximal renal tubules was observed at 2.5 and 6.3 mg/kg or more per day in females and males, respectively. The ability to concentrate urine, as determined by measuring urine osmolarity, was significantly reduced in female rats at 2.5 mg/kg or more and in male rats at 15 mg/kg. Increases in kidney weight to body weight ratios were observed at the two highest doses for each sex. Hepatotoxicity, characterized by increased cytoplasmic basophilia of hepatocytes and liver weight, was seen in males at the two highest doses, and an increased liver weight was seen in females at the highest dose (15.6 mg/kg per day).

Long-Term Toxicity and Carcinogenicity

Kociba et al. (1977) studied the long-term toxicity of dietary hexachloro-1,3-butadiene in Sprague Dawley rats (39-40 per sex and dose and 90 per sex for controls) at concentrations in feed corresponding to doses of 0, 0.2, 2, or 20 mg/kg per day for up to 2 years. Hexachloro-l,3-butadiene caused multiple toxicologic effects, the kidney being the organ most affected. At the high dose (20 mg/kg per day), hexachloro-1,3-butadiene caused decreased weight gain and survival, increased urinary excretion of coproporphyrin, increased kidney weight, increased incidences of renal tubular epithelial hyperplasia/proliferation, and renal tubular adenomas and adenocarcinomas (two neoplasms metastasized to the lungs). At the mid dose (2 mg/kg per day), toxicity was less pronounced. Effects included increases in urinary coproporphyrin excretion and renal tubular epithelial hyperplasia/proliferation; no renal neoplasms were observed at this dose. At the lowest dose (0.2 mg/kg per day), no compound-related effects were noted.

Theiss et al. (1977) tested hexachloro-l,3-butadiene for carcinogenicity by the pulmonary tumor response in strain A mice. Two groups of males, 6-8 weeks of age, were given intraperitoneal injections of hexachloro-1,3-butadiene at 4 or 8 mg/kg in tricaprylin three times per week for a total of 12-13 injections. All survivors were killed 24 weeks after the first injection and were examined for lung tumors. The tumor incidences were similar in dosed and control groups.

The International Agency for Research on Cancer concluded in 1978 that there is limited evidence that hexachloro-1,3-butadiene is carcinogenic for rats (IARC, 1979).

National Toxicology Program Genetic Toxicity Studies

hexachloro-1,3-butadiene was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 when tested at doses up to

33 µg/plate with a preincubation protocol in the presence or absence of Aroclor 1254-induced male Sprague Dawley rat or Syrian hamster liver S9 (Haworth et al., 1983). In tests for the induction of cytogenetic effects in Chinese hamster ovary (CHO) cells, hexachloro-1,3-butadiene induced sister chromatid exchanges in both the presence and absence of Aroclor 1254-induced male Sprague Dawley rat liver S9 (Galloway et al., 1987). Doses ranged from 1.4 to 14 µg/ml. A positive response was noted at the low dose without S9 and at the mid dose (4.2 µg/ml) with S9; cell cycle delay was observed in the culture at the high dose in the absence of S9. No chromosomal aberrations were induced by hexachloro-1,3-butadiene in cultured CHO cells with or without S9 (Galloway et al., 1987). No induction of sex-linked recessive lethal mutations was observed in *Drosophlla melanogaster* males administered 15 ppm hexachloro-l,3-butadiene by injection or by feeding (Woodruff et al., 1985).

Study Rationale

Hexachloro-1,3-butadiene was included in the first group of 24 priority chemicals (plus isomers of some of these chemicals) selected under Superfund auspices for toxicologic evaluation by the NTP as part of an interagency agreement between the NTP and the Agency for Toxic Substances and Disease Registry. Because no information about mice other than toxicity after a single dose was available at the time of selection, the studies reported herein were designed to characterize the short-term toxicity of hexachloro-l,3-butadiene in B6C3F1 mice.

II. MATERIALS AND METHODS

Procurement and Characterization of hexachloro-1,3-butadiene

Hexachloro-1,3-butadiene was obtained in one lot (lot no. 2107HC) from Aldrich Chemical Company, Inc. (Milwaukee, WI). Reports on purity and identity analyses are on file at the National Institute of Environmental Health Sciences.

Lot no. 2107HC was identified as hexachloro-1,3-butadiene by infrared, ultraviolet/visible, and nuclear magnetic resonance spectroscopy. The infrared and ultraviolet/visible spectra were consistent with those expected for the structure and with the literature spectra (Sadtler Standard Spectra). No peaks were observed in the nuclear magnetic resonance spectrum, consistent with the structure of hexachloro-1,3-butadiene.

The purity of lot no. 2107HC was determined by elemental analysis, potentiometric titration to determine free acid (performed in methanol solution with 0.01 N methanolic sodium hydroxide as the titrant), thin-layer chromatography, and gas chromatography. Thin-layer chromatography was performed on silica gel plates with a hexanes (100%) solvent system or on silanized silica gel plates with a methanol: saturated aqueous sodium chloride (80:20) solvent system; visualization was achieved under ultraviolet light, 254 nm, followed by an *N,N*-dimethyl-p-phenylenediamine hydrochloride in sodium ethoxide spray. Gas chromatography was performed with flame ionization detection, a 70 ml/minute nitrogen flow rate, and a 20% SP2100/0.1% Carbowax 1500 column (system 1) or with a 10% Carbowax 20M-TPA column (system 2).

Cumulative data indicated that lot no. 2107HC was approximately 98% pure. Results of elemental analysis for carbon and chlorine agreed with the theoretical values. Titration indicated less than 0.001 meq of free acid per gram of sample. No impurities were detected by either thin-layer chromatographic system. Gas chromatographic system 1 indicated three impurity peaks with areas greater than 0.1% relative to the major peak area; the combined area of the three impurity peaks was 2.1% relative to the major peak. Gas chromatographic system 2 indicated two impurities, with a combined area of 1% relative to that of the major peak.

Stability studies performed by gas chromatographic system 1 with *n*-undecane as an internal standard indicated that hexachloro-l,3-butadiene is stable as the bulk chemical when stored for 2 weeks at temperatures up to 60° C. Periodic reanalysis by gas chromatography of the bulk chemical at the study laboratory indicated no change in the purity of the chemical throughout the studies.

Preparation and Characterization of Formulated Diets

Diets were formulated by preparing a hexachloro-l,3-butadiene/corn oil/feed premix or a hexachloro-1,3-butadiene/feed premix (for final concentrations of 1,000 or 3,000 ppm) and blending the premix with feed in a twin-shell blender for two 10-minute periods. Hexane extracts of feed mixtures were analyzed by gas chromatography with nickel-63 electron-capture detection and a 3% SP2100 column. Formulated diets of hexachloro-1,3-butadiene at a concentration of 1 ppm were shown to be stable for 3 weeks in the dark at 5° C, but losses of 9%, 16%, and 41% after 1, 3, and 7 days, respectively, occurred when diets were stored open to air and light in an animal cage. Losses of 34%-40% were observed at target concentrations of 50 or 400 ppm hexachloro-l,3-butadiene in formulated diets after 7 days under animal room conditions. Further studies at the analytical chemistry laboratory indicated that under animal room conditions, formulated diets containing 30, 300, or 3,000 ppm hexachloro-1,3-butadiene lost 14%-17% after 3 days. A 9% loss of chemical was observed after 1 day for all three formulations. Consequently, feed was replaced every 2 days during the 2-week and 13-week studies.

Periodic analysis of formulated diets was conducted at the study laboratory and the analytical chemistry laboratory by gas chromatography with the method previously described but with 4-bromochlorobenzene as the internal standard (Table 2). Formulated diets were analyzed two times before the 13-week studies began and two times during the 13-week studies. The formulated diets were within specifications 100% of the time and averaged 102% of the target concentration.

Two-Week Study Design

Male and female B6C3F1 mice were obtained from Frederick Cancer Research Facility. Experimental design details are outlined in Table 3. Groups of five males and five females were fed diets containing 0, 30, 100, 300, 1,000, or 3,000 ppm hexachloro-1,3-butadiene for 15 consecutive days. A necropsy was performed on all animals. Histologic examinations were performed on controls and animals that were exposed at 300, 1,000, or 3,000 ppm. The organs examined are listed in Table 3.

Thirteen-Week Study Design

Groups of 10 male and 10 female mice were fed diets containing 0, 1, 3, 10, 30, or 100 ppm hexachloro-l,3-butadiene for 13 weeks. Experimental design details are outlined in Table 3. At the end of the studies, samples were taken for evaluation of sperm count, motility, and morphology and vaginal cytology.

Source and Specifications of Animals: Male and female B6C3F1 (C57BL/6N, female X C3H/HeN MTV^{--}, male) mice used in these studies were

TABLE 2. MEAN CONCENTRATION OF HEXACHLORO-1.3-BUTADIENE IN FEED IN THE THIRTEEN-WEEK FEED STUDIES

Target Concentration (ppm)	Determined (ppm) (a)
1	1.05 ± 0.047
3	3.09 ± 0.084
10	9.99 ± 0.363
30	30.3 ± 0.666
100	101.3 ± 3.864

(a) Mean ± standard deviation

TABLE 3. EXPERIMENTAL DESIGN AND MATERIALS AND METHODS IN THE FEED STUDIES OF HEXACHLORO-1,3-BUTADIENE

Two-Week Studies	Thirteen-Week Studies
Strain and Species $B6C3F_1$ mice	$B6C3F_1$ mice
Animal Source Frederick Cancer Research Facility (Frederick, MD)	Frederick Cancer Research Facility (Frederick, MD)
Study Laboratory Microbiological Associates, Inc.	Microbiological Associates, Inc.
Size of Study Groups 5 males and 5 females, individually caged	10 males and 10 females, individually caged
Doses 0, 30, 100, 300, 1,000, or 3,000 ppm hexachloro-l,3-butadiene in feed	0, 1, 3, 10, 30, or 100 ppm hexachloro-l,3-butadiene in feed
Method of Animal Distribution Animals distributed to weight classes and then assigned to cages and to groups by a table of random numbers	Same as 2-wk studies
Feed NIH 07 Rat and Mouse Ration (Zeigler Bros., Inc., Gardners, PA); available ad libitum	Same as 2-wk studies
Feed Consumption Measured on d 3 and then 1 X 2 d	Measured 1 X wk
Animal Room Environment Temp-66°-76° F; hum-45%-74%	Temp-69°-75° F; hum--38%-82%; fluorescent light 12 h/d; 12-15 room air changes/h
Time Held Before Study 15d	19d
Age When Placed on Study 6wk	7wk
Duration of Dosing 15d	13wk
Age When Killed 8wk	20 wk
Type and Frequency of Observation Observed 2 X d; weighed initially and on d 7 and d 15	Observed 2 X d; weighed initially and 1 X wk thereafter
Necropsy and Histologic Examinations Necropsy performed on all animals; histologic examinations performed on control, 300-, 1,000-, and 3,000-ppm animals. Bone marrow, kidneys, and liver examined for all animals. Liver, thymus, kidneys, heart, brain, lung, and testis weighed at necropsy	Necropsy performed on all animals; the following tissues examined histologically for all control and 100-ppm animals and all animals dying before the end of the studies: adrenal glands, brain, cecum, colon, duodenum, epididymis/seminal vesicles/prostate/testes or ovaries/uterus, esophagus, femur or sternebrae or vertebrae including marrow, gallbladder, gross lesions, heart, ileum, jejunum, kidneys, liver, lungs and bronchi, mammary gland. mandibular and mesenteric lymph nodes, nasal cavity and turbmales, pancreas, parathyroid glands, pituitary gland, rectum, salivary glands, skin, spleen, stomach, thymus, thyroid gland, trachea, and urinary bladder. Kidneys examined for all animals. Organ weights obtained at necropsy; sperm morphology and vaginal cytology evaluated

produced under strict barrier conditions at Frederick Cancer Research Facility. Animals were progeny of defined microflora-associated parents that were transferred from isolators to barrier-maintained rooms. Mice were shipped to the study laboratory at 4 weeks of age. The animals were quarantined at the study laboratory for 19 days. Mice were placed on study at 7 weeks of age.

Clinical Examinations and Pathology: Details of clinical examinations and pathology procedures are outlined in Table 3. A necropsy was performed on animals found moribund and on those surviving to the end of the studies. Thus, the number of animals from which particular organs or tissues were examined microscopically varies and is not necessarily equal to the number of animals that were placed on study.

Organs and tissues were examined for gross lesions. Tissues were preserved in 10% neutral buffered formalin and routinely processed for preparation of histologic sections for microscopic examination. Tissues and groups examined are listed in Table 3.

Upon completion of the histologic evaluation by the laboratory pathologist, 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, and the 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).

Statistical Methods: Organ weight to body weight ratios were analyzed by the multiple comparison methods of Dunn (1964) and Shirley (1977). Jonckheere's test (Jonckheere, 1954) was used to evaluate the significance of dose-response trends and to determine whether Dunn's or Shirley's test was more appropriate for pairwise comparisons.

III. RESULTS

Two-Week Studies

All mice that received feed containing 1,000 or 3,000 ppm hexachloro-1,3-butadiene died before the end of the studies (Table 4). Male and female mice that received 100 or 300 ppm lost weight. Compound-related clinical signs seen in mice that received 300 ppm or more included lethargy, rough hair coats, hunched position, and incoordination. Marked reductions in organ weight were observed in the thymus (28% and 49% of controls in the 300-ppm females and males, respectively) and heart (69% and 75% of controls in the 300-ppm females and males, respectively). Lesions clearly attributed to toxicity of hexachloro-l,3-butadiene were seen in the kidney of mice of each sex. At the two highest doses, at which all mice died during the first week of the studies, there was severe necrosis of the cortex and outer medulla of the kidney. In the surviving dosed mice from the lower dose groups, necrosis was less severe and regeneration was prominent in the outer stripe of the outer medulla (pars recta). Changes in other tissues were not clearly related to the toxicity of the compound. Most of these lesions occurred only in the highest dose mice that died during the first week of the studies. Lymphoid necrosis and depletion (atrophy) in the spleen, thymus, and lymph nodes and depletion (atrophy) and necrosis of the red pulp of the spleen were present in mice that died early. Testicular degeneration, characterized by the presence of syncytial giant cell formation of spermatocytes, was present in the two highest dose groups of male mice. Cytoplasmic vacuolization and necrosis of individual hepatocytes occurred primarily in the two highest dose groups; necrosis was very minimal in the mice that lived to the end of the studies and consisted of only a few necrotic hepatocytes in the section of liver. Minimal-to-mild depletion of the bone marrow in the

TABLE 4. SURVIVAL, MEAN BODY WEIGHTS, AND FEED CONSUMPTION OF MICE IN THE TWO-WEEK FEED STUDIES OF HEXACHLORO-1,3-BUTADIENE

Concentration (ppm)	Survival (a)	Mean Body Weights (grams) Initial (b)	Mean Body Weights (grams) Final (b)	Final Weight Relative to Controls (percent)	Feed Consumption (c)	Estimated Intake Dose (d)
MALE						
0	5/5	19.0 ± 0.3	21.2 ± 0.3		118	
30	5/5	19.3 ± 0.4	19.3 ± 0.4	91.0	100	3
100	5/5	19.2 ± 0.4	17.5 ± 0.5	82.5	119	12
300	5/5	19.0 ± 0.4	16.9 ± 0.4	79.7	133	40
1,000	(e)0/5	18.5 ± 0.4	(f)	(f)	(g)19	19
3,000	(h)0/5	19.1 ± 0.3	(f)	(f)	(g)8	24
FEMALE						
0	5/5	16.1 ± 0.5	18.1 ± 0.6		105	
30	5/5	16.2 ± 0.4	16.6 ± 0.5	91.7	159	5
100	5/5	15.1 ± 0.4	12.5 ± 0.4	69.1	159	16
300	5/5	15.5 ± 0.5	12.3 ± 0.6	68.0	162	49
1,000	(i)0/5	15.2 ± 0.3	(f)	(f)	(g)30	30
3,000	(j)0/5	14.6 ± 0.4	(f)	(f)	(g)12	36

(a) Number surviving/number initially in group
(b) Initial group mean body weight ± standard error of the mean; final refers to the last scheduled body weight measurement.
(c) Grains per kilogram body weight per day, averaged over the 2-week period; not corrected for scatter.
(d) Milligrams per kilogram per day based on average feed consumption
(e) Day of death: 4,7,7,7,7
(f) No data are reported due to 100% mortality in this group.
(g) Feed consumption for week 1 only, due to 100% mortality in this group
(h) Day of death: 4,5,5,6,6
(i) Day of death: 3,3,4,4,4
(j) Day of death: 3,4,4,5,5

femur was a subtle change characterized by a decrease in hematopoietic cells of the bone marrow. This lesion was observed in two to five male and female mice per dose group in animals given diets containing 300 ppm or more hexachloro-l,3-butadiene.

Thirteen-Week Studies

One male mouse that received 1 ppm hexachloro-l,3-butadiene died before the end of the study (Table 5). Mean body weights of male mice that received 30 or 100 ppm and female mice that received 100 ppm were lower than those of controls throughout most of the studies (Figure 2). The final mean body weight of male mice that received 30 or 100 ppm was 90% or 84% that of controls. The final mean body weight of females that received 100 ppm was 85% that of controls. In Table 6, only significant organ weight changes are summarized and presented together with the mean body weights at the terminal kill. The most significant dose-related organ weight changes were reductions (up to 26%) of kidney weight in the three highest dose male groups and the highest dose female group. The reduction (12%) of the heart weight of the 100-ppm males may also be of toxicologic significance. No compound-related clinical signs were observed.

TABLE 5. SURVIVAL, MEAN BODY WEIGHTS, AND FEED CONSUMPTION OF MICE IN THE THIRTEEN-WEEK FEED STUDIES OF HEXACHLORO-1,3-BUTADIENE

Concentration (ppm)	Survival (a)	Mean Body Weights (grams) Initial (b)	Mean Body Weights (grams) Final (b)	Final Weight Relative to Controls (percent)	Feed Consumption (c)	Estimated Intake Dose (d)
MALE						
0	10/10	17.8 ± 0.6	27.2 ± 1.0		3.2	
1	(e)9/10	19.3 ± 0.4	28.3 ± 0.7	104.0	3.5	0.1
3	10/10	19.0 ± 0.3	29.7 ± 0.5	109.2	3.5	0.4
10	(f) 10/10	19.0 ± 0.4	27.2 ± 0.8	100.0	3.5	1.5
30	10/10	19.5 ± 0.2	24.5 ± 0.5	90.1	3.6	4.9
100	10/10	18.7 ± 0.4	22.9 ± 0.4	84.2	3.5	16.8
FEMALE						
0	10/10	15.8 ± 0.3	24.0 ± 0.5		3.6	
1	10/10	16.3 ± 0.5	23.9 ± 0.7	99.6	3.6	0.2
3	10/10	16.5 ± 0.3	24.8 ± 0.7	103.3	3.7	0.5
10	10/10	15.6 ± 0.4	23.0 ± 0.2	95.8	3.4	1.8
30	10/10	16.7 ± 0.4	24.2 ± 0.5	100.8	3.1	4.5
100	10/10	16.0 ± 0.4	20.4 ± 0.6	85.0	3.5	19.2

(a) Number surviving/number initially in group
(b) Initial group mean body weight ± standard error of the mean. Subsequent calculations are based on animals surviving to the end of the study; final refers to the last scheduled body weight measurement.
(c) Grams per animal per day (averaged for weeks 6 and 12); not corrected for scatter.
(d) Milligrams per kilogram per day based on average feed consumption
(e) Week of death: 7
(f) Includes one missexed animal

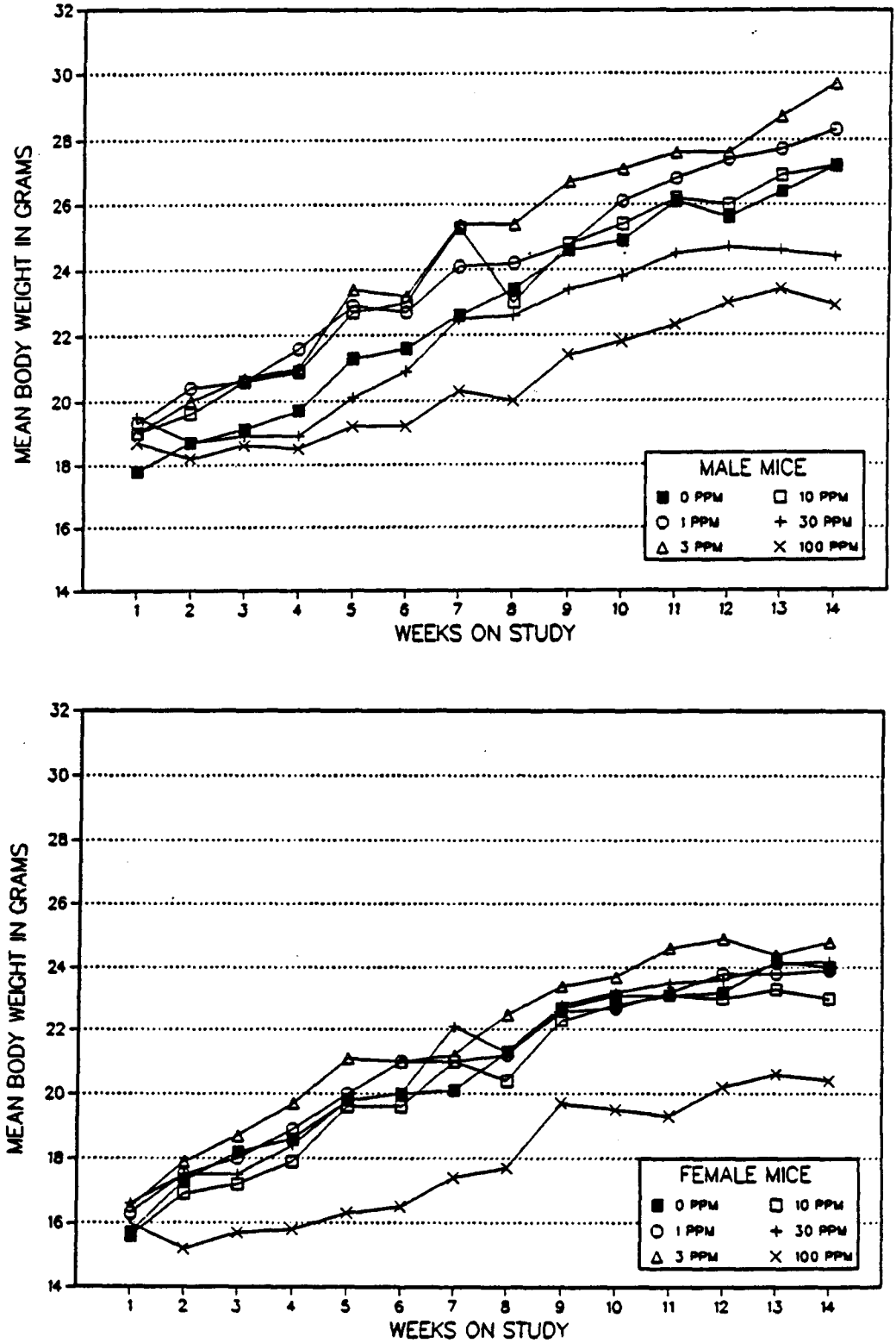

FIGURE 2. GROWTH CURVES FOR MICE FED DIETS CONTAINING HEXACHLORO-1,3-BUTADIENE FOR THIRTEEN WEEKS

TABLE 6. BODY AND ORGAN WEIGHTS OF MICE IN THE THIRTEEN-WEEK FEED STUDIES OF HEXACHLORO-1,3-BUTADIENE (a)

	Control	1 ppm	3 ppm	10 ppm	30 ppm	100 ppm
MALE						
Number weighed (b)	10	9	10	9	10	10
Necropsy body weight (grains)	27.1 ± 0.94	28.3 ± 0.75	28.9 ± 0.45	27.8 ± 0.68	24.7 ± 0.47	**23.5 ± 0.46
Brain						
Absolute	502 ± 10.2	513 ± 14.5	504 ± 11.4	504 ± 9.3	486 ± 17.8	506 ± 6.0
Relative	18.6 ± 0.56	18.3 ± 0.84	17.4 ± 0.42	18.2 ± 0.53	19.9 ± 1.00	**21.6 ± 0.28
Heart						
Absolute	167 ± 6.7	164 ± 4.1	166 ± 7.3	163 ± 7.3	154 ± 4.8	**147 ± 3.0
Relative	6.2 ± 0.21	5.8 ± 0.17	5.7 ± 0.21	5.9 ± 0.21	6.3 ± 0.15	6.3 ± 0.16
Right kidney						
Absolute	317 ± 13.2	324 ± 6.5	331 ± 8.1	288 ± 14.0	**233 ± 6.5	**241 ± 6.4
Relative	11.7 ± 0.33	11.5±0.24	11.5±0.33	**10.3 ± 0.38	**9.5 ± 0.18	**10.3 ± 0.15
Liver						
Absolute	1,531 ± 64.0	1,657 ± 39.8	1,632 ± 64.6	1,670 ± 51.8	1,475 ± 48.4	1,587 ± 33.0
Relative	56.7 ± 2.30	58.8 ± 1.30	56.5 ± 2.35	60.2 ± 1.58	59.9 ± 16.80	**67.7 ± 1.11
Spleen						
Absolute	54 ± 1.2	57 ± 1.1	(c) 56 ± 0.6	**62 ± 1.5	57 ± 1.6	56 ± 1.4
Relative	2.0 ± 0.07	2.0 ± 0.07	(c) 1.9 ± 0.04	*2.3± 0.06	**2.3 ± 0.05	**2.4 ± 0.04
Left testis						
Absolute	120 ± 3.0	118 ± 1.9	–	(d) 121 ± 2.7	–	*113 ± 2.1
Relative	4.5 ± 0.16	4.2 ± 0.09	–	(d) 4.3 ±0.13	–	*4.8 ± 0.08
FEMALE						
Number weighed	10	10	10	10	10	10
Necropsy body weight (grams)	24.3 ± 0.47	23.5 ± 0.64	25.0 ± 0.75	23.4 ± 0.27	24.6 ± 0.46	**20.7 ± 0.50
Brain						
Absolute	517 ± 8.7	515 ± 10.1	525 ± 6.9	523 ± 5.8	529 ± 10.8	527 ± 6.7
Relative	21.3 ± 0.36	22.0 ± 0.58	21.2 ± 0.70	22.4 ± 0.21	21.6 ± 0.26	**25.6 ± 0.53
Right kidney						
Absolute	228 ± 8.9	231 ± 8.0	223 ± 11.4	207 ± 5.4	209 ± 6.0	**175 ± 9.7
Relative	9.4 ± 0.28	9.9 ± 0.25	8.9 ± 0.38	8.8 ± 0.19	8.5 ± 0.14	8.4 ± 0.36

(a) Mean ± standard error in milligrams for absolute weights unless otherwise specified; P values vs. the controls by Dunn's or Shirley's test (Dunn, 1964; Shirley, 1977).
(b) Unless otherwise specified
(c) Spleens of eight animals were weighed.
(d) Testes of seven animals were weighed.
*P<0.05
**P<0.01

A compound-related increase in renal tubular epithelial regeneration was observed. The regeneration was prominent in the outer stripe of the outer medulla and extended into the medullary rays (pars recta) (Table 7). This was characterized by increased basophilia of the tubular cell cytoplasm and an increased number of nuclei; occasional mitoses were seen in these regenerative cells. The motility of sperm from dosed mice was significantly lower, though not dose related, than that from controls (Table 8). No significant changes were observed in the sperm count, the incidence of abnormal sperm, estrual cyclicity, or the average estrous cycle length.

TABLE 7. INCIDENCES OF RENAL TUBULAR REGENERATION IN B6C3F$_1$ MICE IN THE THIRTEEN-WEEK FEED STUDIES OF HEXACHLORO-1,3-BUTADIENE

Concentration (ppm)	Number of Mice with Lesion/Number Examined	
	Male	Female
0	0/10	0/10
1	0/10	1/10
3	0/10	9/10
10	0/9	10/10
30	10/10	10/10
100	10/10	10/10

TABLE 8. REPRODUCTIVE SYSTEM DATA FOR MALE MICE IN THE THIRTEEN-WEEK FEED STUDIES OF HEXACHLORO-1,3-BUTADIENE (a)

	Control	1 ppm	10 ppm	100 ppm
Number examined (b)	10	9	8	10
Abnormal sperm (percent)	1.14 ± 0.079	1.40 ±0.115	(c) 1.33 ± 0.088	1.30 ± 0.172
Caudal weight (mg)	17 ± 1	17 ± 1	17 ± 1	17 ± 1
Right epididymis weight (mg)	48 ± 3	47 ± 1	50 ±2	46 ±2
Sperm density (10^6/g cauda)	1,078 ± 91	1,046 ± 101	(c) 1,122 ± 104	896 ± 102
Sperm motility (percent)	89.4 ± 1.51	**76.9 ± 2.63	**77.5 ± 1.47	**81.3 ± 1.81

(a) Mean ± standard error; P values vs. the controls by Dunn's or Shirley's test (Dunn, 1964; Shirley, 1977).
(b) Unless otherwise specified
(c) Nine animals were examined.
**P<0.01

IV. DISCUSSION AND CONCLUSIONS

Two-Week Studies in B6C3F$_1$ Mice

There were no deaths in the three lowest dose groups; however, retardation of growth was evident in all groups exposed to hexachloro-l,3-butadiene. Whether this effect on growth is chemical related is not clear because of the variability in the measured feed consumption caused by the scattering of feed by mice in these groups. Rather drastic reductions in organ weights (thymus and heart) were probably related primarily to stress and only secondarily to the chemical insult.

Pale kidney cortices noted at necropsy correlated with the renal tubular necrosis seen in all of the mice from the two highest dose groups (i.e., 1,000 and 3,000 ppm). The distribution of the lesions was indicative of an acute toxic effect, with tubular cell necrosis being most prominent in convoluted tubules of the cortex. Convoluted tubules throughout the width of the cortex were affected. No specific segment of the convoluted tubules appeared spared, though not all tubules in each kidney were uniformly affected. Indeed, even in the most severely affected kidneys, some tubules appeared unaffected. Though tubular necrosis was primarily noted in the cortex, necrotic epithelial cells and debris were present in the outer medulla as well. No tubular regeneration was noted in animals from the two highest dose groups, which is consistent with the fact

that all died or were killed early in the studies. In the lower dose groups, in which all animals lived to the end of the studies, toxicity was evident by the presence of necrosis, but the most striking change was the very prominent tubular cell regeneration. Although the cause of death or a moribund condition in the two highest dose groups was not firmly established, it is reasonable to assume that kidney toxicity and renal failure contributed significantly to the animals' condition.

Hepatocellular cytoplasmic vacuolization and, to a much lesser extent, hepatocellular necrosis were noted grossly as a pale liver in all mice in the 3,000-ppm group and in 8/10 in the 1,000-ppm group. The cytoplasmic vacuolization may be due to inanition in the two highest dose groups. The multifocal individual cell necrosis seen in a few animals in the lower dose groups may have been due to chemical toxicity.

Gross and/or microscopic changes were also noted in the spleen, bone marrow, lymph nodes, thymus, and testis of mice from the two highest dose groups which were found dead or killed in a moribund state before the end of the studies. With the exception of the testis, where degenerative changes were characterized by the presence of giant cells, the lesions of all other tissues may be classified as necrosis or depletion. These effects, though related to chemical administration, are most likely due primarily to stress and only secondarily to the acute toxicity of the chemical.

Thirteen-Week Studies in B6C3F$_1$ Mice

No compound-related deaths were observed. A marked reduction of body weight gain was seen in male mice in the 30- and 100-ppm groups and in female mice in the 100-ppm group. Once again, there were no obvious differences in feed consumption among the various groups, thus suggesting a chemical-related growth retardation. In contrast to the extreme reduction in thymus weight in the 2-week studies, the thymus weights of mice in the 13-week studies were comparable in all groups. The most significant dose-related organ weight changes were reductions in kidney weight in the two highest dose groups of males and in the highest dose group of females. A reduction in the heart weight of the 100-ppm males may be of toxicologic significance and may be related to the toxic stress from hexachloro-1,3-butadiene. There were no histologic lesions in the heart.

No gross lesions attributable to hexachloro-1,3-butadiene exposure were noted at necropsy. The only compound-related microscopic lesion observed was tubular regeneration in the renal cortex. This lesion increased in severity with increased dose and was characterized by a diffuse increase in basophilia of the tubular epithelial cytoplasm and by an increase in the number of nuclei. Occasional mitotic figures were also seen. Although kidney lesions in these studies were similar to those noted in the lower dose groups in the 2-week studies, the necrosis accompanying the renal changes in the 2-week studies at 30 and 100 ppm was not evident in these studies. It can be assumed that some adaptation occurred and that the regenerative capacity of kidney tubular epithelium compensated for cell loss as the result of hexachloro-1,3-butadiene toxicity.

Based on the histopathologic evaluation of the kidney, the no-observed-adverse-effect level for male mice may be estimated to be at or slightly below 10 ppm. Such a level was not seen for female mice, even at the lowest exposure level of 1 ppm.

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