National Toxicology Program
Toxicity Report Series
Number 65

NTP Technical Report
on the Toxicity Studies of

3,3′,4,4′-Tetrachloroazobenzene

(CAS No. 14047-09-7)

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

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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. In July 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. 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 laboratory 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 (usually two species, rats and mice). Chemicals selected for NTP toxicology studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. The interpretive conclusions presented in this Toxicity Study Report are based only on the results of these NTP studies. Extrapolation of these results to other species and quantitative risk analyses for humans require wider analyses beyond the purview of these studies. Selection *per se* is not an indicator of a chemical's toxic potential.

Listings of all published NTP reports and ongoing studies are available from NTP Central Data Management, NIEHS, P.O. Box 12233, MD E1-02, Research Triangle Park, NC 27709 (919-541-3419). Other information about NTP studies is available at the NTP's World Wide Web site: http://ntp-server.niehs.nih.gov.

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PEER REVIEW

The draft report on the toxicity studies of 3,3′,4,4′-tetrachloroazobenzene was evaluated 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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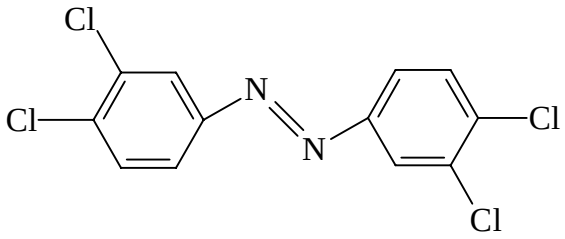
ABSTRACT



3,3′,4,4′-TETRACHLOROAZOBENZENE

CAS No. 14047-09-7

Chemical Formula: $C_{12}H_6Cl_4N_2$ Molecular Weight: 320.0

Synonyms: Azobenzene, 3,3′,4,4′-tetrachloro-(8CI); diazene, bis(3,4-dichlorophenyl)-(9CI); TCAB

3,3′,4,4′-Tetrachloroazobenzene is not commercially manufactured but is formed as an unwanted byproduct in the manufacture of 3,4-dichloroaniline and its herbicidal derivatives Propanil®, Linuron®, and Diuron®. In addition, environmental contamination by 3,3′,4,4′-tetrachloroazobenzene occurs from the degradation of chloranilide herbicides and the photolysis and biolysis of 3,4-dichloroaniline. 3,3′,4,4′-Tetrachloroazobenzene was nominated by the United States Environmental Protection Agency for toxicity testing based on concerns over the potential for human exposure, the structural resemblance to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and the reported dioxin-like effects of 3,3′,4,4′-tetrachloroazobenzene. The toxicity of 3,3′,4,4′-tetrachloroazobenzene was evaluated in 16-day and 13-week gavage studies in male and female F344/N rats and B6C3F_1 mice. In addition to histopathology, evaluations included hematology (rats only), clinical chemistry, thyroid hormone analyses (rats only), cytochrome P_{450}1A immunohistochemical staining in the liver (rats only), and assessments of male reproductive endpoints and estrous cycle length. Genetic toxicology studies included mutagenicity tests in *Salmonella typhimurium* and the determination of micronuclei in mouse bone marrow and peripheral blood erythrocytes.

In the 16-day studies, groups of five male and five female rats received 3,3′,4,4′-tetrachloroazobenzene in corn oil by gavage 5 days a week at doses of 0, 12.5, 32, 80, 200, or 500 mg per kg body weight. Groups of five male and five female mice received 3,3′,4,4′-tetrachloroazobenzene in corn oil by gavage 5 days a week at doses of 0, 1, 3.2, 10, 32, or 100 mg/kg. Major effects included increases in liver, lung, and spleen weights of rats

and liver and heart weights of mice and decreases in thymus weights of rats and mice. No effects were found on survival or mean body weight gains of rats or mice. Incidences of hematopoietic cell proliferation in the spleen were increased in all groups of dosed male rats, in female rats that received 32 mg/kg or greater, and in 100 mg/kg male and female mice. Renal tubule hyaline droplet accumulation in the cytoplasm of renal cortical epithelial cells and chronic nephropathy were observed microscopically in male rats in the 80, 200, and 500 mg/kg groups. Female mice in the 100 mg/kg group had atrophy of the thymus.

In the 13-week studies, groups of 10 male and 10 female rats and mice received 3,3′,4,4′-tetrachloroazobenzene in corn oil by gavage 5 days a week at doses of 0, 0.1, 1, 3, 10, or 30 mg/kg.

In the 13-week rat study, the major effects included a decrease in the mean body weight gain of 30 mg/kg females and final mean body weights of 30 mg/kg males and females, decreased thymus weights of males and females in the 10 and 30 mg/kg groups accompanied by thymic atrophy observed microscopically, increased incidences of hematopoietic cell proliferation in the spleen in 10 and 30 mg/kg males and females, a responsive anemia in 10 and 30 mg/kg males and females at week 13, and decreased platelet counts in 10 and 30 mg/kg males and females on day 21 and at week 13. Spleen weights were increased in 10 and 30 mg/kg males and females. Liver weights were increased in males that received 1 mg/kg or greater and in 10 and 30 mg/kg females. Furthermore, hepatic cytochrome P_{450}1A staining presence and intensity were increased in 30 mg/kg males and females. Sharp decreases in circulating thyroxine concentrations were observed in males and females at all doses. In spite of this sharp decrease, thyroid-stimulating hormone concentrations were marginally increased. Incidences of hyperplasia of the forestomach were increased in males administered 3 mg/kg or greater and females administered 30 mg/kg.

In the 13-week mouse study, the major effects included increases in liver and spleen weights of 10 and 30 mg/kg males and females and increased incidences of hyperplasia of the forestomach in males and females that received 1 mg/kg or greater. Furthermore, a decrease in thymus weight of 30 mg/kg males, an increase in centrilobular hypertrophy of hepatocytes in males that received 3 mg/kg or greater, and an increase in the incidences of hematopoietic cell proliferation in the spleen in males that received 3 mg/kg or greater were observed. A significant decrease in epididymal spermatozoal concentration was observed in 3 and 30 mg/kg males.

3,3′,4,4′-Tetrachloroazobenzene was mutagenic in *S. typhimurium* strain TA97 in the presence of rat liver S9 activation enzymes; no mutagenic activity was detected in strain TA98, TA100, TA1535, or TA1537 with or without S9. *In vivo*, the frequency of micronucleated erythrocytes was significantly increased in peripheral blood samples from male and female mice given 3,3′,4,4′-tetrachloroazobenzene by gavage for 13 weeks. However,

results of a 3-day exposure of up to 200 mg/kg by intraperitoneal injection did not demonstrate induction of micronuclei in bone marrow erythrocytes of male mice.

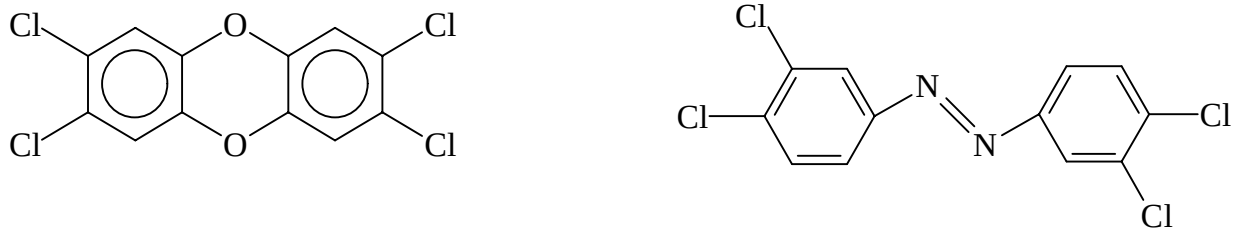
In summary, 3,3′,4,4′-tetrachloroazobenzene caused typical dioxin-like effects, such as thymic atrophy, an increase in liver weights, induction of hepatic cytochrome P_{450}1A, and decreased mean body weight gains. Furthermore, in the 13-week studies, a sharp decrease in circulating thyroxine concentrations was observed even at the lowest dose (0.1 mg/kg) tested in rats. Other effects included a decrease in epididymal spermatozoal concentration in mice, major effects on the hematopoietic system, and increased incidences of hyperplasia of the forestomach in 3 and 30 mg/kg males and 30 mg/kg females. A no-observable-adverse-effect-level (NOAEL) was not reached in rats. The NOAEL in mice was 0.1 mg/kg. Comparison of various dioxin-like effects in these studies with those reported in the literature indicate that 3,3′,4,4′-tetrachloroazobenzene is six to two orders of magnitude less potent than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

INTRODUCTION

CHEMICAL AND PHYSICAL PROPERTIES

3,3′,4,4′-Tetrachloroazobenzene is not commercially manufactured but is formed as an unwanted byproduct in the manufacture of 3,4-dichloroaniline and its herbicidal derivatives Propanil®, Linuron®, and Diuron® (Poland *et al.*, 1976; Sundström *et al.*, 1978; Bunce *et al.*, 1979; Hill *et al.*, 1981). In addition, environmental contamination by 3,3′,4,4′-tetrachloroazobenzene occurs from the degradation of chloroanilide herbicides (acylanilides, phenylcarbamates, and phenylureas) in soil by peroxide-producing microorganisms (Bartha *et al.*, 1968; Bartha and Pramer, 1969; Lay and Ilnicki, 1974). It is also formed by the photolysis and biolysis of 3,4-dichloroaniline (Mansour *et al.*, 1975; Miller *et al.*, 1980).

3,3′,4,4′-Tetrachloroazobenzene is a bright orange, crystalline solid and has a melting point of 158° C (Hsia and Burant, 1979) and a log octanol/water partition coefficient of 5.53 to 6.69 (USEPA, 1985; Hashimoto *et al.*, 1994). The solubility in water is calculated to be 1 µg/L (USEPA, 1985). In the *trans* configuration, 3,3′,4,4′-tetrachloroazobenzene can assume a planar conformation with a molecular shape similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Figure 1; Poland *et al.*, 1976).



2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

3,3′,4,4′-Tetrachloroazobenzene

FIGURE 1
Molecular Structures of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and 3,3′,4,4′-Tetrachloroazobenzene

PRODUCTION, USE, AND HUMAN EXPOSURE

Propanil has been reported to contain higher concentrations of 3,3′,4,4′-tetrachloroazobenzene than other herbicides (Sundström *et al.*, 1978; Bunce *et al.*, 1979; Hill *et al.*, 1981). The concentration of 3,3′,4,4′-tetrachloroazobenzene ranges from 1,000 to 2,700 µg/g in Propanil, 6 to 28 µg/g in Diuron, 8 to 9 µg/g in Linuron, and 9 to 8,600 µg/g in 3,4-dichloroaniline. With a production volume of 10 million pounds of Propanil per year, the resultant 3,3′,4,4′-tetrachloroazobenzene production amount could be as high as 12,000 kg per year (McMillan *et al.*, 1991). With a production volume of 100,000 to 1,000,000 pounds of 3,4-dichloroaniline per year, the resultant 3,3′,4,4′-tetrachloroazobenzene production amount could be as high as 3,900 kg per year (USEPA, 1985). Because 3,4-dichloroaniline is used as a precursor to dyes and, to a limited extent, as a heat transfer fluid in addition to its use in the manufacture of herbicides (USEPA, 1985), 3,3′,4,4′-tetrachloroazobenzene might be present in products other than herbicides.

Exposure of humans to 3,3′,4,4′-tetrachloroazobenzene may occur in rice fields; analyses of soil samples from a rice field plot treated with 6.7 kg Propanil/hectare indicated a 3,3′,4,4′-tetrachloroazobenzene concentration of 0.09 ppm (Kearney *et al.*, 1970). Six of 99 soil samples from the rice-growing states of Arkansas, California, Louisiana, Mississippi, and Texas contained 0.01 to 0.05 ppm 3,3′,4,4′-tetrachloroazobenzene, whereas no residual concentration of Propanil was detected (Carey *et al.*, 1980). Rice plants grown in an artificial medium containing 3,3′,4,4′-tetrachloroazobenzene are able to absorb and translocate it to the aerial portions of the plants (Still, 1969). 3,3′,4,4′-Tetrachloroazobenzene was detected in the roots and shoots of soybean plants grown in soil treated with 25 ppm 3,3′,4,4′-tetrachloroazobenzene (Worobey, 1984). Furthermore, human exposure to 3,3′,4,4′-tetrachloroazobenzene has been reported in various manufacturing plants producing 3,4-dichloroaniline or herbicides derived from 3,4-dichloroaniline (Taylor *et al.*, 1977).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

Male Sprague-Dawley rats administered a single gavage dose of 10 mg ^{14}C-labeled 3,3′,4,4′-tetrachloroazobenzene excreted approximately 27% of the radiolabel in the urine over 5 days (Burant and Hsia, 1984). Male F344 rats administered gavage doses of 3.2 or 32 mg 3,3′,4,4′-tetrachloroazobenzene/kg body weight excreted 39% to 45% in the urine in a 96-hour time period (Pillai *et al.*, 1996). The oral bioavailability of 3,3′,4,4′-tetrachloroazobenzene in this study was calculated to be 30%. Inhalation by rats (strain not specified) and dermal application to male albino rabbits of 3,3′,4,4′-tetrachloroazobenzene resulted in various systemic toxic effects, indicating that 3,3′,4,4′-tetrachloroazobenzene is absorbed after inhalation or dermal exposure (USEPA/OTS, 1983; USEPA, 1985).

The highest concentrations of 3,3′,4,4′-tetrachloroazobenzene in male Sprague-Dawley rats administered a single gavage dose were located in the adrenal gland, epididymal fat, kidney, liver, lung, lymph nodes, pancreas, and urinary bladder (Burant and Hsia, 1984). The liver-to-fat ratio of 3,3′,4,4′-tetrachloroazobenzene was 0.63. The lowest concentrations were found in the brain. In a single-dose gavage disposition study of radiolabeled 3,3′,4,4′-tetrachloroazobenzene in male F344 rats, radiolabel accumulated in the adipose tissue and kidney, as shown by tissue-to-blood ratios greater than 1 (Pillai *et al.*, 1996). The liver-to-fat ratios of 3,3′,4,4′-tetrachloroazobenzene ranged from 0.09 to 0.22 at doses of 32 and 3.2 mg/kg, respectively. Again, the lowest concentrations were found in the brain.

The *in vitro* metabolism of 3,3′,4,4′-tetrachloroazobenzene was studied in an NADPH-generating system and microsomes from male Sprague-Dawley rats exposed to 3,3′,4,4′-tetrachloroazoxybenzene by intraperitoneal injection (Hsia and Kreamer, 1981). The major metabolite was identified as a 3,3′,4,4′-tetrachloroazobenzene phenol. In addition, two minor metabolites were identified as 3,3′,4,4′-tetrachlorohydrazobenzene and an N-hydroxylated derivative of 3,3′,4,4′-tetrachlorohydrazobenzene. In male F344 rats, the major urinary metabolites characterized after exposure to 3,3′,4,4′-tetrachloroazobenzene were sulfate conjugates of mono- or dichloroaniline derivatives. In addition, some of these metabolites were N-acetylated (Hsia and Kreamer, 1981).

In male Sprague-Dawley rats administered radiolabeled 3,3′,4,4′-tetrachloroazobenzene by gavage, 66% of the dose was excreted in urine and feces after 24 hours (Burant and Hsia, 1984). The pattern indicated a biphasic elimination, consisting of an early rapid phase with a half-life of 18 hours and a slow terminal phase with a half-life greater than 20 days. The major route of excretion was via the feces (about twice as much as was excreted in the urine). In male F344 rats intravenously administered 3.2 or 32 mg/kg radiolabeled 3,3′,4,4′-tetrachloroazobenzene, 33% of the total dose was excreted in the bile 6 hours after administration (Pillai *et al.*, 1996), which is higher than the fecal elimination of 21% of the total dose after 24 hours. This finding suggests some enterohepatic recirculation of 3,3′,4,4′-tetrachloroazobenzene. Male F344 rats excreted 53% to 56% of a gavage dose of 3,3′,4,4′-tetrachloroazobenzene via the feces within 48 hours (Pillai *et al.*, 1996). Less than 6% of the administered radioactivity remained in the tissues after 96 hours.

Humans

No absorption, distribution, metabolism, or excretion studies of 3,3′,4,4′-tetrachloroazobenzene in humans have been found in a review of the literature.

TOXICITY

Experimental Animals

A 120-day study of 100 ppm 3,3′,4,4′-tetrachloroazobenzene administered in the feed to male Sprague-Dawley rats resulted in a 9.4% decrease in mean body weight compared to the controls at the end of the study (Hsia *et al.*, 1980). The total consumption of 3,3′,4,4′-tetrachloroazobenzene per animal in this experiment was calculated to be 25.2 mg. Hematocrit and hemoglobin concentration were decreased. 3,3′,4,4′-Tetrachloroazobenzene exposure resulted in an increase in relative liver weight, cytochrome P_{448} concentration, microsomal aryl hydrocarbon hydroxylase activity, and aspartate aminotransferase activity.

A 60-day study with male Sprague-Dawley rats administered 25 mg/kg 3,3′,4,4′-tetrachloroazobenzene per week by intraperitoneal injection resulted in a decrease in body weight, a decrease in relative thymus weight, and an increase in relative liver weight (39% above controls) (Hsia *et al.*, 1981). Histological alterations were found in the liver, lung, lymph nodes, spleen, and thymus. In the livers of dosed animals, hepatocyte swelling and cytoplasmic vacuoles were observed. The cortex of the thymus, the outer cortical areas of the mesenteric lymph nodes, and the periarterial lymphatic sheaths of the spleen were atrophied. In addition, the lungs of dosed rats contained thickened alveolar walls and foamy macrophages.

Two 25 mg/kg intraperitoneal doses of 3,3′,4,4′-tetrachloroazobenzene per week to male weanling Sprague-Dawley rats resulted in a delayed wasting syndrome characterized by reduced feed consumption, a significant reduction in mean body weight, and death after 6 weeks (Hsia and Kreamer, 1985).

In studies of 3,3′,4,4′-tetrachloroazobenzene hepatotoxicity conducted by Schrankel *et al.* (1980), male Sprague-Dawley rats were given four daily intraperitoneal injections of 25 mg/kg and examined on day 5. Female ICR outbred Swiss albino mice were administered five daily 20 mg/kg intraperitoneal injections immediately following the lactation stage and were examined on day 6. In both studies, hepatocytes were enlarged and contained abundant cytoplasmic vacuoles. Proliferation of smooth endoplasmic reticulum was observed; membranous arrays occurred frequently. The hepatic mitotic index was increased in dosed animals compared to the controls. In addition, potential genotoxicity of 3,3′,4,4′-tetrachloroazobenzene was suggested by Schrankel *et al.* (1980) because of the occasional appearance of atypical mitotic figures.

Two intraperitoneal injections of 25 mg/kg 3,3′,4,4′-tetrachloroazobenzene were administered (on days 0 and 4) to 8-week-old male Sprague-Dawley rats. Eleven days after the first injection, thymus weights were decreased and liver and spleen weights were increased compared to those of the controls (Hsia *et al.*, 1982).

The inner surface of the ears of two adult female New Zealand rabbits per group was painted daily for 5 days with 0.1 mL of a 0.001 to 0.08 mg/mL solution of 3,3′,4,4′-tetrachloroazobenzene in methyl isobutyl ketone (for a total dose of 0.5 to 32 µg 3,3′,4,4′-tetrachloroazobenzene) (Hill *et al.*, 1981). The rabbits were killed between 14 and 22 days after their first treatment. A dose-dependent increase in the severity of hyperkeratosis was observed. The hyperkeratosis occurred at the lowest dose, 0.5 µg, suggesting that the chloracnegenic effect for 3,3′,4,4′-tetrachloroazobenzene was as potent as that for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Hill *et al.*, 1981).

3,3′,4,4′-Tetrachloroazobenzene has been shown to bind to the aryl hydrocarbon receptor with a specific binding affinity of one-fifth that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Poland *et al.*, 1976; Schneider *et al.*, 1995). 3,3′,4,4′-Tetrachloroazobenzene induces hepatic aryl hydrocarbon hydroxylase activity in chicken embryos, with an ED_{50} of 2 nmol/kg (Poland *et al.*, 1976).

Male Sprague-Dawley rats dosed with a single intraperitoneal injection of 100 mg/kg 3,3′,4,4′-tetrachloroazobenzene had a 120-fold increase in hepatic microsomal 7-ethoxyresorufin-*O*-deethylase activity and threefold increases in 7-pentoxyresorufin-*O*-dependentylase and 7-benzyloxyresorufin alkylase activities (McMillan *et al.*, 1990). The porphyrinogenic effect of Propanil in chick embryo liver cell cultures has been attributed to 3,3′,4,4′-tetrachloroazobenzene (Mensink and Strik, 1982).

Humans

In manufacturing plants producing Diuron and dichloroaniline, 89 workers were exposed to 3,3′,4,4′-tetrachloroazobenzene and, to a lesser extent, 3,3′,4,4′-tetrachloroazoxybenzene, for 1 to 7 years (Scarisbrick and Martin, 1981). Thirty people in the exposed cohort had chloracne. Alanine aminotransferase activities and total cholesterol concentrations were greater in the exposed workers with chloracne than in exposed workers without chloracne or in unexposed workers. Triglyceride concentrations were elevated in exposed workers, with or without chloracne, in comparison to unexposed workers. Exposure resulted in chloracne in 30 people in one facility in 1989 (Dr. A. Smith, University of Leicester, United Kingdom, personal communication).

Morse *et al.* (1979) reported that 38% of 102 workers in an Arkansas Propanil manufacturing plant had chloracne that was attributed to 3,3′,4,4′-tetrachloroazobenzene exposure. The highest incidence (61%) occurred in the production workers.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Experimental Animals

The embryotoxicity and teratogenicity of 3,3′,4,4′-tetrachloroazobenzene in chick embryos was studied by Schrankel *et al.* (1982). Doses ranging from 0.0001 to 100 µg 3,3′,4,4′-tetrachloroazobenzene dissolved in corn oil per egg were injected into the air cell on day 4 of incubation. In an additional group of eggs, 0.05 µg 3,3′,4,4′-tetrachloroazobenzene per egg was injected on days 11, 12, or 13 of incubation. The majority of embryo deaths occurred before day 13 of incubation for all groups treated with 0.005 to 100 µg. Eggs that were injected on days 11, 12, or 13 of incubation had a lower incidence of embryo mortality than those injected on day 4. The LD_{50} was calculated to be 44 ng of 3,3′,4,4′-tetrachloroazobenzene. Numerous malformations were detected in hatched chicks and in embryos that died prior to hatching. Rump edema was the major abnormality observed in treated embryos. In addition, altered feather pattern, lack of down, hemorrhage, external viscera, reduced body size, failure to withdraw the yolk sac, beak malformation, dilation of blood vessels, and monomicropthalmia were observed (Schrankel *et al.*, 1982).

Humans

No studies of the reproductive or developmental effects 3,3′,4,4′-tetrachloroazobenzene in humans have been found in a review of the literature.

CARCINOGENICITY

Experimental Animals

The carcinogenic potential of 3,3′,4,4′-tetrachloroazobenzene has been investigated in *in vitro* studies. Hsia *et al.* (1977) reported on the ability of 3,3′,4,4′-tetrachloroazobenzene to induce focal morphologic changes, so-called type III foci, typical of transformed cells in C3H/10T1/2 cells. Transplantation of cells from type III foci into syngeneic C3H mice resulted in the formation of neoplasms (Reznikoff *et al.*, 1973).

In a 60-week feed study by the National Cancer Institute, rats (strain not specified) were exposed to 4 mg 3,3′,4,4′-tetrachloroazobenzene per week for the first 3 weeks and 10 mg per week for the following 37 weeks (Bartha and Pramer, 1970). At the end of the 60-week period, no neoplasms were observed in exposed rats, although fatty degeneration in the liver was reported. Additional details of this study were not provided; no other *in vivo* studies of 3,3′,4,4′-tetrachloroazobenzene carcinogenicity were found in the literature.

Humans

No epidemiologic studies of 3,3′,4,4′-tetrachloroazobenzene in humans have been found in a review of the literature.

GENETIC TOXICITY

There is very little published information on the genotoxicity of 3,3′,4,4′-tetrachloroazobenzene. It was not mutagenic in any of several strains of *Salmonella typhimurium*, with or without S9 metabolic activation enzymes, in standard plate incorporation assays (Gilbert *et al.*, 1980; McMillan *et al.*, 1988). Gilbert *et al*. (1980) reported weak, inconsistent mutagenicity of 3,3′,4,4′-tetrachloroazobenzene in a *S. typhimurium* fluctuation test in strains TA1532 and TA1538 tested in the presence of Aroclor 1254-induced S9. 3,3′,4,4′-Tetrachloroazobenzene did not induce unscheduled DNA synthesis (indicative of DNA damage and subsequent repair) in primary hepatocytes of rats in the absence of pretreatment with hepatic mixed-function oxidase inducers (McMillan *et al.*, 1988). However, 3,3′,4,4′-tetrachloroazobenzene was reported to induce dose-related increases in unscheduled DNA synthesis in primary hepatocyte cultures derived from rats pretreated with Aroclor 1254, phenobarbital, or 3,3′,4,4′-tetrachloroazobenzene (Shaddock *et al.*, 1989). Hsia and Kreamer (1979) found that a dose-dependent increase in unscheduled DNA synthesis was induced by 3,3′,4,4′-tetrachloroazobenzene in a freshly isolated suspension of rat hepatocytes. Finally, negative results were obtained with 3,3′,4,4′-tetrachloroazobenzene in a test for mutation induction at the HGPRT locus in cultured Chinese hamster ovary cells, with and without S9 (McMillan *et al.*, 1988).

STUDY RATIONALE AND DESIGN

3,3′,4,4′-Tetrachloroazobenzene and the related chemical 3,3′,4,4′-tetrachloroazoxybenzene were nominated by the United States Environmental Protection Agency (USEPA) for testing based on concerns over the potential for human exposure from consumption of contaminated crops and in occupational settings. In addition, the USEPA was concerned about the structural resemblance of 3,3′,4,4′-tetrachloroazobenzene to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin as well as the dioxin-like effects observed with 3,3′,4,4′-tetrachloroazobenzene exposure.

Because the oral route is the most likely route of exposure through consumption of contaminated plant material, 3,3′,4,4′-tetrachloroazobenzene was administered by gavage. Male and female F344/N rats and B6C3F$_1$ mice were dosed for 16 days or 13 weeks. Endpoints evaluated included survival, body and organ weights, clinical findings of toxicity, and gross and microscopic pathology. In the 13-week studies, hematology (rats only), clinical chemistry, sperm motility, and vaginal cytology parameters were also measured. In the rats, plasma

thyroid hormone and hepatic cytochrome P_{450}1A were also measured because alterations in circulating thyroid hormone and hepatic cytochrome P_{450}1A are affected by dioxin-like compounds at low exposure concentrations. Furthermore, 3,3′,4,4′-tetrachloroazobenzene was tested for the induction of mutations in *S. typhimurium*, the induction of micronuclei in mouse bone marrow cells, and for increases in the frequency of micronuclei in peripheral blood erythrocytes of mice.

The doses used in the 16-day studies were based on a 3,3′,4,4′-tetrachloroazobenzene study that reported slightly decreased mean body weights in rats administered 100 ppm in feed (Hsia *et al.*, 1980), equivalent to 0.5 mg/kg per day for rats consuming 17 grams of feed per day, and on 3,3′,4,4′-tetrachloroazoxybenzene studies that reported decreased mean body and thymus weights in mice administered 10 mg/kg per day (Bleavins *et al.*, 1985a,b). Two higher and two lower doses were chosen for each study. Doses for the 13-week studies were based on the results of the 16-day studies.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF 3,3′,4,4′-TETRACHLOROAZOBENZENE

3,3′,4,4′-Tetrachloroazobenzene was obtained from AccuStandard, Inc. (New Haven, CT) in one lot (G920331A). Information on identity, purity, and stability was provided by the manufacturer; identity was confirmed by the study laboratory. Reports on analyses performed in support of the 3,3′,4,4′-tetrachloroazobenzene studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a bright orange crystalline solid, was identified as 3,3′,4,4′-tetrachloroazobenzene by infrared spectroscopy. The spectrum was consistent with a literature reference (Hsia and Burant, 1979). Gas chromatography indicated a purity of approximately 98%.

Information supplied by the manufacturer indicated that 3,3′,4,4′-tetrachloroazobenzene is stable as a bulk chemical when stored at room temperature. Throughout the studies, the bulk chemical was stored at room temperature in a well-ventilated area.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared once during the 16-day studies and every 2 weeks during the 13-week studies by mixing 3,3′,4,4′-tetrachloroazobenzene with corn oil; formulations were stored no longer than 3 weeks. Homogeneity and stability studies of 0.1 and 2.5 mg/mL formulations and homogeneity studies of a 100 mg/mL formulation were performed by the study laboratory using high-performance liquid chromatography. Homogeneity was confirmed and the stability of the dose formulations was confirmed for up to 21 days at room temperature when stored in dosing bottles.

Periodic analyses of the dose formulations of 3,3′,4,4′-tetrachloroazobenzene were conducted at the study laboratory using high-performance liquid chromatography and ultraviolet spectroscopy. All dose formulations administered to rats and mice were within 10% of the target concentrations. Animal room samples from the 16-day and 13-week studies were generally within 10% of the target concentration, although some samples from the two highest dose levels in the 16-day studies contained a small amount of insoluble chemical in the suspension, making it difficult to ensure adequate homogeneity.

16-DAY STUDIES

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Taconic Farms (Germantown, NY). Upon receipt, the rats and mice were 4 weeks old. Rats were quarantined for 12 or 13 days and mice were quarantined for 14 days. Rats and mice were 6 weeks old on the first day of the studies. Groups of five male and five female rats received 3,3′,4,4′-tetrachloroazobenzene in corn oil by gavage 5 days a week at doses of 0, 12.5, 32, 80, 200, or 500 mg/kg and groups of five male and five female mice received 3,3′,4,4′-tetrachloroazobenzene in corn oil by gavage at doses of 0, 1, 3.2, 10, 32, or 100 mg/kg. Feed and water were available *ad libitum*. Rats and female mice were housed five per cage, and male mice were housed individually. Clinical findings were recorded and animals were weighed initially, on day 8, and at the end of the studies. At the beginning of the studies, two male and two female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. Details of the study design and animal maintenance are summarized in Table 1.

A necropsy was performed on all rats and mice. The heart, right kidney, liver, lung, spleen, right testis, thymus, and uterus were weighed. Histopathologic examinations of selected tissues were performed on all vehicle control rats and mice, the highest dose groups of rats and mice with at least 60% survivors, and all higher dose groups at the end of the studies. Table 1 lists the tissues and organs examined.

13-WEEK STUDIES

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Taconic Farms (Germantown, NY). On receipt, the rats and mice were 4 weeks old. Animals were quarantined for 12 to 15 days and were 6 weeks old on the first day of the studies. Before initiation of the studies, five male and five female rats and mice were randomly selected for parasite evaluation and gross observation for evidence of disease. Blood samples were collected from five male and five female rats and mice at the beginning of the 13-week studies. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b). All results were negative.

Groups of 10 male and 10 female rats and mice received 3,3′,4,4′-tetrachloroazobenzene in corn oil by gavage at doses of 0, 0.1, 1, 3, 10, or 30 mg/kg. Feed and water were available *ad libitum*. Rats and female mice were housed five per cage, and male mice were housed individually. Clinical findings were recorded weekly for rats and mice. The animals were weighed initially, weekly, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table 1.

Special study groups of 10 male and 10 female rats per dose were included in the 13-week study and were designated for interim hematology and clinical chemistry evaluations (days 3 and 21) and cytochrome P_{450} immunohistochemistry (day 21). Rats in these groups were housed with the core study animals. The left liver lobe was collected from groups of 10 rats in the special studies on day 21 and at the end of the study from the core study rats for immunohistochemical determination of cytochrome P_{450}1A. The tissues were fixed in 4% paraformaldehyde at 4 C for 19 to 23 hours. The tissues were then washed in cold phosphate-buffered saline for 6 hours at 4 C with a change at 3 hours and were stored in 70% ethanol until processed into paraffin blocks and sectioned, and slides were prepared. Liver tissue samples were stained with anti-P_{450}1A antibodies (Oxford Biomedical, Oxford, MI). Sections of a liver from a rat exposed to 3,3′,4,4′-tetrachloroazoxybenzene served as the quality control in cytochrome P_{450}1A determinations. The presence and intensity of cytochrome P_{450}1A staining in the liver lobe were then rated.

Hematology and clinical chemistry studies were performed on special study rats on days 3 and 21 and on all core study rats at the end of the study. At the end of the 13-week study, clinical chemistry analyses were performed on all mice. At all time points, rats and mice were anesthetized with a CO_2/O_2 mixture and blood was collected from the retroorbital sinus. Blood for hematology determinations was placed in tubes containing potassium EDTA as the anticoagulant. Manual hematocrit determinations were performed using an Adams CT2900 Microhematocrit centrifuge (Clay Adams, Sparks, MD). All other hematology parameters were measured using a Serono-Baker 9000 automated cell counter (Serono-Baker Diagnostics, Allentown, PA). Leukocyte differentials, nucleated erythrocyte counts, and morphological evaluation of blood cells were determined by light microscopic evaluations of blood films stained with a modified Wright's stain using an Ames Hema-Tek II Slide Stainer (Miles Laboratory, Ames Division, Elkhart, IN). Smears made from preparations of equal volumes of new methylene blue (Sigma Chemical Company, St. Louis, MO) and whole blood were incubated for at least 20 minutes at room temperature and examined microscopically for the quantitative determination of reticulocytes.

Blood for clinical chemistry determinations was placed in tubes with no anticoagulant and allowed to clot at room temperature, and the serum was separated. All clinical chemistry endpoints except total triiodothyronine, total thyroxine, and thyroid-stimulating hormone concentrations were determined using a Hitachi® 717 chemistry analyzer (Boehringer Mannheim Diagnostics, Indianapolis, IN). Reagents were obtained from the manufacturer with the exception of the reagents for sorbitol dehydrogenase and total bile acid determinations, which were obtained from Sigma Chemical Company. Total triiodothyronine, total thyroxine, and thyroid-stimulating hormone concentrations were measured by radioimmunoassay techniques. DPC Coat-A-Count reagent kits (Diagnostic Products Corporation, Los Angeles, CA) were used for the total triiodothyronine and total thyroxine assays. Thyroid-stimulating hormone concentrations were measured using a double-antibody technique and rat-

specific reagents obtained from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (Bethesda, MD). The parameters measured for clinical pathology determinations are listed in Table 1.

At the end of the 13-week studies, samples were collected for sperm motility and vaginal cytology evaluations of core study rats and mice receiving 0, 3, 10, and 30 mg/kg. The parameters evaluated are listed in Table 1. Methods used were those described in the NTP's sperm morphology and vaginal cytology evaluations protocol (NTP, 1991). For 12 consecutive days prior to the scheduled terminal sacrifice, the vaginal vaults of the females were moistened with saline, if necessary, and samples of vaginal fluid and cells were stained. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus, estrus, and metestrus). Male animals were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk (rats) or modified Tyrode's buffer (mice) was applied to slides and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then heat fixed at 65 C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the 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.

A necropsy was performed on all core study animals. The heart, right kidney, liver, lung, spleen, right testis, thymus, and uterus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 to 6 µm, and stained with hematoxylin and eosin. A complete histopathologic examination was performed on all vehicle controls, the highest dose groups of rats and mice with at least 60% survivors, and all higher groups at the end of the 13-week studies. Target organs were examined until a no-effect-level was observed. Table 1 lists the tissues and organs examined.

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of 3,3′,4,4′-Tetrachloroazobenzene

16-Day Studies	13-Week Studies
Study Laboratory Microbiological Associates, Inc. (Bethesda, MD)	Microbiological Associates, Inc. (Bethesda, MD)
Strain and Species Rats: F344/N Mice: $B6C3F_1$	Rats: F344/N Mice: $B6C3F_1$
Animal Source Taconic Farms (Germantown, NY)	Taconic Farms (Germantown, NY)
Time Held Before Studies Rats: 12 or 13 days Mice: 14 days	Rats: 12 days (males) or 13 days (females) Mice: 14 days (males) or 15 days (females)
Average Age When Studies Began 6 weeks	6 weeks
Date of First Dose Rats: 12 October 1992 Mice: 13 October 1992	Rats: 19 January 1993 (males) or 20 January 1993 (females) Mice: 21 January 1993 (males) or 22 January 1993 (females)
Duration of Dosing 16 days (5 days/week) for a total of 12 doses	91 days (5 days/week)
Date of Last Dose Rats: 27 October 1992 Mice: 28 October 1992	Rats: 19 April 1993 (males) or 20 April 1993 (females) Mice: 21 April 1993 (males) or 22 April 1993 (females)
Necropsy Dates Rats: 28 October 1992 Mice: 29 October 1992	Rats: 20 April 1993 (males) or 21 April 1993 (females) Mice: 22 April 1993 (males) or 23 April 1993 (females)
Average Age at Necropsy 8 weeks	19 weeks
Size of Study Groups 5 males and 5 females	10 males and 10 females
Method of Distribution Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 16-day studies
Animals per Cage Rats: 5 Mice: 1 (males) or 5 (females)	Rats: 5 Mice: 1 (males) or 5 (females)
Method of Animal Identification Tail tattoo	Tail tattoo
Diet NIH-07 open formula pelleted diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed weekly	Same as 16-day studies

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of 3,3′,4,4′-Tetrachloroazobenzene

16-Day Studies	13-Week Studies
Water Tap water (Washington Suburban Sanitary Commission Potomac Plant) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*	Same as 16-day studies
Cages Polycarbonate (Lab Products, Maywood, NJ), rotated every 2 weeks	Same as 16-day studies
Bedding Sani-Chips® (P.J. Murphy Forest Products, Montville, NJ), changed twice weekly for rats and female mice and weekly for male mice	Same as 16-day studies
Racks Stainless steel (Lab Products, Rochelle Park, NJ), rotated every 2 weeks	Same as 16-day studies
Animal Room Environment Temperature: 72 ± 3 F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Temperature: 72 ± 3 F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour
Doses Rats: 0, 12.5, 32, 80, 200, or 500 mg/kg in corn oil by gavage (dosing volume = 5 mL/kg body weight) Mice: 0, 1, 3.2, 10, 32, or 100 mg/kg in corn oil by gavage (dosing volume = 10 mL/kg body weight)	0, 0.1, 1, 3, 10, or 30 mg/kg in corn oil by gavage (dosing volume = 5 mL for rats or 10 mL for mice per kg body weight)
Type and Frequency of Observation Observed twice daily; animals were weighed and clinical findings were recorded initially, on day 8, and at the end of the studies.	Observed twice daily; animals were weighed initially, weekly, and at the end of the studies; clinical findings were recorded weekly.
Method of Sacrifice 70%:30% CO_2:O_2	Same as 16-day studies
Necropsy Necropsy was performed on all animals. Organs weighed were heart, right kidney, liver, lung, spleen, right testis, thymus, and uterus.	Necropsy was performed on all core study animals. Organs weighed were heart, right kidney, liver, lung, spleen, right testis, thymus, and uterus.
Clinical Pathology None	Blood was collected from the retroorbital sinus of special study rats on days 3 and 21 and all core study rats and mice surviving to the end of the studies for hematology (rats only) and clinical chemistry. ***Hematology:*** automated and manual hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and nucleated erythrocyte counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; platelet count; and total leukocyte counts and differentials ***Clinical chemistry:*** urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, bile acids, thyroid-stimulating hormone (rats only), total triiodothyronine (rats only), and total thyroxine (rats only)

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of 3,3′,4,4′-Tetrachloroazobenzene

16-Day Studies	13-Week Studies
Histopathology	
Histopathology was performed on all vehicle control animals, the highest dose groups of rats and mice with at least 60% survivors, and animals in all higher dose groups at the end of the studies. In addition to gross lesions and tissue masses, the following tissues were examined: gallbladder (mice), kidney, liver, lymph nodes (mesenteric), spleen, stomach (including forestomach and glandular stomach), and thymus.	Complete histopathology was performed on core study vehicle control animals, the highest dose groups of rats and mice with at least 60% survivors, and animals in all higher dose groups at the end of the studies. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone (including marrow), brain, clitoral gland, esophagus, gallbladder (mice), heart, large intestine (cecum, colon, and rectum), small intestine (duodenum, jejunum, and ileum), kidney, liver, lung (and mainstem bronchi), lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (including forestomach and glandular stomach), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, and uterus. Target organs examined to a no-observed-effect-level were forestomach, kidney (males only), liver, spleen, and thymus for rats. Target organs examined in all lower dose groups of mice were forestomach, glandular stomach (males only), liver, and spleen.
Sperm Motility and Vaginal Cytology	
None	At the end of the studies, sperm samples were collected from all core study male animals in the 0, 3, 10, and 30 mg/kg groups for sperm motility evaluations. The following parameters were evaluated: spermatid heads per testis and per gram testis, spermatid counts, and epididymal spermatozoal motility and concentration. The left cauda epididymis, left epididymis, and left testis were weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies from all core study females administered 0, 3, 10, or 30 mg/kg for vaginal cytology evaluations. The parameters evaluated were the percentage of cycle spent in the various estrous stages and estrous cycle length.
Hepatic Cytochrome P_{450}1A Staining	
None	Cytochrome P_{450}1A presence and staining intensity were determined in special study rats on day 21 and in core study rats at the end of the study.

STATISTICAL METHODS

Calculation and Analysis of Lesion Incidences

The incidences of lesions as presented in Appendix A are given as the number of animals bearing such lesions at a specific anatomic site and the number of animals with that site examined microscopically. The Fisher exact test, a procedure based on the overall proportion of affected animals, was used to determine significance (Gart *et al.*, 1979).

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and vehicle control groups in the analysis of continuous variables. Organ and body weight data, which have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology, clinical chemistry, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related 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 a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical 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. Average severity values were analyzed for significance with the Mann-Whitney U test (Hollander and Wolfe, 1973).

Because vaginal cytology 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 a normality assumption. 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 dose levels.

QUALITY ASSURANCE METHODS

The 13-week studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice 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.

GENETIC TOXICOLOGY

Salmonella Mutagenicity Test Protocol

Testing was performed as reported by Mortelmans *et al*. (1986). 3,3′,4,4′-Tetrachloroazobenzene was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains TA97, TA98, TA100, TA1535, and TA1537 either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37 C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37 C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and of least five doses of 3,3′,4,4′-tetrachloroazobenzene. In the absence of toxicity, 10,000 µg/plate was selected as the high dose. All positive trials were repeated under the conditions that elicited the positive response.

In this assay, a positive response 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, is not reproducible, or is 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 is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

Mouse Bone Marrow Micronucleus Test Protocol

Preliminary range-finding studies were performed. Factors affecting dose selection included chemical solubility and toxicity and the extent of cell cycle delay induced by 3,3′,4,4′-tetrachloroazobenzene exposure. The standard three-exposure protocol is described in detail by Shelby *et al*. (1993). Groups of five male B6C3F_1 mice were injected intraperitoneally three times at 24-hour intervals with 3,3′,4,4′-tetrachloroazobenzene dissolved in corn oil at dose levels up to 200 mg/kg; the total dosing volume was 0.4 mL. Solvent control animals were injected with 0.4 mL of corn oil only. The positive control animals received injections of cyclophosphamide. The animals were killed 24 hours after the third injection, and blood smears were prepared from bone marrow cells obtained from the femurs. Air-dried smears were fixed and stained; 2,000 polychromatic erythrocytes (PCEs) were scored for the frequency of micronucleated cells in each of five animals per dose group.

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 PCEs was analyzed by

a statistical software package that tested for increasing trend over dose groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each dosed group and the vehicle control group (ILS, 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 is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single dose group is less than or equal to 0.025 divided by the number of dose groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitudes of those effects.

Mouse 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. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange and coded. Slides were scanned to determine the frequency of micronuclei in 2,000 normochromatic erythrocytes in each of five animals per dose group. Results were analyzed by the same methods described in the mouse bone marrow micronucleus test protocol.

RESULTS

RATS

16-DAY STUDY

All rats survived to the end of the study (Table 2). The final mean body weights and body weight gains of male and female rats were similar to those of the vehicle controls (Table 2). No chemical-related clinical findings were observed in dosed male or female rats.

TABLE 2
Survival and Body Weights of Rats in the 16-Day Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	123 ± 6	196 ± 8	73 ± 2	
12.5	5/5	125 ± 4	191 ± 7	66 ± 5	98
32	5/5	126 ± 4	186 ± 9	60 ± 6	95
80	5/5	128 ± 3	195 ± 6	68 ± 5	100
200	5/5	125 ± 4	190 ± 5	65 ± 3	97
500	5/5	127 ± 3	198 ± 3	70 ± 1	101
Female					
0	5/5	104 ± 2	137 ± 4	33 ± 3	
12.5	5/5	102 ± 2	132 ± 2	30 ± 1	96
32	5/5	101 ± 2	131 ± 2	29 ± 1	95
80	5/5	101 ± 2	130 ± 2	29 ± 1	95
200	5/5	101 ± 4	131 ± 4	30 ± 2	96
500	5/5	102 ± 2	134 ± 2	32 ± 2	98

[a] Number of animals surviving at 16 days/number initially in group
[b] Weights and weight changes are given as mean ± standard error. Differences from the vehicle control group are not significant by Dunnett's test.

The absolute and relative thymus weights of all dosed groups of male and female rats were less than those of the vehicle controls (Tables 3 and C1). The absolute spleen weights of 500 mg/kg males and females and relative spleen weights of males receiving 80 mg/kg or greater and females receiving 32 mg/kg or greater were greater than in the vehicle controls. The relative right kidney weight of males that received 80 mg/kg or greater and of all dosed groups of females were significantly greater than those of the vehicle controls. The absolute liver weights of male and female rats receiving 80 mg/kg or greater and the relative liver weights of male and female rats in all dosed groups were greater than those of the vehicle controls. The absolute and relative lung weights of males receiving 32 mg/kg or greater and females in the 500 mg/kg group were significantly greater than those of the vehicle controls. For male and female rats, significant dose-dependent trends were observed for the increases in absolute and relative liver, lung, and spleen weights, relative kidney weights and for the decreases in absolute and relative thymus weights. An increase in the absolute kidney weight of male mice also occurred with a significant trend, although the dose groups were not significantly different from the vehicle control group.

No treatment-related gross lesions were observed. In the kidney, renal tubule hyaline droplet accumulation in the cytoplasm of renal cortical epithelial cells and chronic nephropathy were observed microscopically in males in the 80, 200, and 500 mg/kg groups. Hematopoietic cell proliferation of the spleen was observed in all groups of dosed males and in females receiving 32 mg/kg or greater.

The dose selection for the 13-week studies in rats was based on the lower thymus weights observed in male and female rats administered 12.5 mg/kg or greater. Because 3,3′,4,4′-tetrachloroazobenzene is expected to bioaccumulate, although to a lesser extent than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the doses for the 13-week study in rats were chosen to be minimally immunotoxic. Because it was unknown whether animals would develop a tolerance to 3,3′,4,4′-tetrachloroazobenzene (i.e., by increased metabolism), the doses chosen for the 13-week study in rats were 0, 0.1, 1, 3, 10, and 30 mg/kg.

TABLE 3
Selected Organ Weight Data for Rats in the 16-Day Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	12.5 mg/kg	32 mg/kg	80 mg/kg	200 mg/kg	500 mg/kg
n	5	5	5	5	5	5
Male						
Necropsy body wt	196 ± 8	191 ± 7	186 ± 9	195 ± 6	190 ± 5	198 ± 3
R. Kidney						
Absolute	0.867 ± 0.029	0.890 ± 0.045	0.873 ± 0.045	0.991 ± 0.031	0.959 ± 0.040	0.994 ± 0.031
Relative	4.44 ± 0.09	4.65 ± 0.09	4.70 ± 0.07	5.09 ± 0.11**	5.04 ± 0.11**	5.03 ± 0.13**
Liver						
Absolute	9.970 ± 0.198	10.828 ± 0.777	10.723 ± 0.507	11.751 ± 0.484*	11.950 ± 0.459**	12.462 ± 0.282**
Relative	51.13 ± 1.24	56.39 ± 1.86*	57.79 ± 0.49**	60.20 ± 1.07**	62.97 ± 1.70**	63.07 ± 1.19**
Lung						
Absolute	1.140 ± 0.038	1.145 ± 0.082	1.395 ± 0.072*	1.365 ± 0.076*	1.368 ± 0.081*	1.420 ± 0.076*
Relative	5.84 ± 0.09	5.97 ± 0.23	7.52 ± 0.10**	7.02 ± 0.41**	7.21 ± 0.38**	7.19 ± 0.40**
Spleen						
Absolute	0.586 ± 0.011	0.593 ± 0.027	0.607 ± 0.024	0.656 ± 0.034	0.645 ± 0.023	0.662 ± 0.016*
Relative	3.01 ± 0.11	3.11 ± 0.13	3.28 ± 0.07	3.36 ± 0.10*	3.40 ± 0.11*	3.35 ± 0.05*
Thymus						
Absolute	0.518 ± 0.015	0.396 ± 0.012**	0.353 ± 0.008**	0.351 ± 0.011**	0.329 ± 0.006**	0.345 ± 0.005**
Relative	2.67 ± 0.14	2.08 ± 0.08**	1.92 ± 0.12**	1.80 ± 0.05**	1.74 ± 0.05**	1.74 ± 0.03**
Female						
Necropsy body wt	137 ± 4	132 ± 2	131 ± 2	130 ± 2	131 ± 4	134 ± 2
R. Kidney						
Absolute	0.635 ± 0.012	0.648 ± 0.006	0.646 ± 0.013	0.649 ± 0.019	0.665 ± 0.025	0.680 ± 0.031
Relative	4.63 ± 0.09	4.93 ± 0.07*	4.94 ± 0.11*	4.98 ± 0.08*	5.07 ± 0.12**	5.08 ± 0.03**
Liver						
Absolute	6.370 ± 0.224	6.651 ± 0.121	6.958 ± 0.148	7.217 ± 0.175*	7.783 ± 0.374**	8.043 ± 0.241**
Relative	46.40 ± 1.12	50.61 ± 1.10*	53.18 ± 0.91**	55.39 ± 1.05**	59.21 ± 1.54**	59.99 ± 1.01**
Lung						
Absolute	0.987 ± 0.055	0.870 ± 0.023	0.940 ± 0.014	0.963 ± 0.021	1.103 ± 0.079	1.159 ± 0.096*[b]
Relative	7.20 ± 0.41	6.61 ± 0.16	7.19 ± 0.12	7.40 ± 0.26	8.41 ± 0.57	8.66 ± 0.78*[b]
Spleen						
Absolute	0.414 ± 0.011	0.397 ± 0.011	0.436 ± 0.008	0.432 ± 0.013	0.445 ± 0.017	0.458 ± 0.013*
Relative	3.02 ± 0.05	3.02 ± 0.08	3.34 ± 0.08*	3.32 ± 0.14*	3.39 ± 0.07**	3.42 ± 0.08**
Thymus						
Absolute	0.380 ± 0.007	0.307 ± 0.007**	0.279 ± 0.006**	0.259 ± 0.014**	0.271 ± 0.012**	0.222 ± 0.013**
Relative	2.77 ± 0.05	2.34 ± 0.08**	2.13 ± 0.06**	1.99 ± 0.13**	2.07 ± 0.11**	1.66 ± 0.10**

* Significantly different (P 0.05) from the vehicle control group by Williams' test
** P 0.01
Trend is significantly increased (P 0.01) by Jonckheere's test.
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] n = 4

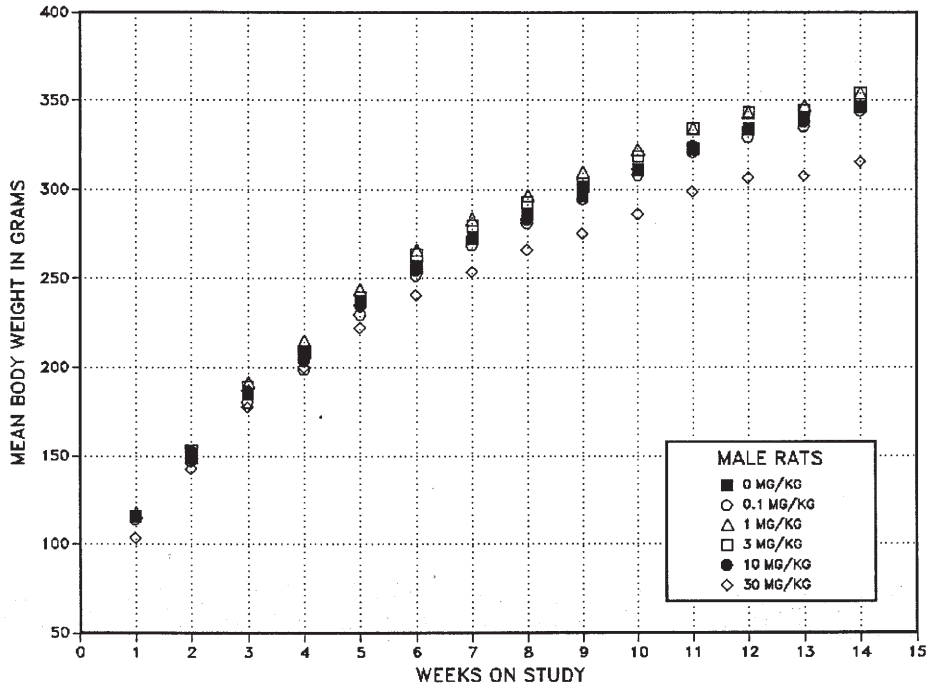
13-WEEK STUDY

All rats survived to the end of the study (Table 4). The final mean body weight of 30 mg/kg males and the final mean body weight and body weight gain of 30 mg/kg females were significantly less than those of the vehicle controls (Table 4 and Figure 2). This was a dose-dependent effect. No treatment-related clinical findings were observed.

TABLE 4
Survival and Body Weights of Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	116 ± 3	347 ± 7	231 ± 5	
0.1	10/10	114 ± 3	345 ± 7	231 ± 6	99
1	10/10	118 ± 3	355 ± 7	237 ± 6	102
3	10/10	116 ± 3	354 ± 8	238 ± 6	102
10	10/10	117 ± 2	345 ± 6	229 ± 6	100
30	10/10	103 ± 6	315 ± 5**	212 ± 8	91
Female					
0	10/10	101 ± 2	196 ± 3	95 ± 3	
0.1	10/10	102 ± 2	200 ± 3	98 ± 3	102
1	10/10	100 ± 2	192 ± 3	91 ± 2	98
3	10/10	102 ± 2	191 ± 2	90 ± 3	98
10	10/10	100 ± 2	189 ± 3	89 ± 3	97
30	10/10	100 ± 3	182 ± 4**	82 ± 2**	93

** Significantly different (P 0.01) from the vehicle control group by Williams' or Dunnett's test
[a] Number of animals surviving at 13 weeks/number initially in group
[b] Weights and weight changes are given as mean ± standard error.



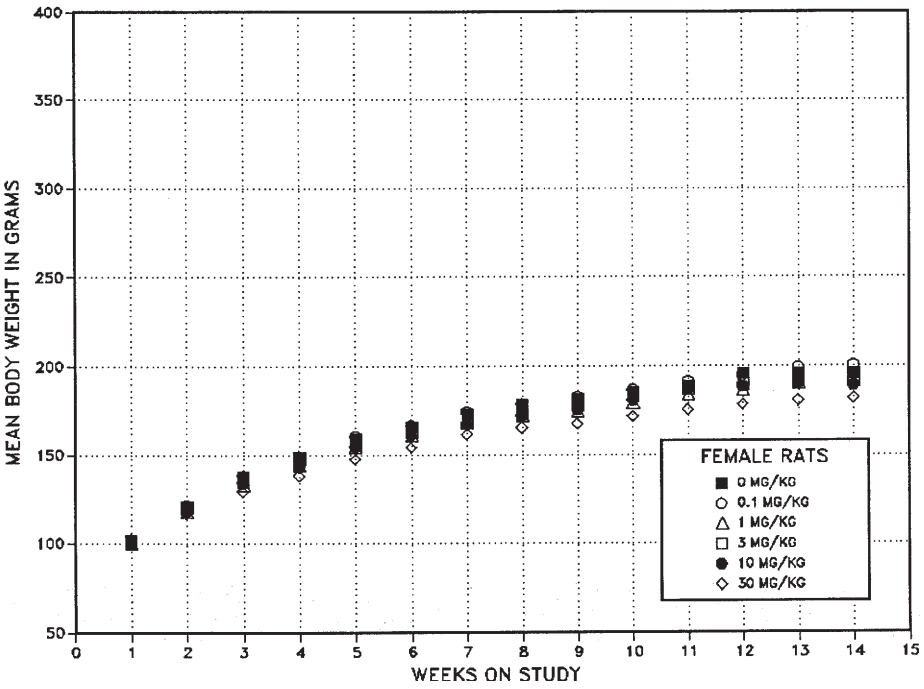


FIGURE 2
Body Weights of Rats Administered 3,3′,4,4′ -Tetrachloroazobenzene by Gavage for 13 Weeks

The hematology and clinical chemistry data for rats are listed in Tables 5 and B1. At week 13, a dose-related anemia occurred in the 10 and 30 mg/kg male and female rats. The anemia was evidenced by minimal to mild decreases in erythrocyte counts, hemoglobin concentrations, and hematocrit values. The anemia was characterized as macrocytic, normochromic, and responsive. Evidence of a macrocytosis was demonstrated by minimal increases in mean cell volumes; normochromic erythrocytes were evidenced by the lack of change in the mean cell hemoglobin concentrations in males. An erythropoietic response was demonstrated by minimal increases in reticulocyte counts. A minimal decrease in mean cell hemoglobin concentration occurred in the 10 and 30 mg/kg female rats at 13 weeks. Reticulocytosis can cause slight increases in mean cell hemoglobin; the increase of mean cell hemoglobin would be consistent with the occurrence of reticulocytosis. On day 21 and at week 13, a thrombocytopenia, evidenced by decreased platelet counts, occurred in male and female rats in the 10 and 30 mg/kg dose groups and occurred with a negative trend.

Decreases in total thyroxine were observed in all dose groups of males and females on day 21 and at 13 weeks. On day 21 these decreases ranged from 29% to 95%; at 13 weeks decreases ranged from 25% to greater than 99%. In an apparent response to decreased thyroid hormone concentrations, thyroid-stimulating hormone concentrations were moderately increased on day 21 in the 3, 10, and 30 mg/kg males and the 10 and 30 mg/kg females. These increases were less pronounced in females than in males. On day 21 and at week 13, dose-dependent increases in total protein and albumin concentrations were observed in the 10 and 30 mg/kg male and female rats; these changes also occurred in the lower dose groups, but with less consistency. On day 21 and at week 13, alanine aminotransferase activities were generally decreased in the 30 mg/kg males and females. Alterations of other clinical chemistry or hematology variables were either inconsistent between dose groups or genders, did not demonstrate a significant trend, or were within physiological values and were not considered toxicologically significant.

TABLE 5
Selected Hematology and Clinical Chemistry Data for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Male						
Hematology						
n						
Day 3	10	10	9	10	9	10
Day 21	10	10	9	9	10	10
Week 13	9	10	8	10	9	10
Automated hematocrit (%)						
Day 3	37.7 ± 0.7	37.7 ± 0.5	37.4 ± 0.7	38.7 ± 0.6	38.5 ± 0.4	38.9 ± 0.4
Day 21	40.4 ± 0.4	41.1 ± 0.3	39.7 ± 0.4	40.3 ± 0.5	41.0 ± 0.4	40.0 ± 0.5
Week 13	43.4 ± 0.7	44.5 ± 0.4	43.0 ± 0.9	43.1 ± 0.7	40.2 ± 0.3**	37.4 ± 0.4**
Manual hematocrit (%)						
Day 3	44.9 ± 0.9	44.1 ± 0.4	44.3 ± 0.8	45.1 ± 0.7	44.7 ± 0.5	45.0 ± 0.5
Day 21	45.4 ± 0.5	46.1 ± 0.6	45.9 ± 0.6	45.6 ± 0.4	46.0 ± 0.5	45.2 ± 0.6
Week 13	47.3 ± 0.8	48.2 ± 0.4	46.8 ± 0.9	46.5 ± 0.5	43.7 ± 0.4**	41.3 ± 0.3**
Hemoglobin (g/dL)						
Day 3	13.8 ± 0.3	13.9 ± 0.2	13.8 ± 0.2	14.3 ± 0.2	14.1 ± 0.1	14.2 ± 0.2
Day 21	14.8 ± 0.1	15.0 ± 0.1	14.8 ± 0.2	14.9 ± 0.1	15.0 ± 0.2	14.7 ± 0.2
Week 13	15.5 ± 0.2	15.7 ± 0.1	15.3 ± 0.3	15.0 ± 0.2	14.1 ± 0.1**	13.1 ± 0.1**
Erythrocytes (10^6/µL)						
Day 3	6.33 ± 0.14	6.27 ± 0.08	6.21 ± 0.12	6.47 ± 0.11	6.43 ± 0.08	6.45 ± 0.08
Day 21	6.66 ± 0.10	6.92 ± 0.08	6.67 ± 0.09	6.84 ± 0.09	7.00 ± 0.10	6.81 ± 0.09
Week 13	8.57 ± 0.13	8.77 ± 0.09	8.53 ± 0.18	8.51 ± 0.15	7.77 ± 0.06**	6.79 ± 0.08**
Reticulocyte (10^6/µL)						
Day 3	0.11 ± 0.03	0.28 ± 0.03**	0.26 ± 0.03*	0.25 ± 0.03*	0.23 ± 0.02	0.28 ± 0.08**
Day 21	0.18 ± 0.03	0.16 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.19 ± 0.01
Week 13	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.17 ± 0.02**
Mean cell volume (fL)						
Day 3	59.5 ± 0.5	60.2 ± 0.3	60.3 ± 0.4	59.9 ± 0.2	60.0 ± 0.4	60.4 ± 0.2
Day 21	60.8 ± 0.8	59.5 ± 0.3	59.6 ± 0.4	58.9 ± 0.3*	58.5 ± 0.4**	58.7 ± 0.2**
Week 13	50.6 ± 0.1	50.7 ± 0.1	50.5 ± 0.1	50.7 ± 0.2	51.8 ± 0.1**	55.0 ± 0.2**
Mean cell hemoglobin (pg)						
Day 3	21.8 ± 0.2	22.2 ± 0.1	22.3 ± 0.2	22.2 ± 0.1	22.0 ± 0.1	22.0 ± 0.1
Day 21	22.2 ± 0.3	21.7 ± 0.1	22.2 ± 0.2	21.9 ± 0.2	21.5 ± 0.2	21.6 ± 0.2
Week 13	18.1 ± 0.1	17.9 ± 0.1	17.9 ± 0.1	17.7 ± 0.1	18.1 ± 0.1	19.3 ± 0.1**
Mean cell hemoglobin concentration (g/dL)						
Day 3	36.7 ± 0.2	36.9 ± 0.3	36.9 ± 0.2	37.0 ± 0.2	36.6 ± 0.2	36.4 ± 0.2
Day 21	36.6 ± 0.3	36.5 ± 0.2	37.3 ± 0.2	37.1 ± 0.3	36.7 ± 0.2	36.7 ± 0.2
Week 13	35.7 ± 0.3	35.2 ± 0.2	35.5 ± 0.3	34.9 ± 0.2	35.0 ± 0.2	35.0 ± 0.2
Platelets (10^3/µL)						
Day 3	803.5 ± 60.3	940.8 ± 15.1	959.0 ± 21.2	908.7 ± 26.1	955.1 ± 14.6	966.3 ± 28.5
Day 21	868.1 ± 14.8	868.6 ± 8.2	858.2 ± 8.5	839.0 ± 24.3	792.9 ± 14.1**	715.1 ± 13.2**
Week 13	745.0 ± 33.9	730.6 ± 8.9	776.0 ± 48.0	713.2 ± 25.8	619.2 ± 7.0**	496.0 ± 30.2**

TABLE 5
Selected Hematology and Clinical Chemistry Data for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Male (continued)						
Clinical Chemistry						
n						
Day 3	10	10	10	10	10	10
Day 21	10	10	9	9	10	10
Week 13	10	10	10	10	10	10
Total protein (g/dL)						
Day 3	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.5 ± 0.1	5.4 ± 0.1	5.4 ± 0.1
Day 21	6.5 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.7 ± 0.1	6.8 ± 0.1**	6.9 ± 0.1**
Week 13	6.9 ± 0.1	7.0 ± 0.1	7.2 ± 0.1	7.4 ± 0.1**	7.3 ± 0.0**	7.3 ± 0.1*
Albumin (g/dL)						
Day 3	4.0 ± 0.1	3.9 ± 0.1	3.9 ± 0.0	4.1 ± 0.0	4.0 ± 0.1	4.0 ± 0.0
Day 21	4.7 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.9 ± 0.1*	5.0 ± 0.1**
Week 13	5.0 ± 0.1	5.0 ± 0.0	5.1 ± 0.1	5.2 ± 0.0	5.2 ± 0.0*	5.3 ± 0.0**
Alanine aminotransferase (IU/L)						
Day 3	49 ± 2	48 ± 2	47 ± 1	50 ± 1	47 ± 2	44 ± 2
Day 21	55 ± 2	57 ± 1	52 ± 1	53 ± 1	51 ± 1	48 ± 1**
Week 13	47 ± 2	49 ± 2	46 ± 3	46 ± 2	42 ± 1	42 ± 1
Sorbitol dehydrogenase (IU/L)						
Day 3	19 ± 2	18 ± 1	16 ± 1	21 ± 1	19 ± 1	18 ± 2
Day 21	17 ± 2	17 ± 1	15 ± 1	18 ± 1	18 ± 1	17 ± 1
Week 13	16 ± 1	20 ± 1*	20 ± 1*	20 ± 1*	21 ± 1**	19 ± 1*
Thyroid-stimulating hormone (ng/mL)						
Day 21	1.8 ± 0.3	1.4 ± 0.3	1.8 ± 0.2	3.4 ± 0.5*	3.2 ± 0.4*	3.0 ± 0.4*
Week 13	2.0 ± 0.3	1.8 ± 0.2	1.9 ± 0.2	2.3 ± 0.3	2.7 ± 0.3	3.4 ± 0.5*
Total triiodothyronine (ng/dL)						
Day 21	117 ± 6	95 ± 8	90 ± 4*[b]	90 ± 7*	107 ± 6[b]	94 ± 8[c]
Week 13	140 ± 8	113 ± 3*	121 ± 6*	116 ± 6*	119 ± 4*	102 ± 6**
Total thyroxine (µg/dL)						
Day 21	4.2 ± 0.1	3.0 ± 0.2**	1.8 ± 0.2**	1.3 ± 0.2**[b]	1.8 ± 0.1**	1.3 ± 0.2**[b]
Week 13	3.4 ± 0.2	2.1 ± 0.2**	0.6 ± 0.2**	0.5 ± 0.1**	0.5 ± 0.1**	0.1 ± 0.1**

TABLE 5
Selected Hematology and Clinical Chemistry Data for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Female						
Hematology						
n						
Day 3	10	10	10	10	10	10
Day 21	10	10	9	10	10	9
Week 13	10	10	10	10	10	10
Automated hematocrit (%)						
Day 3	41.0 ± 0.6	40.6 ± 0.8	42.2 ± 0.3	42.1 ± 0.5	42.1 ± 0.6	40.1 ± 0.5
Day 21	42.6 ± 0.4	43.6 ± 0.3	43.2 ± 0.4	43.2 ± 0.6	43.9 ± 0.4	43.0 ± 0.4
Week 13	42.5 ± 0.6	42.2 ± 0.3	42.3 ± 0.5	41.8 ± 0.2	40.5 ± 0.4**	38.5 ± 0.2**
Manual hematocrit (%)						
Day 3	46.1 ± 0.7	45.3 ± 1.0	46.7 ± 0.5	45.0 ± 0.4	46.3 ± 0.8	45.3 ± 0.4
Day 21	46.1 ± 0.5	46.5 ± 0.4	45.1 ± 0.4	46.3 ± 0.6	45.6 ± 0.3	44.4 ± 0.4
Week 13	45.4 ± 0.4	45.4 ± 0.4	45.1 ± 0.4	44.4 ± 0.2	43.3 ± 0.6**	40.8 ± 0.4**
Hemoglobin (g/dL)						
Day 3	15.0 ± 0.2	14.9 ± 0.3	15.4 ± 0.1	15.2 ± 0.1	15.4 ± 0.2	14.6 ± 0.2
Day 21	15.4 ± 0.1	15.8 ± 0.2	15.6 ± 0.1	15.9 ± 0.2	15.6 ± 0.1	15.1 ± 0.1
Week 13	15.3 ± 0.2	15.2 ± 0.1	15.1 ± 0.1	15.0 ± 0.1	14.3 ± 0.1**	13.4 ± 0.1**
Erythrocytes (10^6/µL)						
Day 3	6.90 ± 0.13	6.82 ± 0.16	7.14 ± 0.09	7.13 ± 0.10	7.10 ± 0.11	6.73 ± 0.09
Day 21	6.99 ± 0.09	7.20 ± 0.04	7.08 ± 0.07	7.24 ± 0.10	7.29 ± 0.08*	7.15 ± 0.08
Week 13	7.69 ± 0.11	7.60 ± 0.06	7.59 ± 0.09	7.49 ± 0.04	7.14 ± 0.07**	6.48 ± 0.04**
Reticulocyte (10^6/µL)						
Day 3	0.15 ± 0.01	0.18 ± 0.02	0.15 ± 0.01	0.22 ± 0.03	0.17 ± 0.01	0.19 ± 0.02
Day 21	0.11 ± 0.02	0.12 ± 0.01	0.14 ± 0.02	0.16 ± 0.01**	0.14 ± 0.02	0.18 ± 0.01**
Week 13	0.06 ± 0.01	0.10 ± 0.01**	0.10 ± 0.01**	0.09 ± 0.02*	0.12 ± 0.02**	0.13 ± 0.01**
Mean cell volume (fL)						
Day 3	59.4 ± 0.4	59.6 ± 0.2	59.2 ± 0.3	59.1 ± 0.3	59.3 ± 0.3	59.7 ± 0.3
Day 21	60.9 ± 0.3	60.6 ± 0.2	61.0 ± 0.2	59.7 ± 0.2*	60.2 ± 0.2	60.2 ± 0.2
Week 13	55.2 ± 0.2	55.5 ± 0.1*	55.7 ± 0.2*	55.7 ± 0.1**	56.7 ± 0.1**	59.5 ± 0.1**
Mean cell hemoglobin (pg)						
Day 3	21.7 ± 0.2	21.9 ± 0.2	21.5 ± 0.1	21.4 ± 0.2	21.6 ± 0.1	21.7 ± 0.1
Day 21	22.0 ± 0.1	22.0 ± 0.1	22.0 ± 0.1	21.9 ± 0.2	21.4 ± 0.1**	21.2 ± 0.2**
Week 13	19.9 ± 0.1	20.0 ± 0.1	19.9 ± 0.1	20.0 ± 0.1	20.0 ± 0.1	20.7 ± 0.1**
Mean cell hemoglobin concentration (g/dL)						
Day 3	36.5 ± 0.3	36.8 ± 0.2	36.4 ± 0.2	36.2 ± 0.2	36.5 ± 0.1	36.3 ± 0.2
Day 21	36.2 ± 0.2	36.3 ± 0.2	36.1 ± 0.2	36.7 ± 0.2	35.5 ± 0.2	35.2 ± 0.2*
Week 13	36.1 ± 0.2	35.9 ± 0.1	35.7 ± 0.2	35.9 ± 0.1	35.3 ± 0.1**	34.7 ± 0.2**
Platelets (10^3/µL)						
Day 3	927.9 ± 18.3	920.2 ± 39.6	918.5 ± 21.7	910.2 ± 31.6	866.0 ± 20.2	896.7 ± 14.1
Day 21	803.3 ± 16.1	775.8 ± 16.2	836.7 ± 15.5	771.4 ± 23.0	683.3 ± 25.0**	686.8 ± 19.3**
Week 13	801.4 ± 66.8	801.4 ± 45.4	741.3 ± 34.4	697.9 ± 23.3	687.9 ± 28.4*	641.0 ± 10.9**

TABLE 5
Selected Hematology and Clinical Chemistry Data for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Female (continued)						
Clinical Chemistry						
n						
Day 3	10	10	9	10	10	10
Day 21	10	10	9	10	10	9
Week 13	10	10	10	10	10	10
Total protein (g/dL)						
Day 3	5.5 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	5.7 ± 0.1	5.6 ± 0.1
Day 21	6.1 ± 0.1	6.3 ± 0.1*	6.3 ± 0.1*	6.3 ± 0.1*	6.4 ± 0.1*	6.7 ± 0.1**
Week 13	7.0 ± 0.1	6.9 ± 0.3	7.1 ± 0.1	7.2 ± 0.1	7.3 ± 0.1*	7.3 ± 0.1*
Albumin (g/dL)						
Day 3	4.2 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.3 ± 0.0	4.2 ± 0.1
Day 21	4.6 ± 0.1	4.8 ± 0.1*	4.8 ± 0.1*	4.7 ± 0.1	5.0 ± 0.1**	5.1 ± 0.1**
Week 13	4.9 ± 0.1	4.8 ± 0.2	5.1 ± 0.1	5.1 ± 0.1	5.3 ± 0.1*	5.5 ± 0.1**
Alanine aminotransferase (IU/L)						
Day 3	47 ± 3	51 ± 3	45 ± 3	45 ± 2	45 ± 3	43 ± 3
Day 21	48 ± 1	48 ± 1	45 ± 1	45 ± 1	43 ± 1**	41 ± 2**
Week 13	54 ± 7	50 ± 5	49 ± 3	45 ± 5	39 ± 2	31 ± 1**
Sorbitol dehydrogenase (IU/L)						
Day 3	19 ± 1	22 ± 1	21 ± 2	20 ± 1	22 ± 1	18 ± 2
Day 21	17 ± 1	18 ± 1	21 ± 2	21 ± 1*	23 ± 2**	32 ± 2**
Week 13	22 ± 2	23 ± 2	25 ± 2	24 ± 2	24 ± 2	27 ± 2
Thyroid-stimulating hormone (ng/mL)						
Day 21	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.2	1.7 ± 0.2**	1.3 ± 0.2*
Week 13	1.0 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.4 ± 0.2
Total triiodothyronine (ng/dL)						
Day 21	117 ± 6	110 ± 9	85 ± 6**	92 ± 6*	111 ± 5	89 ± 4*
Week 13	131 ± 5	112 ± 5*	107 ± 6**	102 ± 5**	101 ± 5**	99 ± 6**
Total thyroxine (µg/dL)						
Day 21	3.2 ± 0.2	2.3 ± 0.2*	0.8 ± 0.1**	0.6 ± 0.1**	0.7 ± 0.1**	0.2 ± 0.1**
Week 13	2.3 ± 0.2	1.7 ± 0.2*	0.5 ± 0.1**	0.2 ± 0.0**	0.1 ± 0.0**	0.0 ± 0.0**

* Significantly different (P 0.05) from the vehicle control group by Dunn's or Shirley's test
** P 0.01
Trend is significantly increased (P 0.01) by Jonckheere's test.
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n = 8
[c] n = 9

The absolute kidney weight of 10 mg/kg males and the relative kidney weights of 10 and 30 mg/kg males and females were significantly greater than those of the vehicle controls (Tables 6 and C2). The absolute and relative heart weights of 30 mg/kg males were significantly greater than those of the vehicle controls. In general, the absolute and relative liver weights of males receiving 1 mg/kg or greater and females receiving 10 or 30 mg/kg were significantly greater than those of the vehicle controls. Relative to the vehicle controls, the absolute and relative spleen weights were generally significantly increased in males and females receiving 10 or 30 mg/kg. The absolute and relative thymus weights of male and female rats in the 10 and 30 mg/kg groups were significantly less than those of the vehicle control groups.

TABLE 6
Selected Organ Weight Data for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
n	10	10	10	10	10	10
Male						
Necropsy body wt	347 ± 7	345 ± 7	355 ± 7	354 ± 8	346 ± 6	315 ± 5**
Heart						
Absolute	1.047 ± 0.018	1.029 ± 0.026	1.053 ± 0.023	1.059 ± 0.016	1.062 ± 0.025	1.142 ± 0.018**
Relative	3.02 ± 0.03	2.99 ± 0.04	2.97 ± 0.04	3.00 ± 0.06	3.07 ± 0.04	3.62 ± 0.04**
R. Kidney						
Absolute	1.264 ± 0.034	1.253 ± 0.043	1.304 ± 0.027	1.293 ± 0.022	1.411 ± 0.037*	1.325 ± 0.030
Relative	3.64 ± 0.04	3.63 ± 0.07	3.68 ± 0.05	3.65 ± 0.03	4.08 ± 0.07**	4.20 ± 0.06**
Liver						
Absolute	12.756 ± 0.293	12.660 ± 0.482	13.760 ± 0.377	14.045 ± 0.407*	14.948 ± 0.350**	15.221 ± 0.466**
Relative	36.77 ± 0.26	36.65 ± 0.77	38.82 ± 0.75*	39.62 ± 0.57**	43.19 ± 0.44**	48.17 ± 0.87**
Spleen						
Absolute	0.700 ± 0.015	0.728 ± 0.024	0.749 ± 0.021	0.757 ± 0.025	0.808 ± 0.027**	0.807 ± 0.016**
Relative	2.02 ± 0.03	2.11 ± 0.05	2.12 ± 0.06	2.14 ± 0.05	2.33 ± 0.05**	2.56 ± 0.04**
Thymus						
Absolute	0.348 ± 0.021	0.344 ± 0.012	0.362 ± 0.016	0.325 ± 0.028	0.288 ± 0.009*	0.221 ± 0.014**
Relative	1.00 ± 0.04	1.00 ± 0.05	1.02 ± 0.05	0.92 ± 0.07	0.83 ± 0.02*	0.70 ± 0.04**
Female						
Necropsy body wt	196 ± 3	200 ± 3	192 ± 3	191 ± 2	189 ± 3	182 ± 4**
R. Kidney						
Absolute	0.710 ± 0.012	0.739 ± 0.019	0.705 ± 0.018	0.717 ± 0.012	0.747 ± 0.012	0.743 ± 0.022
Relative	3.62 ± 0.03	3.69 ± 0.07	3.68 ± 0.06	3.75 ± 0.04	3.95 ± 0.03**	4.08 ± 0.05**
Liver						
Absolute	7.050 ± 0.163	7.238 ± 0.252	6.885 ± 0.149	7.119 ± 0.183	7.643 ± 0.160*	8.177 ± 0.215**
Relative	36.01 ± 0.71	36.10 ± 1.05	35.92 ± 0.60	37.21 ± 0.74	40.39 ± 0.62**	44.97 ± 0.63**
Spleen						
Absolute	0.472 ± 0.010	0.528 ± 0.012*	0.503 ± 0.008	0.491 ± 0.013	0.507 ± 0.015	0.529 ± 0.016*
Relative	2.41 ± 0.05	2.64 ± 0.06*	2.62 ± 0.03	2.57 ± 0.06	2.68 ± 0.07**	2.92 ± 0.11**
Thymus						
Absolute	0.291 ± 0.015	0.284 ± 0.014	0.255 ± 0.014	0.251 ± 0.016	0.229 ± 0.016**	0.163 ± 0.015**
Relative	1.49 ± 0.09	1.42 ± 0.07	1.33 ± 0.07	1.31 ± 0.08	1.21 ± 0.07*	0.90 ± 0.08**

* Significantly different (P 0.05) from the vehicle control group by Williams' or Dunnett's test
** P 0.01
Trend is significantly increased (P 0.01) by Jonckheere's test.
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

No differences were found in sperm or vaginal cytology parameters between dosed and vehicle control rats (Tables D1 and D2).

The presence and intensity of hepatic cytochrome P_{450}1A staining in male and female rats in the 30 mg/kg groups and females in the 10 mg/kg group were increased compared to the vehicle controls (Table E1). This change reflects an increase in Ah receptor-mediated cytochrome P_{450}1A enzyme activity.

No gross lesions related to 3,3′,4,4′-tetrachloroazobenzene treatment were observed. Microscopically, effects of 3,3′,4,4′-tetrachloroazobenzene administration to rats were observed in the spleen, forestomach, thymus, and kidney (Tables 7, A1, and A2).

Increased spleen weights in males and females corresponded microscopically to hematopoietic cell proliferation in the red pulp. Hematopoietic cell proliferation was characterized by an increase in the number of blood cell precursors, primarily of the erythroid series. The severity of this change was minimal to mild and the incidence was significantly increased in 10 and 30 mg/kg males and females, although this effect was also observed in 3 mg/kg males. The incidence of pigmentation of the spleen, consisting of yellow-brown granules in the cytoplasm of red pulp macrophages, was also increased in 30 mg/kg females relative to the vehicle controls. This pigment was interpreted to be hemosiderin.

In the stomach, squamous hyperplasia of the forestomach epithelium was a treatment-related effect. This change was characterized by an increased thickness of the epithelium at the limiting ridge, often accompanied by increased keratin (hyperkeratosis). Mild hyperplasia was present in most rats administered 30 mg/kg, with fewer incidences of minimal hyperplasia occurring at lower doses.

Reduced thymus weights corresponded microscopically to minimal to mild thymic atrophy, consisting of thinning of the cortex due to reduced numbers of cortical lymphocytes. This effect was significant in 10 and 30 mg/kg males and 30 mg/kg females, although thymic atrophy was also observed in 3 and 10 mg/kg females.

In male F344/N rats, minimal chronic nephropathy is a common spontaneous lesion characterized by one or more scattered foci of regenerative tubules with thickened basement membranes within the cortex. The severity of nephropathy (increased number of foci) was increased in 30 mg/kg male rats and was interpreted to be a treatment-related effect. This change likely accounted for the increased kidney weights in this group.

TABLE 7
Incidence of Selected Nonneoplastic Lesions in Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Male						
Spleen[a]	10	0	9	10	10	10
Hematopoietic Cell Proliferation[b]	0	0	0	3 (1.0)[c]	8** (1.0)	10** (1.4)
Stomach, Forestomach	10	0	0	10	10	10
Epithelium, Hyperplasia, Focal	0	0	0	4* (1.0)	3 (1.0)	8** (1.8)
Thymus	9	0	0	10	10	10
Atrophy	0	0	0	0	5* (1.0)	10** (1.9)
Kidney	10	10	10	10	10	10
Nephropathy	9 (1.0)	10 (1.0)	10 (1.0)	10 (1.0)	10 (1.1)	10 (1.4)*
Female						
Spleen	10	1	0	10	10	10
Hematopoietic Cell Proliferation	0	0	0	0	5* (1.2)	9** (1.2)
Pigmentation	0	0	0	0	2 (1.0)	8** (1.6)
Stomach, Forestomach	10	0	1	10	10	10
Epithelium, Hyperplasia, Focal	0	0	1 (1.0)	1 (1.0)	2 (1.0)	10** (1.9)
Thymus	10	0	10	9	8	9
Atrophy	0	0	0	2 (1.5)	3 (1.0)	9** (1.1)

* Significantly different (P 0.05) from the vehicle control group by the Fisher exact test (incidences) or by the Mann-Whitney U test (severity of nephropathy)
** P 0.01
[a] Number of animals with organ examined microscopically
[b] Number of animals with lesions
[c] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

MICE

16-DAY STUDY

All mice survived to the end of the study (Table 8). Final mean body weights and body weight gains of dosed and vehicle control males and females were generally similar (Table 8). No clinical findings of toxicity were observed in male or female mice.

TABLE 8
Survival and Body Weights of Mice in the 16-Day Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	24.0 ± 0.5	26.3 ± 0.3	2.3 ± 0.3	
1	5/5	23.5 ± 0.6	25.8 ± 0.6	2.3 ± 0.2	98
3.2	5/5	24.1 ± 0.5	26.3 ± 0.4	2.2 ± 0.2	100
10	5/5	23.8 ± 0.6	26.4 ± 0.7	2.6 ± 0.3	100
32	5/5	23.9 ± 0.5	26.4 ± 0.6	2.6 ± 0.3	100
100	5/5	23.9 ± 0.8	26.6 ± 1.0	2.8 ± 0.2	101
Female					
0	5/5	18.8 ± 0.3	20.1 ± 0.3	1.3 ± 0.2	
1	5/5	18.9 ± 0.4	18.7 ± 0.6	0.2 ± 0.3**	93
3.2	5/5	18.7 ± 0.2	20.2 ± 0.4	1.5 ± 0.2	100
10	5/5	18.2 ± 0.5	19.5 ± 0.3	1.2 ± 0.4	97
32	5/5	19.0 ± 0.8	19.7 ± 0.4	0.7 ± 0.4	98
100	5/5	19.0 ± 0.4	20.3 ± 0.4	1.3 ± 0.2	101

** Significantly different (P 0.01) from the vehicle control group by Dunnett's test
[a] Number of animals surviving at 16 days/number initially in group
[b] Weights and weight changes are given as mean ± standard error.

The absolute and relative thymus weights of male and female mice in the 10, 32, and 100 mg/kg groups were significantly less than those of the vehicle controls (Tables 9 and C3). The absolute and relative liver weights of males and females in the 10, 32, and 100 mg/kg groups were generally significantly greater than those of the vehicle controls. Relative to the vehicle controls, the absolute and relative heart weights were significantly increased in males receiving 32 or 100 mg/kg. The decreases in thymus weights and the increases in liver and heart weights occurred with significant trends. In females, a significant positive trend was observed in absolute and lung weights, and significant negative trends were observed in absolute and relative uterus weights.

TABLE 9
Selected Organ Weight Data for Mice in the 16-Day Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	1 mg/kg	3.2 mg/kg	10 mg/kg	32 mg/kg	100 mg/kg
n	5	5	5	5	5	5
Male						
Necropsy body wt	26.3 ± 0.3	25.8 ± 0.6	26.3 ± 0.4	26.4 ± 0.7	26.4 ± 0.6	26.6 ± 1.0
Heart						
Absolute	0.130 ± 0.002	0.132 ± 0.004	0.138 ± 0.003	0.136 ± 0.004	0.142 ± 0.005*	0.144 ± 0.006*
Relative	4.92 ± 0.05	5.13 ± 0.10	5.23 ± 0.15	5.17 ± 0.04	5.37 ± 0.06**	5.43 ± 0.13**
Liver						
Absolute	1.627 ± 0.043	1.659 ± 0.055	1.691 ± 0.036	1.808 ± 0.054*	1.893 ± 0.030**	2.034 ± 0.066**
Relative	61.85 ± 1.68	64.38 ± 1.32	64.25 ± 0.79	68.55 ± 0.91**	71.70 ± 1.37**	76.45 ± 0.91**
Thymus						
Absolute	0.059 ± 0.003	0.052 ± 0.004	0.053 ± 0.002	0.040 ± 0.005**	0.040 ± 0.002**	0.035 ± 0.001**
Relative	2.25 ± 0.15	2.04 ± 0.20	2.00 ± 0.05	1.52 ± 0.19**	1.51 ± 0.06**	1.31 ± 0.04**
Female						
Necropsy body wt	20.1 ± 0.3	18.7 ± 0.6	20.2 ± 0.4	19.5 ± 0.3	19.7 ± 0.4	20.3 ± 0.4
Liver						
Absolute	1.208 ± 0.036	1.132 ± 0.025	1.259 ± 0.020	1.261 ± 0.038	1.337 ± 0.065*	1.594 ± 0.038**
Relative	60.17 ± 1.42	60.65 ± 1.93	62.51 ± 0.88	64.75 ± 1.41*	67.81 ± 1.93**	78.60 ± 0.99**
Thymus						
Absolute	0.078 ± 0.005	0.062 ± 0.004*	0.070 ± 0.005	0.059 ± 0.002**	0.051 ± 0.005**	0.052 ± 0.002**
Relative	3.87 ± 0.20	3.32 ± 0.18	3.47 ± 0.19	3.06 ± 0.13**	2.59 ± 0.20**	2.57 ± 0.10**

* Significantly different (P 0.05) from the vehicle control group by Williams' test
** P 0.01
Trend is significantly increased (P 0.01) by Jonckheere's test.
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

No gross lesions were observed that were considered to be treatment related. Microscopically, hematopoietic cell proliferation of the spleen was observed in 100 mg/kg male and female mice. Females in the 100 mg/kg group also had atrophy of the thymus.

The dose selection for the 13-week study was based on lower thymus weights observed in males and females administered 10 mg/kg or greater. Because 3,3′,4,4′-tetrachloroazobenzene is expected to bioaccumulate, although to a lesser extent than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the doses for the 13-week study were chosen to be minimally immunotoxic. Because it was unknown whether the mice would develop a tolerance to 3,3′,4,4′-tetrachloroazobenzene (i.e., by increased metabolism), the doses for the 13-week study in mice were 0, 0.1, 1, 3, 10, and 30 mg/kg.

13-WEEK STUDY

All mice survived to the end of the study (Table 10). Final mean body weights and body weight gains of dosed and vehicle control mice were generally similar (Table 10 and Figure 3). No treatment-related clinical findings were observed.

TABLE 10
Survival and Body Weights of Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	10/10	23.6 ± 0.4	37.3 ± 1.1	13.7 ± 1.0	
0.1	10/10	23.4 ± 0.4	35.3 ± 0.7	11.9 ± 0.5	95
1	10/10	23.7 ± 0.3	37.4 ± 1.2	13.7 ± 1.1	100
3	10/10	23.9 ± 0.5	37.2 ± 1.3	13.3 ± 1.0	100
10	10/10	23.2 ± 0.5	38.1 ± 1.1	14.9 ± 0.9	102
30	10/10	23.7 ± 0.6	37.7 ± 1.6	14.0 ± 1.2	101
Female					
0	10/10	19.0 ± 0.4	26.0 ± 0.7	7.0 ± 0.4	
0.1	10/10	18.9 ± 0.2	27.8 ± 0.6	8.9 ± 0.6*	107
1	10/10	19.0 ± 0.3	28.6 ± 0.7*	9.6 ± 0.7**	110
3	10/10	19.0 ± 0.4	26.4 ± 0.5	7.4 ± 0.3	102
10	10/10	18.3 ± 0.4	26.4 ± 0.9	8.1 ± 0.5	102
30	10/10	18.8 ± 0.2	26.2 ± 0.5	7.4 ± 0.4	101

* Significantly different (P 0.05) from the vehicle control group by Dunnett's test
** P 0.01
[a] Number of animals surviving at 13 weeks/number initially in group
[b] Weights and weight changes are given as mean ± standard error.

The clinical chemistry data for mice are listed in Table B2. Minimal increases in albumin concentrations of 3 mg/kg males and 10 and 30 mg/kg females compared to the vehicle controls were observed at week 13.

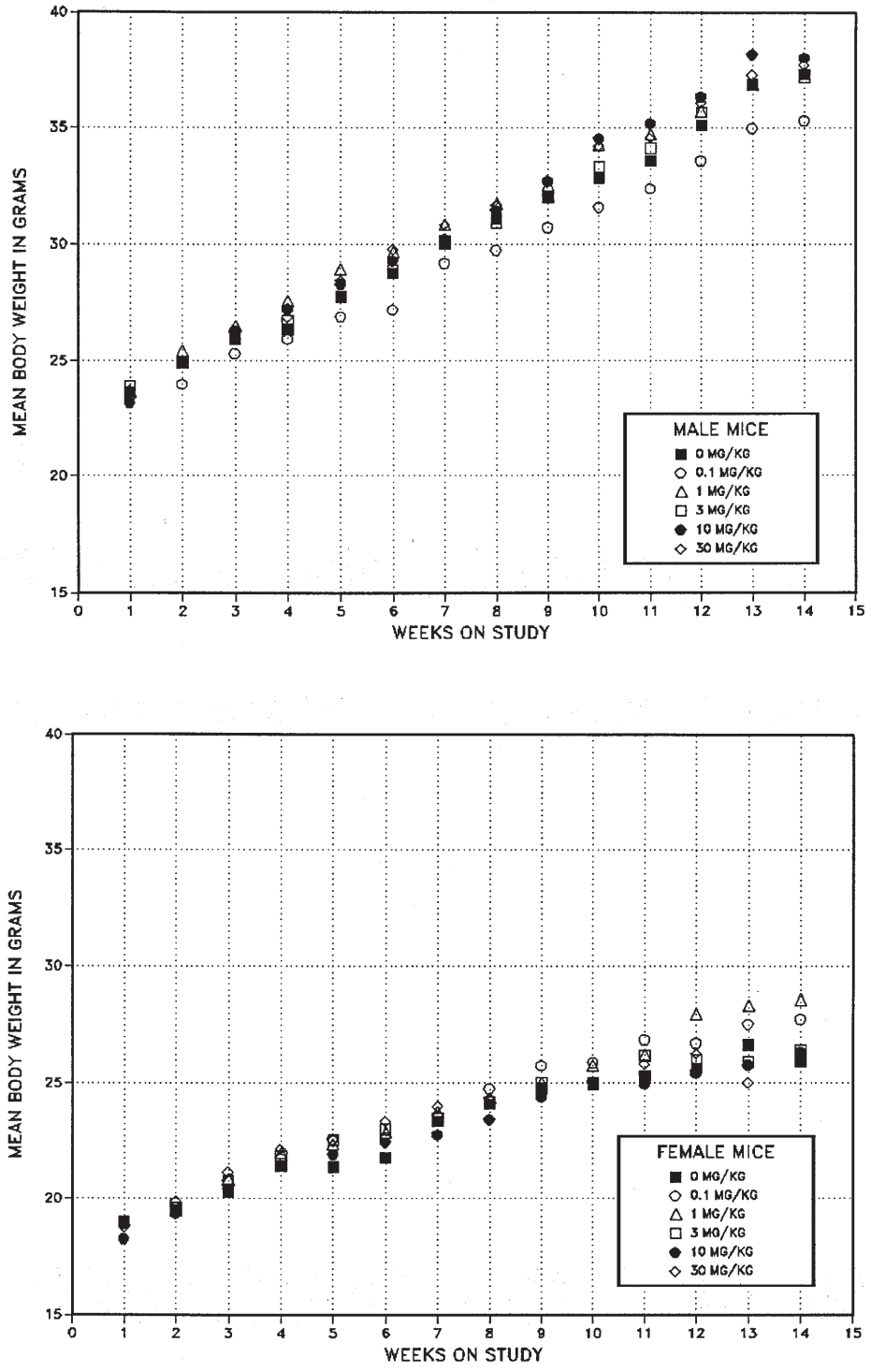


FIGURE 3
Body Weights of Mice Administered 3,3′,4,4′ -Tetrachloroazobenzene by Gavage for 13 Weeks

In general, the absolute and relative liver and spleen weights of male and female mice were significantly greater in the 10 and 30 mg/kg groups than those of the vehicle controls (Tables 11 and C4). The relative liver weight of 3 mg/kg males was also significantly greater than that of the vehicle controls. The absolute and relative heart weights of 30 mg/kg females were significantly greater than those of the vehicle controls. The absolute and relative thymus weights of 30 mg/kg males were significantly less than those of the vehicle controls. These changes occurred with significant trends; an increase in the absolute heart weight of male mice, although not significantly different from the vehicle controls, also occurred with a significant trend.

TABLE 11
Selected Organ Weight Data for Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
n	10	10	10	10	10	10
Male						
Necropsy body wt	37.3 ± 1.1	35.3 ± 0.7	37.4 ± 1.2	37.2 ± 1.3	38.1 ± 1.1	37.7 ± 1.6
Liver						
Absolute	1.661 ± 0.052	1.609 ± 0.053	1.715 ± 0.058	1.830 ± 0.064	1.929 ± 0.075*[b]	2.038 ± 0.104**
Relative	44.64 ± 1.15	45.50 ± 0.86	46.15 ± 1.75	49.62 ± 2.17*	50.77 ± 1.90**[b]	53.91 ± 0.73**
Spleen						
Absolute	0.075 ± 0.002	0.071 ± 0.002	0.076 ± 0.003	0.076 ± 0.004	0.083 ± 0.002*	0.087 ± 0.002**
Relative	2.02 ± 0.07	2.03 ± 0.05	2.06 ± 0.09	2.04 ± 0.10	2.19 ± 0.06	2.32 ± 0.08**
Thymus						
Absolute	0.043 ± 0.003	0.043 ± 0.004	0.042 ± 0.003[b]	0.034 ± 0.002	0.037 ± 0.003[b]	0.029 ± 0.002**
Relative	1.15 ± 0.08	1.22 ± 0.11	1.13 ± 0.09[b]	0.92 ± 0.05	0.96 ± 0.07[b]	0.77 ± 0.06**
Female						
Necropsy body wt	26.0 ± 0.7	27.8 ± 0.6	28.6 ± 0.7*	26.4 ± 0.5	26.4 ± 0.9	26.2 ± 0.5
Heart						
Absolute	0.121 ± 0.003	0.124 ± 0.002	0.124 ± 0.003	0.122 ± 0.003	0.123 ± 0.002	0.136 ± 0.003**
Relative	4.66 ± 0.07	4.48 ± 0.14	4.37 ± 0.14	4.62 ± 0.07	4.71 ± 0.13	5.18 ± 0.07**
Liver						
Absolute	1.083 ± 0.034	1.139 ± 0.030	1.198 ± 0.024	1.168 ± 0.037	1.275 ± 0.048**	1.380 ± 0.041**
Relative	41.79 ± 0.97	41.03 ± 0.67	42.03 ± 0.88	44.17 ± 0.87	48.44 ± 1.41**	52.67 ± 0.97**
Spleen						
Absolute	0.083 ± 0.004	0.081 ± 0.002	0.092 ± 0.003	0.081 ± 0.002	0.094 ± 0.003*	0.106 ± 0.003**
Relative	3.17 ± 0.08	2.94 ± 0.10	3.22 ± 0.12	3.07 ± 0.05	3.56 ± 0.09**	4.06 ± 0.13**

* Significantly different (P 0.05) from the vehicle control group by Williams' or Dunnett's test
** Significantly different (P 0.01) from the vehicle control group by Williams' test
Trend is significantly increased (P 0.01) by Jonckheere's test.
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] n = 9

The epididymal spermatozoal concentrations of males in the 3 and 30 mg/kg groups were significantly less than those of the vehicle controls, and this change occurred with a significant negative trend (Table D3). No differences in vaginal cytology parameters were observed between dosed and vehicle control females (Table D4).

No treatment-related gross lesions were observed at necropsy. Microscopically, effects of 3,3′,4,4′-tetrachloroazobenzene administration were observed in the spleen, forestomach, and liver (Tables 12, A3, and A4).

Enhanced hematopoietic cell proliferation in the splenic red pulp relative to the vehicle controls was observed in dosed male and female mice. The incidences were significantly increased in males that received 3 mg/kg or greater, although the severities were minimal. This change likely contributed to increased spleen weights.

In the stomach of males and females, minimal to mild squamous hyperplasia of the forestomach epithelium was considered to be treatment related. This change was observed in all groups of dosed males, and the incidences were significant in the 1, 10, and 30 mg/kg groups; in females, the incidences of this lesion were significant in the 1, 3, 10, and 30 mg/kg groups.

Increased liver weights in male mice corresponded microscopically to centrilobular hypertrophy of hepatocytes; incidences were significantly increased in male mice that received 3 mg/kg or greater. This change consisted of enlarged hepatocytes with abundant eosinophilic cytoplasm surrounding the central vein of the liver lobule. The incidence of this lesion increased with increasing dose, although the severity did not. Hypertrophy was not detected in dosed female mice, although liver weights were likewise increased.

TABLE 12
Incidence of Selected Nonneoplastic Lesions in Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control		0.1 mg/kg		1 mg/kg		3 mg/kg		10 mg/kg		30 mg/kg	
Male												
Spleen[a]	10		10		10		10		10		10	
Red Pulp, Hematopoietic Cell Proliferation[b]	4	(1.0)[c]	8	(1.0)	7	(1.1)	9*	(1.0)	10**	(1.0)	10**	(1.1)
Stomach, Forestomach	10		10		10		10		10		10	
Epithelium, Hyperplasia	0		2	(2.0)	6**	(1.2)	3	(1.0)	4*	(1.5)	6**	(1.5)
Liver	10		10		10		10		10		10	
Centrilobular Hypertrophy	0		0		2	(1.0)	4*	(1.0)	8**	(1.0)	9**	(1.0)
Female												
Spleen	10		10		10		10		10		10	
Red Pulp, Hematopoietic Cell Proliferation	5	(1.0)	9	(1.0)	10	(1.2)	10	(1.0)	10	(1.0)	10	(1.3)
Stomach, Forestomach	10		10		10		10		10		10	
Epithelium, Hyperplasia	0		0		7**	(1.3)	7**	(1.4)	5*	(1.4)	7**	(1.6)

* Significantly different (P 0.05) from the vehicle control group by the Fisher exact test
** P 0.01
[a] Number of animals with organ/tissue examined microscopically
[b] Number of animals with lesions
[c] Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked

GENETIC TOXICOLOGY

3,3′,4,4′-Tetrachloroazobenzene was mutagenic in *Salmonella typhimurium* strain TA97 when testing was carried out in the presence of 30% induced rat liver S9 (Table F1); no mutagenic activity was detected in strain TA98, TA100, or TA1535 with or without S9 or in strain TA1537 with S9. No induction of micronuclei was noted in bone marrow erythrocytes of male mice treated with 3,3′,4,4′-tetrachloroazobenzene by intraperitoneal injection (three times at 24-hour intervals) (Table F2). However, results of a 13-week peripheral blood micronucleus test, in which 3,3′,4,4′-tetrachloroazobenzene was administered once daily by gavage, were positive in male and female mice (Table F3). The response observed in male mice in this latter test was stronger than that observed in females. For males and females, trend tests yielded significant P values, but only in male mice in the two highest dose groups (10 and 30 mg/kg) were the micronucleus frequencies found to be significantly different from the vehicle control values.

DISCUSSION

3,3′,4,4′-Tetrachloroazobenzene was nominated by the United States Environmental Protection Agency for toxicologic evaluation based on concerns over the potential for human exposure, the structural resemblance to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and the dioxin-like effects of 3,3′,4,4′-tetrachloroazobenzene. A summary of the effects of 3,3′,4,4′-tetrachloroazobenzene in F344/N rats and B6C3F_1 mice is given in Table 13.

The common mechanism of action of dioxin-like compounds, such as polychlorinated dibenzo-*p*-dioxins, dibenzofurans, biphenyls, and naphthalenes, involves an initial binding to the aryl hydrocarbon (Ah) receptor. Binding to this receptor is a necessary but not sufficient step in the cascade of effects that occurs after exposure to dioxin-like compounds (Birnbaum, 1994). 3,3′,4,4′-Tetrachloroazobenzene binds to the aryl hydrocarbon receptor with an affinity of about one-fifth that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Poland *et al.*, 1976; Schneider *et al.*, 1995). This is in the same order of magnitude as most potent dioxin-like compounds (Safe, 1990; Kafafi *et al.*, 1993; Schneider *et al.*, 1995). Typical dioxin-like effects in rodents include dermal lesions, body weight loss, thymic atrophy, impairment of immune responses, hepatotoxicity, reproductive and developmental toxicity, endocrine responses, induction of cytochrome P_{450}1A1, tissue-specific hypo- and hyperplastic responses, and carcinogenesis (Poland and Knutson, 1982; Safe, 1990).

The effects in the current 13-week studies, which are not entirely consistent with dioxin-like compounds, included the magnitude of decreased circulating thyroid hormone concentrations of male and female rats, even at the lowest dose (0.1 mg/kg). A decrease in epididymal spermatozoal concentration in mice was observed at the lowest dose tested (3 mg/kg). In addition, effects on the hematopoietic system occurred at doses that caused no histopathologic alterations in the liver. Liver lesions typically occur at lower doses than hematopoietic changes with dioxin-like compounds. Furthermore, increases in the incidences of hyperplasia of the forestomach epithelium were treatment related in rats and mice.

TABLE 13
Summary of Selected Treatment-Related Effects in the 13-Week Gavage Studies of 3,3′,4,4′-Tetrachloroazobenzene in F344/N Rats and B6C3F$_1$ Mice

Endpoint	Affected Dose Groups (mg/kg)			
	Male Rats	Female Rats	Male Mice	Female Mice
Terminal body weight (decrease)	30	30	NS[a]	NS
Body weight gain (decrease)	NS	30	NS	NS
Liver				
Weight (increase)	3↑	10↑	10↑	10↑
Centrilobular hypertrophy of hepatocytes (increased incidence)	NO[b]	NO	3↑	NO
Hepatic cytochrome P_{450}1A concentration (increase)	30	10↑	—[c]	—
Thymus				
Weight (decrease)	10↑	10↑	30	NS
Atrophy (increased incidence)	10↑	30	NO	NO
Spleen				
Weight (increase)	10↑	30	30	10↑
Hematopoietic cell proliferation (increased incidence)	10↑	10↑	3↑	NS
Responsive anemia	10↑	10↑	—	—
Platelet count (decrease)	10↑	10↑	—	—
Total T_3 and T_4 concentrations[d] (decrease)	0.1↑[e]	0.1↑[e]	—	—
Forestomach				
Epithelial hyperplasia (increased incidence)	3 and 30	30	1, 10, and 30	1↑
Epididymal spermatozoal concentration (decrease)	NS	—	3[e] and 30	—

↑ All higher doses were affected
[a] NS = not significantly affected
[b] NO = not observed
[c] Not applicable or not analyzed
[d] T_3 = triiodothyronine; T_4 = thyroxine
[e] Lowest dose tested for this effect

A decrease in mean body weight gain after exposure to 3,3′,4,4′-tetrachloroazobenzene in rats was observed in a 120-day feed study in male Sprague-Dawley rats (Hsia *et al.*, 1980). Assuming an average body weight of 400 grams, the daily intake in the Hsia *et al.*, study was estimated to be about 0.5 mg 3,3′,4,4′-tetrachloroazobenzene/kg body weight per day. At this exposure concentration, the decrease in mean body weight gain was about the same in the current 13-week gavage study as in rats that received 30 mg/kg per day. This suggests that male Sprague-Dawley rats are more prone to body weight loss induced by 3,3′,4,4′-tetrachloroazobenzene than male F344/N rats. This is further demonstrated by the death of male weanling Sprague-Dawley rats administered two 25 mg/kg intraperitoneal injections per week for up to 6 weeks (Hsia and Kreamer, 1985). One of the mechanisms that may play a role in body weight loss after exposure to dioxin-like compounds is reduced feed consumption, regulated by inhibition of key enzymes of gluconeogenesis in the liver (Weber *et al.*, 1991). These authors demonstrated that the induction of appetite suppression was preceded by the inhibition of hepatic phosphoenolpyruvate carboxylase, which caused a reduction in gluconeogenesis followed by a progressive increase in plasma tryptophan concentrations. Tryptophan effectively decreases feed

consumption (Fernstrom, 1983, 1985). Decreases in mean body weight gains and final body weights were not observed in the present 16-day or 13-week mouse studies.

In the 16-day studies, the liver weights were generally increased in male and female mice that received 10 mg/kg or greater, doses lower than those that induced this effect in rats. This could be due to a difference in the half-life of 3,3′,4,4′-tetrachloroazobenzene in these species, as has been observed for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds (Van den Berg *et al.*, 1994). In general, mice have a shorter half-life for dioxin-like compounds than rats, leading to an earlier steady-state level of the administered compounds. The lowest-observed-effect levels for the increases in liver weights in rats and mice were about the same in the 13-week studies. In contrast to the effects on liver weights, histopathologic changes consisting of centrilobular hypertrophy of hepatocytes were observed only in male mice in the 13-week study. This lesion also occurred in male and female mice administered the structural analogue 3,3′,4,4′-tetrachloroazoxybenzene (NTP, 1998). Typical dioxin-like effects on the liver in rodents include cytoplasmic vacuolization, increased incidences of anisokaryosis, and necrosis (Kociba *et al.*, 1976; Chu *et al.*, 1994, 1995). Increased incidences of these lesions were not observed in the current studies. The hepatic centrilobular hypertrophy in male mice, however, resembles the hepatic response in rats to the highly lipophilic, nondioxin-like polychlorinated biphenyl, 2,2′,4,4′,5,5′-hexachlorobiphenyl (Chu *et al.*, 1996; Peng *et al.*, 1997). Centrilobular hypertrophy is often a reflection of enzyme induction and proliferation of smooth endoplasmic reticulum (Butler, 1996). In rats, the absence of histopathologic lesions in the liver was confirmed by the minimal effects on various clinical chemistry parameters. In mice, no alterations were observed in clinical chemistry parameters associated with hepatic effects; because only centrilobular hypertrophy was observed, it was not necessarily expected that these clinical chemistry parameters would be affected. 3,3′,4,4′-Tetrachloroazobenzene administration increased hepatic cytochrome P_{450}1A protein concentrations in rats as determined by immunohistologic staining. An increase in cytochrome P_{450}1A1 and P_{450}1A2 activities, respectively, often measured as ethoxyresorufin-*O*-deethylase and methyoxyresorufin-*O*-demeethylase activities, is a typical dioxin-like effect and is mediated through the Ah receptor (Safe, 1990, 1994).

In the 16-day studies in rats and mice, a marked decrease in thymus weights occurred even at the lowest doses tested, with no differences in survival or mean body weight gains. In the 13-week studies, thymic atrophy was observed in rats and, again, occurred at doses causing no change in survival or mean body weight gains, suggesting a direct lymphocytotoxic effect and not a stress mechanism. Thymic atrophy was observed in male Sprague-Dawley rats administered 3,3′,4,4′-tetrachloroazobenzene (Hsia *et al.*, 1981, 1982) and is one of the hallmarks of toxicity of dioxin-like compounds (Safe, 1990; De Waal *et al.*, 1997). The thymic atrophy in the current studies consisted of thinning of the cortex due to reduced numbers of cortical lymphocytes. An important feature in dioxin-mediated thymic toxicity is the disruption of epithelial cells in the cortex. Histologically, exposure to dioxin-like compounds results in depletion of the cortical area and a loss of demarcation between

the cortex and medulla. Thymic atrophy was also observed with 3,3′,4,4′-tetrachloroazoxybenzene (NTP, 1998), again at lower doses in rats than in mice.

The immunotoxicity of dioxin-like compounds in mice has been associated with an Ah receptor-dependent mechanism (Vecchi *et al.*, 1983; Davis and Safe, 1988, 1990). In rats, thymic atrophy has been associated with an Ah receptor-mediated mechanism, because the rank order of dioxin-like compounds for thymic atrophy is the same as that based on other toxic and biochemical endpoints (Ahlborg *et al.*, 1992, 1994). It has been suggested that thymic atrophy is mainly due to inhibition of development of the prethymic and early intrathymic stem cell compartment, primarily mediated by activation of hemopoietic cells (Silverstone *et al.*, 1997).

In rats, thymic atrophy occurred at the same doses at which decreases in leukocyte, segmented neutrophil, lymphocyte, and eosinophil counts were observed. In a 13-week feed study with 2,3,4,7,8-pentachlorodibenzofuran, the decreases in leukocyte counts and increased incidences of thymic atrophy were equally sensitive endpoints (Plüss *et al.*, 1988). This suggests that the lymphocytotoxic effects are interrelated and are not specific to one organ. Another possible involvement in thymic atrophy could be autoimmunity, as suggested for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Holladay *et al.*, 1991; Vos and Van Loveren, 1995; De Waal *et al.*, 1997). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin has been found to increase lupus-like nephritis in a mouse model, suggesting an association between autoimmunity and exposure to dioxin-like compounds (Silverstone *et al.*, 1996). Hexachlorobenzene, a compound with dioxin-like and nondioxin-like activities, resulted in autoimmunity (Schielen *et al.*, 1993). In most autoimmune-related cases, an estrogen-dependent factor is involved (Ahmed *et al.*, 1985). The thymic atrophy, however, generally occurred at the same doses in male and female rats in the 13-week study. This was also observed for 3,3′,4,4′-tetrachloroazoxybenzene (NTP, 1998).

The hematology results of this gavage study demonstrated that 3,3′,4,4′-tetrachloroazobenzene, administered at 10 and 30 mg/kg, induced decreased platelet counts and a minimal to mild responsive anemia. Other dioxin-like compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 2,3,4,7,8-pentachlorodibenzofuran, and 3,3′,4,4′-tetrachloroazoxybenzene also caused a responsive anemia (Kociba *et al.*, 1976; Plüss *et al.*, 1988; NTP, 1998) and decreased platelet counts (Weisberg and Zinkl, 1973; Kociba *et al.*, 1976; Plüss *et al.*, 1988). The development of an anemia has also been reported for 3,3′,4,4′-tetrachloroazobenzene and 3,3′,4,4′-tetrachloroazoxybenzene administered to rats by dosed feed (Hsia *et al.*, 1980). In contrast to the present study, the hematological changes in the feed study suggested a nonresponsive anemia, and an aplastic anemia in the early stages was indicated. In the present study, however, the increased reticulocyte counts and the splenic hematopoietic cell proliferation would be consistent with an erythropoietic response. Additionally, the mean cell volume was increased suggesting the erythrocytes were macrocytic. Macrocytosis could be attributed to the increased numbers of larger reticulocytes in the circulation and thus, also would be consistent with an

erythropoietic response to anemia. It should be noted that the anemia induced in male Sprague Dawley rats by Hsia *et al.* (1980) was of minimal severity and similar to that which occurred for the 10 mg/kg male F344/N rats of the present study; the 10 mg/kg male rats did not demonstrate increased reticulocyte counts but did show increased splenic hematopoietic cell proliferation. Hsia *et al.* (1980) did not consider reticulocyte counts or microscopic examination of tissues for the evaluation of an erythropoietic response and this, in part, may help explain the differences between that report and the results of the present study.

The etiology of the anemia in the current studies is unknown. Increased incidences of hemosiderin accumulation in the spleen of female rats in the 13-week study suggested an increase in erythrocyte injury. A golden brown pigment was observed in the liver, kidney, and lungs of rats exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Kociba *et al.*, 1976). A decrease in the erythrocytic activity of enzymes of carbohydrate metabolism might explain the development of a responsive anemia and increased splenic hemosiderin, because this decrease can result in increased susceptibility of erythrocytes to oxidative stress or chemical-induced injury (Dhur *et al.*, 1989; Grossman *et al.*, 1995; Kanno *et al.*, 1995). 3,3′,4,4′-Tetrachloroazoxybenzene has been shown to decrease hepatic enzyme activities involved in carbohydrate metabolism (Hsia and Kreamer, 1985).

The anemia might also be related to the sulfate conjugates of mono- or dichloroaniline, the major urinary metabolites of 3,3′,4,4′-tetrachloroazobenzene in male rats (Pillai *et al.*, 1996). Two metabolites of dichloroaniline, 6-hydroxy-3,4-dichloroaniline and *N*-hydroxy-3,4-dichloroaniline, have been shown to cause a hemolytic anemia (McMillan *et al.*, 1991). The decreases in platelet counts in 10 and 30 mg/kg male and female rats were minimal to mild in severity, consistent with the absence of gross or microscopic evidence of hemorrhage. The mechanism by which 3,3′,4,4′-tetrachloroazobenzene causes thrombocytopenia is unknown. Dioxin-like compounds are known to cause decreased platelet counts (Zinkl *et al.*,1973; Kociba *et al.*, 1976; Plüss *et al.*, 1988; Viluksela *et al.*, 1997). It has been suggested that an immune-mediated etiology be explored as a possible mechanism (Weissberg and Zinkl, 1973). An immune-mediated erythrocyte injury or production of erythrocytes with a shortened life-span could also be involved in the anemia (Jain, 1986). Silverstone *et al.* (1996) have shown an increase in lupus-like nephritis in a mouse model after exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. An increase in serum immunoglobulin concentrations has been reported after 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure (Burns *et al.*, 1996). This strengthens the basis for an immunologic component in the observed anemia and thrombocytopenia in rats.

Marked decreases in circulating thyroid hormone concentrations were found in rats exposed to 3,3′,4,4′-tetrachloroazobenzene for 21 days or 13 weeks. This effect has also been observed after exposure to other dioxin-like compounds (Bastomsky, 1977; Brouwer and van den Berg, 1986; Henry and Gasiewicz, 1986; Morse *et al.*, 1993; Van Birgelen *et al.*, 1995a). The same magnitudes of decrease in total thyroxine and triiodothyronine concentrations were also observed after exposure to 3,3′,4,4′-tetrachloroazoxybenzene (NTP,

1998). The apparent magnitude of the thyroxine concentration decrease and relatively weak thyroid-stimulating hormone response, however, is unusual. A weak thyroid-stimulating hormone response has been observed previously after exposure to dioxin-like compounds (Barter and Klaassen, 1994; Morse *et al.*, 1996).

Decreases in circulating thyroid hormone concentrations are associated with induction of hepatic thyroid hormone glucuronyl transferase (Bock, 1991; Schrenk *et al.*, 1991; Barter and Klaassen, 1992; Van Birgelen *et al.*, 1995a). Furthermore, a destabilization of the complex of thyroxine and transthyretin by metabolites of polychlorinated biphenyls has been proposed to be involved in the decrease in circulating thyroxine concentrations (Brouwer and van den Berg, 1986; Brouwer *et al.*, 1988). Another theory is that changes in thyroid gland function and morphology result in an interference with the synthesis and excretion of thyroxine (Collins and Capen, 1980; Chu *et al.*, 1994).

The decrease in thyroxine concentrations was so drastic that, along with an increase in glucuronidation by an Ah receptor-regulated mechanism, metabolites of 3,3′,4,4′-tetrachloroazobenzene very likely played a role, as has been found for other dioxin-like compounds (Brouwer and van den Berg, 1986; Lans *et al.*, 1993). The pattern of metabolites as found in the urine by Pillai *et al.* (1996) after *in vitro* metabolism of 3,3′,4,4′-tetrachloroazobenzene (Hsia and Kreamer, 1981) suggests that sulfone-like structures and hydroxylated metabolites might be involved in the destabilization of the complex transthyretin and thyroxine. Methyl sulfones of polychlorinated biphenyls decrease thyroid hormone concentrations in mink and rats (Lund *et al.*, 1997, Kato *et al.*, 1998). Hydroxylated metabolites of polychlorinated biphenyls bind to transthyretin, thereby destabilizing the transthyretin-thyroxine complex and eventually decreasing circulating thyroxine levels (Brouwer and van den Berg, 1986; Lans *et al.*, 1993).

Pre- and postnatal exposures to dioxin-like compounds in humans have been correlated with alterations in circulating thyroid hormone concentrations and (neuro)developmental effects (Jacobson *et al.*, 1990a,b; Koopman-Esseboom *et al.*, 1994; Huisman *et al.*, 1995; Jacobson and Jacobson, 1996; Lonky *et al.*, 1996; Nagayama *et al.*, 1997; Patandin *et al.*, 1997a,b). Decreased thyroid hormone concentrations are associated with permanent alterations in behavior and brain maturation in the offspring (Porterfield and Hendrich, 1993). No developmental studies with 3,3′,4,4′-tetrachloroazobenzene have been reported in the literature.

No histopathologic changes were observed in the thyroid gland after exposure to 3,3′,4,4′-tetrachloroazobenzene or 3,3′,4,4′-tetrachloroazoxybenzene (NTP, 1998). This is unusual in light of the severity of the decrease in circulating thyroid hormone concentrations. Although thyroid-stimulating hormone concentrations were slightly increased, this increase was insufficient to alter the morphology of the thyroid gland. A possible explanation might be that 3,3′,4,4′-tetrachloroazobenzene or its metabolites mimic thyroxine, suppressing the feedback

mechanism to stimulate thyroid-stimulating hormone production and release. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin has been shown to mimic the action of thyroxine in the development of tadpoles (McKinney *et al.*, 1985). Of interest for this hypothesis is the high accumulation of azoxybenzene in the thyroid gland in rats (Kujawa *et al.*, 1989).

Incidences of hyperplasia of the forestomach were increased in groups of dosed rats and mice in the current 13-week studies with mice being more sensitive for this response. Hyperplasia of the forestomach was also observed in rats and mice administered 3,3′,4,4′-tetrachloroazoxybenzene (NTP, 1998). This effect has been associated with irritation of the forestomach in rats and mice in gavage studies (Brown and Hardisty, 1990). Most 13-week studies with dioxin-like compounds in which histopathologic evaluations were performed were feed studies. Gastric ulcers were observed in *Macaca mulatta* monkeys given "toxic fat" containing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in feed (Allen and Carstens, 1967). Albino rats exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by gavage also had an increased incidence of stomach ulcers (Gupta *et al.*, 1973).

3,3′,4,4′-Tetrachloroazobenzene administration reduced the epididymal spermatozoal concentration in male mice. A similar decrease was generally observed with 3,3′,4,4′-tetrachloroazoxybenzene (NTP, 1998). In a 13-week feed study with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in Sprague-Dawley rats, a decrease in testicular spermatogenic activity and occlusive stasis of spermatozoa in the epididymides was observed in one of five rats (Kociba *et al.*, 1976). In rats, various dioxin-like compounds have been shown to reduce epididymal sperm numbers after *in utero* or lactational exposure (Mably *et al.*, 1992; Gray *et al.*, 1993, 1997; Waalkens-Berendsen *et al.*, 1994; Sommer *et al.*, 1996). In addition, a nondioxin-like PCB, 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153), has been shown to reduce epididymal sperm numbers (Waalkens-Berendsen *et al.*, 1996).

3,3′,4,4′-Tetrachloroazobenzene was mutagenic in *Salmonella typhimurium* strain TA97, but only with 30% rat liver S9 activation enzymes. 3,3′,4,4′-Tetrachloroazobenzene did not induce micronuclei in bone marrow erythrocytes in male mice treated three times with 3,3′,4,4′-tetrachloroazobenzene. In the 13-week study, however, 3,3′,4,4′-tetrachloroazobenzene resulted in an increase in the frequency of micronuclei in peripheral blood erythrocytes in male and female mice. The discordancy between the short- and long-term micronucleus test results has also been observed with phenolphthalein, salicylazosulfapyridine, diisopropylcarbodiimide, and 3,3′,4,4′-tetrachloroazoxybenzene (Bishop *et al.*, 1990; Dietz *et al.*, 1992; Witt *et al.*, 1995, 1996, 1998; NTP, 1998). 3,3′,4,4′-Tetrachloroazobenzene-induced DNA damage was observed in primary rat hepatocytes, but only after pretreatment with hepatic mixed-function oxidase inducers (Shaddock *et al.*, 1989). These data suggest that an enhanced metabolism is necessary for 3,3′,4,4′-tetrachloroazobenzene-induced genetic toxicity.

For dioxin-like compounds such as 3,3′,4,4′-tetrachloroazobenzene, a relative potency value can be estimated for each compound that acts through the Ah receptor. This value expresses the potency of that specific

compound compared to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxic Equivalency Factor (TEF) values are consensus relative potency values derived from all available studies that compare the TEF chemical to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the most potent dioxin-like compound, which is assigned a TEF value of one (USEPA, 1989; van Zorge *et al*., 1989; Kutz *et al*., 1990; Safe, 1990, 1994; Ahlborg *et al*., 1992, 1994; Feeley and Grant, 1993; Birnbaum and DeVito, 1995). TEF values are based on repeat-dose *in vivo* studies, single-exposure studies, structure-activity considerations, and data from *in vitro* studies, with preference for repeat-dose studies (Ahlborg *et al*., 1994). Multiplying the TEF value of a specific compound by the concentration of that compound in a mixture results in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TEQs) of that compound. The sum of all TEQs for every dioxin-like compound in a mixture gives the total TEQ of that specific mixture. Table 14 summarizes a range of relative potency values for 3,3′,4,4′-tetrachloroazobenzene, based on *in vitro* and *in ovo* experiments, a 13-week gavage study in female B6C3F$_1$ mice, and comparisons of data from the current rat study with the literature.

3,3′,4,4′-Tetrachloroazobenzene has a high binding affinity to the Ah receptor and therefore a great likelihood of having a potency of the same order of magnitude as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Once administered *in vivo* however, the apparent relative potency decreases considerably. 3,3′,4,4′-Tetrachloroazobenzene has a short half-life in comparison to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Van den Berg *et al*., 1994; Pillai *et al*., 1996). In repeat-dose experiments, 3,3′,4,4′-tetrachloroazobenzene reaches steady-state concentrations fairly quickly, whereas 2,3,7,8-tetrachlorodibenzo-*p*-dioxin will still accumulate. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin will thus appear more potent over time in comparison to 3,3′,4,4′-tetrachloroazobenzene. This has been shown with 2,3,7,8-tetrachlorodibenzofuran and 3,3′,4,4′-tetrachlorobiphenyl (Ahlborg *et al*., 1994; DeVito and Birnbaum, 1995). Comparing the results of the 13-week gavage study with 3,3′,4,4′-tetrachloroazobenzene in F344/N rats to a 13-week feed experiment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in female Sprague-Dawley rats shows again that 3,3′,4,4′-tetrachloroazobenzene is of lower potency (Table 14). Although a comparison between different strains of rats in different laboratories is not optimal for estimating a relative potency value (Ahlborg *et al*., 1992, 1994), 3,3′,4,4′-tetrachloroazobenzene is about five to six orders of magnitude less potent than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin using thymic atrophy as the endpoint (Figure 4; Van Birgelen *et al*., 1995b). This is in the same order of magnitude as the range of 3×10^{-6} to 10^{-5} for the relative potency value based on dermal cytochrome P$_{450}$1A1 induction in the 13-week gavage study in female B6C3F$_1$ mice (Hébert *et al*., 1993). In this latter study, 3,3′,4,4′-tetrachloroazobenzene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin were tested in parallel. Figure 5 shows that the decrease in thyroxine concentrations in F344/N rats in the current gavage study at a dose of 0.1 mg/kg 3,3′,4,4′-tetrachloroazobenzene per day is similar to that induced by 1 µg/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in feed per day in female Sprague-Dawley rats (Van Birgelen *et al*., 1995b), indicating that 3,3′,4,4′-tetrachloroazobenzene is about two orders of magnitude less potent than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin based on this endpoint.

TABLE 14
Relative Potency Estimates for 3,3′,4,4′-Tetrachloroazobenzene Based on *In Vitro* and *In Ovo* Experiments, a 13-Week Gavage Study in Female B6C3F$_1$ Mice, and Comparisons of Data from the Current Rat Study with the Literature[a]

Effect	Concentration Inducing Effect		Relative Potency for TCAB	Reference
	TCDD	TCAB		
***In Vitro* Experiments**				
Binding affinity to the Ah receptor (nM)	0.27	1.1	0.2	Poland *et al.*, 1976
EC_{50} for binding to the mouse hepatic Ah receptor (nM)	1.22	6.03	0.2	Schneider *et al.*, 1995
***In Ovo* Experiments**				
ED_{50} (nmol/kg) for induction of aryl hydrocarbon hydroxylase in chicken embryos	0.31	2.0	0.2	Poland *et al.*, 1976
LD_{50} (ng/egg) in chicken embryos[b]	0.2	44	0.005	Higginbotham *et al.*, 1968; Schrankel *et al.*, 1982
Mouse Study				
Cytochrome P_{450}1A1 induction in the skin in a 13-week gavage study in female B6C3F$_1$ mice with TCDD and TCAB			0.000003–0.00001	Hébert *et al.*, 1993
Rat Studies				
Decreased thyroxine concentrations in 13-week gavage studies[c]			About two orders of magnitude less than TCDD	Van Birgelen *et al.*, 1995b and current study
Thymic atrophy in 13-week gavage studies[c]			About five to six orders of magnitude less than TCDD	Van Birgelen *et al.*, 1995b and current study

[a] Data are presented as toxic equivalents. TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCAB = 3,3′,4,4′-tetrachloroazobenzene
[b] Experiments were not performed at the same laboratory.
[c] The TCDD feed study used female Sprague-Dawley rats, and the TCAB study data are from the current gavage study.

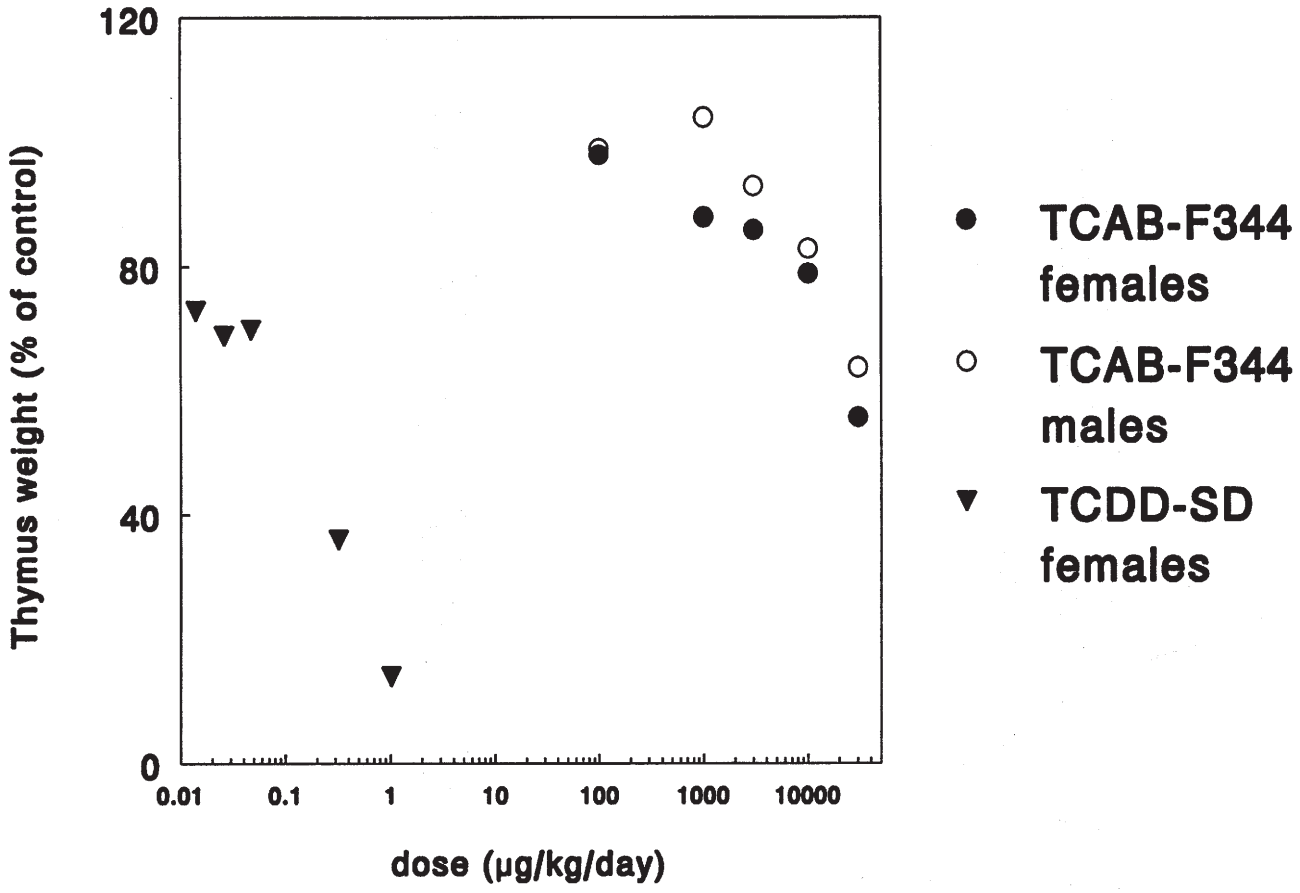


FIGURE 4
Decrease in Thymus Weights (% of Controls) in the 13-Week Studies of 3,3′,4,4′-Tetrachloroazobenzene (TCAB) and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD).
The 3,3′,4,4′-tetrachloroazobenzene data for male and female F344/N rats were obtained from the current gavage study. The 13-week 2,3,7,8-tetrachlorodibenzo-*p*-dioxin feed study was performed in female Sprague-Dawley (SD) rats (Van Birgelen *et al*., 1995b).

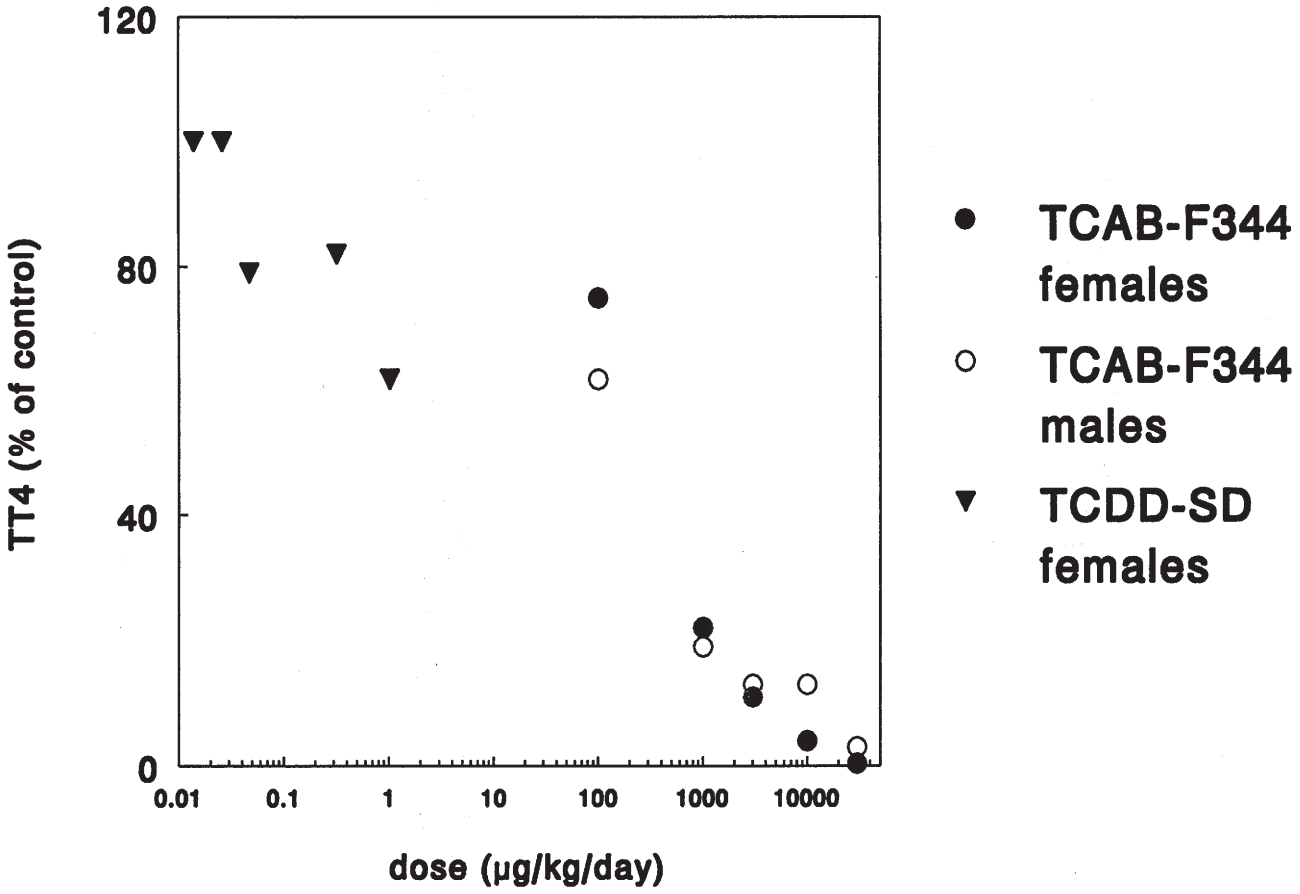


FIGURE 5
Decrease in Total Circulating Thyroxine (TT4) Concentrations (% of Controls) in the 13-Week Studies of 3,3′,4,4′-Tetrachloroazobenzene (TCAB) and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD).
The 3,3′,4,4′-tetrachloroazobenzene data for male and female F344/N rats were obtained from the current gavage study. The 13-week 2,3,7,8-tetrachlorodibenzo-*p*-dioxin feed study was performed in female Sprague-Dawley (SD) rats (Van Birgelen *et al*., 1995b).

This difference in potency for inducing a decrease in circulating thyroxine concentrations can very likely be explained by the involvement of multiple mechanisms, as has been found with a mixture of dioxin-like compounds (van Birgelen *et al.*, 1997). This mixture caused a marked decrease in circulating thyroid hormone concentrations when compared to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. This synergistic effect has been attributed to the involvement of metabolites of polychlorinated biphenyls and multiple isozymes of thyroxine glucuronidation enzymes (van Birgelen *et al.*, 1997; Birnbaum *et al.*, 1998). Because 3,3′,4,4′-tetrachloroazobenzene is quickly metabolized and induces dioxin-like effects, it is likely that metabolites of 3,3′,4,4′- tetrachloroazobenzene and Ah receptor-associated glucuronidation of thyroxine are involved in the decrease in circulating thyroid hormone concentrations.

Based on the production levels of Propanil® and dichloroaniline, the resultant 3,3′,4,4′-tetrachloroazobenzene production amount might be as high as 16,000 kg per year in the United States (Sundström *et al.*, 1978; Bunce *et al.*, 1979; Hill *et al.*, 1981; USEPA, 1985; McMillan *et al.*, 1991). Assuming 3,3′,4,4′-tetrachloroazobenzene is indeed six to two orders of magnitude less potent than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, this could lead to an annual release of 0.016 to 160 kg of toxic equivalents into the environment due to 3,3′,4,4′-tetrachloroazobenzene alone. For comparison, the annual atmospheric emission in Germany, including all incinerators, traffic, and industry, is estimated to be 1 kg of toxic equivalents for dioxin-like compounds (Fiedler *et al.*, 1990). In the United States, the sum of the atmospheric emissions of 10 high-emission solid waste incinerators was estimated to be 3.6 kg of toxic equivalents in 1993 (Webster and Connett, 1996). This implies a possibly large impact of 3,3′,4,4′-tetrachloroazobenzene on the release of all dioxin-like compounds. Human and environmental exposure assessments of 3,3′,4,4′-tetrachloroazobenzene are thus obligatory to estimate the impact of 3,3′,4,4′-tetrachloroazobenzene on the total exposure to dioxin-like compounds.

Hematologic, splenic, and thymic effects were observed in rats administered 3,3′,4,4′-tetrachloroazobenzene for 13 weeks; however, no histopathologic alterations were observed in the liver. This is in sharp contrast to the pattern of effects observed in rodents exposed to other dioxin-like compounds to which the liver is one of the most sensitive organs (Kociba *et al.*, 1976; Chu *et al.*, 1994, 1995). This discrepancy might be explained by the fact that the liver-to-fat ratio of other dioxin-like compounds in rodents is at least one order of magnitude higher than that for 3,3′,4,4′-tetrachloroazobenzene (Table 15). A low liver-to-fat ratio means that relatively more of the test compound can be distributed to organs other than the liver before an effective concentration in the liver is reached. The liver is the most commonly affected organ in 2-year bioassays in rodents with dioxin-like compounds (IARC, 1997). Based on the difference in the liver-to-fat ratio between 3,3′,4,4′-tetrachloroazobenzene and other dioxin-like compounds, 3,3′,4,4′-tetrachloroazobenzene could induce

TABLE 15
Liver-to-Fat Ratios of Dioxin-Like Compounds in Rats and Mice

Compound	Liver-to-Fat Ratio	Reference
Rats		
3,3′,4,4′-Tetrachloroazobenzene	0.1–0.2	Pillai *et al*. (1996)
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	2–6	Abraham *et al*. (1989); Van Birgelen *et al*. (1995b)
1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin	13	Abraham *et al*. (1989)
Hexachlorinated dibenzo-*p*-dioxins	34	Abraham *et al*. (1989)
1,2,3,4,6,7,8-Heptachlorodibenzo-*p*-dioxin	66	Abraham *et al*. (1989)
2,3,7,8-Tetrachlorodibenzofuran	2	Abraham *et al*. (1989)
2,3,4,7,8-Pentachlorodibenzofuran	43	Abraham *et al*. (1989)
Mice		
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	0.5–2.5	DeVito *et al*. (1995)
1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin	5–9	DeVito *et al*. (1995)
2,3,7,8-Tetrachlorodibenzofuran	1–5	DeVito *et al*. (1995)
2,3,4,7,8-Pentachlorodibenzofuran	7–47	DeVito *et al*. (1995)

neoplasms mainly in organs other than the liver. No data from a 2-year bioassay are available to strengthen this hypothesis.

In summary, 3,3′,4,4′-tetrachloroazobenzene caused typical dioxin-like effects, such as thymic atrophy, an increase in liver weights, induction of hepatic cytochrome P_{450}1A, and decreased mean body weight gains. Furthermore, in the 13-week studies, a sharp decrease in circulating thyroxine concentrations was observed even at the lowest dose (0.1 mg/kg) tested in rats. Other effects included a decrease in epididymal spermatozoal concentration in mice, major effects on the hematopoietic system, and increased incidences of hyperplasia of the forestomach in 3 and 30 mg/kg males and 30 mg/kg females. A no-observable-adverse-effect-level (NOAEL) was not reached in rats. The NOAEL in mice was 0.1 mg/kg. Comparison of various dioxin-like effects in these studies with those reported in the literature indicate that 3,3′,4,4′-tetrachloroazobenzene is six to two orders of magnitude less potent than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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APPENDIX A
SUMMARY OF NONNEOPLASTIC LESIONS

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)			(10)	(10)	(10)
Hepatodiaphragmatic nodule				1 (10%)		
Inflammation, focal	1 (10%)					
Mesentery						(1)
Fat, hemorrhage, focal						1 (100%)
Fat, necrosis, focal						1 (100%)
Pancreas	(10)					(10)
Acinus, atrophy, focal						3 (30%)
Stomach, forestomach	(10)			(10)	(10)	(10)
Epithelium, hyperplasia, focal				4 (40%)	3 (30%)	8 (80%)
Cardiovascular System						
Heart	(10)					(10)
Cardiomyopathy	6 (60%)					
Endocrine System						
None						
General Body System						
None						
Genital System						
Preputial gland	(10)					(10)
Inflammation, chronic	4 (40%)					2 (20%)
Inflammation, chronic, focal	2 (20%)					
Bilateral, cyst						1 (10%)
Hematopoietic System						
Spleen	(10)		(9)	(10)	(10)	(10)
Hematopoietic cell proliferation				3 (30%)	8 (80%)	10 (100%)
Thymus	(9)			(10)	(10)	(10)
Atrophy					5 (50%)	10 (100%)
Integumentary System						
None						

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(10)				(1)	(10)
Hemorrhage, focal					1 (100%)	
Infiltration cellular, focal, lymphocyte	4 (40%)				1 (100%)	6 (60%)
Infiltration cellular, focal, histiocyte	1 (10%)					1 (10%)
Inflammation, focal	2 (20%)					3 (30%)
Alveolar epithelium, hyperplasia, focal	4 (40%)					3 (30%)
Special Senses System						
None						
Urinary System						
Kidney	(10)	(10)	(10)	(10)	(10)	(10)
Nephropathy	9 (90%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Animals examined microscopically	10	3[b]	10	10	10	10
Alimentary System						
Liver	(10)	(1)	(1)		(10)	(10)
Hepatodiaphragmatic nodule		1 (100%)	1 (100%)			1 (10%)
Inflammation, chronic, focal	1 (10%)				1 (10%)	1 (10%)
Necrosis, focal	1 (10%)					
Mesentery					(1)	
Fat, necrosis, focal					1 (100%)	
Pancreas	(10)					(10)
Acinus, atrophy, focal	1 (10%)					
Stomach, forestomach	(10)		(1)	(10)	(10)	(10)
Epithelium, hyperplasia, focal			1 (100%)	1 (10%)	2 (20%)	10 (100%)
Cardiovascular System						
Heart	(10)					(10)
Myocardium, degeneration, focal	1 (10%)					
Endocrine System						
None						
General Body System						
None						
Genital System						
Clitoral gland	(10)					(10)
Inflammation, focal	1 (10%)					
Ovary	(10)					(10)
Cyst						1 (10%)
Uterus	(10)	(1)		(1)		(10)
Hydrometra	1 (10%)	1 (100%)				2 (20%)

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Hematopoietic System						
Lymph node, mandibular	(10)					(10)
Hemorrhage	1 (10%)					
Lymph node, mesenteric	(10)					(10)
Inflammation, granulomatous						2 (20%)
Spleen	(10)	(1)		(10)	(10)	(10)
Hematopoietic cell proliferation					5 (50%)	9 (90%)
Pigmentation					2 (20%)	8 (80%)
Thymus	(10)		(10)	(9)	(8)	(9)
Atrophy				2 (22%)	3 (38%)	9 (100%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(10)					(10)
Hemorrhage, focal	3 (30%)					2 (20%)
Infiltration cellular, focal, lymphocyte	7 (70%)					5 (50%)
Inflammation, chronic active, focal						1 (10%)
Inflammation, focal						3 (30%)
Inflammation, focal, granulomatous	1 (10%)					
Alveolar epithelium, hyperplasia, focal	3 (30%)					6 (60%)
Special Senses System						
None						
Urinary System						
Kidney	(10)					(10)
Mineralization, focal	10 (100%)					7 (70%)

[a] Number of animals examined microscopically at the site and the number of animals with lesion
[b] Animals in this group were examined only when lesions were observed grossly.

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Esophagus	(10)					(9)
Muscularis, inflammation, chronic, focal	1 (10%)					
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Inflammation, focal						1 (10%)
Centrilobular, hypertrophy			2 (20%)	4 (40%)	8 (80%)	9 (90%)
Hepatocyte, vacuolization cytoplasmic	1 (10%)		1 (10%)		1 (10%)	3 (30%)
Stomach, forestomach	(10)	(10)	(10)	(10)	(10)	(10)
Epithelium, hyperplasia		2 (20%)	6 (60%)	3 (30%)	4 (40%)	6 (60%)
Stomach, glandular	(10)	(10)	(10)	(10)	(10)	(10)
Cyst						1 (10%)
Cardiovascular System						
Blood vessel	(10)					(10)
Adventitia, inflammation	1 (10%)					
Heart	(10)					(10)
Inflammation, focal						1 (10%)
Endocrine System						
None						
General Body System						
None						
Genital System						
None						
Hematopoietic System						
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Red pulp, hematopoietic cell proliferation	4 (40%)	8 (80%)	7 (70%)	9 (90%)	10 (100%)	10 (100%)
Red pulp, pigmentation						1 (10%)
Integumentary System						
None						

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
None						
Special Senses System						
None						
Urinary System						
Kidney	(10)					(10)
Inflammation, chronic, focal	1 (10%)					1 (10%)

[a] Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Disposition Summary						
Animals initially in study	10	10	10	10	10	10
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Animals examined microscopically	10	10	10	10	10	10
Alimentary System						
Liver	(10)	(10)	(10)	(10)	(10)	(10)
Hepatocyte, vacuolization cytoplasmic		2 (20%)	5 (50%)	1 (10%)	1 (10%)	2 (20%)
Stomach, forestomach	(10)	(10)	(10)	(10)	(10)	(10)
Epithelium, hyperplasia			7 (70%)	7 (70%)	5 (50%)	7 (70%)
Cardiovascular System						
None						
Endocrine System						
None						
General Body System						
None						
Genital System						
None						
Hematopoietic System						
Spleen	(10)	(10)	(10)	(10)	(10)	(10)
Red pulp, hematopoietic cell proliferation	5 (50%)	9 (90%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						

TABLE A4
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Respiratory System						
Lung	(10)					(10)
Alveolar epithelium, hyperplasia, focal						2 (20%)
Special Senses System						
None						
Urinary System						
Kidney	(10)					(10)
Inflammation, chronic, focal	1 (10%)					1 (10%)

[a] Number of animals examined microscopically at the site and the number of animals with lesion

APPENDIX B
HEMATOLOGY AND CLINICAL CHEMISTRY RESULTS

TABLE B1
Hematology and Clinical Chemistry Data for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Male						
Hematology						
n						
Day 3	10	10	9	10	9	10
Day 21	10	10	9	9	10	10
Week 13	9	10	8	10	9	10
Automated hematocrit (%)						
Day 3	37.7 ± 0.7	37.7 ± 0.5	37.4 ± 0.7	38.7 ± 0.6	38.5 ± 0.4	38.9 ± 0.4
Day 21	40.4 ± 0.4	41.1 ± 0.3	39.7 ± 0.4	40.3 ± 0.5	41.0 ± 0.4	40.0 ± 0.5
Week 13	43.4 ± 0.7	44.5 ± 0.4	43.0 ± 0.9	43.1 ± 0.7	40.2 ± 0.3**	37.4 ± 0.4**
Manual hematocrit (%)						
Day 3	44.9 ± 0.9	44.1 ± 0.4	44.3 ± 0.8	45.1 ± 0.7	44.7 ± 0.5	45.0 ± 0.5
Day 21	45.4 ± 0.5	46.1 ± 0.6	45.9 ± 0.6	45.6 ± 0.4	46.0 ± 0.5	45.2 ± 0.6
Week 13	47.3 ± 0.8	48.2 ± 0.4	46.8 ± 0.9	46.5 ± 0.5	43.7 ± 0.4**	41.3 ± 0.3**
Hemoglobin (g/dL)						
Day 3	13.8 ± 0.3	13.9 ± 0.2	13.8 ± 0.2	14.3 ± 0.2	14.1 ± 0.1	14.2 ± 0.2
Day 21	14.8 ± 0.1	15.0 ± 0.1	14.8 ± 0.2	14.9 ± 0.1	15.0 ± 0.2	14.7 ± 0.2
Week 13	15.5 ± 0.2	15.7 ± 0.1	15.3 ± 0.3	15.0 ± 0.2	14.1 ± 0.1**	13.1 ± 0.1**
Erythrocytes (10^6/μL)						
Day 3	6.33 ± 0.14	6.27 ± 0.08	6.21 ± 0.12	6.47 ± 0.11	6.43 ± 0.08	6.45 ± 0.08
Day 21	6.66 ± 0.10	6.92 ± 0.08	6.67 ± 0.09	6.84 ± 0.09	7.00 ± 0.10	6.81 ± 0.09
Week 13	8.57 ± 0.13	8.77 ± 0.09	8.53 ± 0.18	8.51 ± 0.15	7.77 ± 0.06**	6.79 ± 0.08**
Reticulocytes (10^6/μL)						
Day 3	0.11 ± 0.03	0.28 ± 0.03**	0.26 ± 0.03*	0.25 ± 0.03*	0.23 ± 0.02	0.28 ± 0.02**
Day 21	0.18 ± 0.03	0.16 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.19 ± 0.01
Week 13	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.17 ± 0.02**
Nucleated erythrocytes (10^3/μL)						
Day 3	0.09 ± 0.04	0.21 ± 0.06	0.18 ± 0.05	0.15 ± 0.05	0.09 ± 0.03	0.17 ± 0.03
Day 21	0.04 ± 0.02	0.07 ± 0.02	0.03 ± 0.02	0.05 ± 0.03	0.06 ± 0.03	0.06 ± 0.03
Week 13	0.03 ± 0.02	0.04 ± 0.02	0.10 ± 0.04	0.03 ± 0.02	0.02 ± 0.02	0.03 ± 0.01
Mean cell volume (fL)						
Day 3	59.5 ± 0.5	60.2 ± 0.3	60.3 ± 0.4	59.9 ± 0.2	60.0 ± 0.4	60.4 ± 0.2
Day 21	60.8 ± 0.8	59.5 ± 0.3	59.6 ± 0.4	58.9 ± 0.3*	58.5 ± 0.4**	58.7 ± 0.2**
Week 13	50.6 ± 0.1	50.7 ± 0.1	50.5 ± 0.1	50.7 ± 0.2	51.8 ± 0.1**	55.0 ± 0.2**
Mean cell hemoglobin (pg)						
Day 3	21.8 ± 0.2	22.2 ± 0.1	22.3 ± 0.2	22.2 ± 0.1	22.0 ± 0.1	22.0 ± 0.1
Day 21	22.2 ± 0.3	21.7 ± 0.1	22.2 ± 0.2	21.9 ± 0.2	21.5 ± 0.2	21.6 ± 0.2
Week 13	18.1 ± 0.1	17.9 ± 0.1	17.9 ± 0.1	17.7 ± 0.1	18.1 ± 0.1	19.3 ± 0.1**
Mean cell hemoglobin concentration (g/dL)						
Day 3	36.7 ± 0.2	36.9 ± 0.3	36.9 ± 0.2	37.0 ± 0.2	36.6 ± 0.2	36.4 ± 0.2
Day 21	36.6 ± 0.3	36.5 ± 0.2	37.3 ± 0.2	37.1 ± 0.3	36.7 ± 0.2	36.7 ± 0.2
Week 13	35.7 ± 0.3	35.2 ± 0.2	35.5 ± 0.3	34.9 ± 0.2	35.0 ± 0.2	35.0 ± 0.2
Platelets (10^3/μL)						
Day 3	803.5 ± 60.3	940.8 ± 15.1	959.0 ± 21.2	908.7 ± 26.1	955.1 ± 14.6	966.3 ± 28.5
Day 21	868.1 ± 14.8	868.6 ± 8.2	858.2 ± 8.5	839.0 ± 24.3	792.9 ± 14.1**	715.1 ± 13.2**
Week 13	745.0 ± 33.9	730.6 ± 8.9	776.0 ± 48.0	713.2 ± 25.8	619.2 ± 7.0**	496.0 ± 30.2**

TABLE B1
Hematology and Clinical Chemistry Data for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Male (continued)						
Hematology (continued)						
n						
Day 3	10	10	9	10	9	10
Day 21	10	10	9	9	10	10
Week 13	9	10	8	10	9	10
Leukocytes (10^3/μL)						
Day 3	8.80 ± 0.38	8.66 ± 0.52	8.46 ± 0.52	8.46 ± 0.35	8.54 ± 0.60	9.56 ± 0.30
Day 21	9.21 ± 0.40	9.46 ± 0.38	9.57 ± 0.47	8.78 ± 0.40	9.63 ± 0.30	7.41 ± 0.24*
Week 13	10.76 ± 0.40	10.52 ± 0.29	10.95 ± 0.40	10.16 ± 0.50	9.44 ± 0.41*	7.23 ± 0.31**
Segmented neutrophils (10^3/μL)						
Day 3	1.16 ± 0.16	1.24 ± 0.13	0.97 ± 0.23	0.90 ± 0.11	1.18 ± 0.27	0.91 ± 0.07
Day 21	1.56 ± 0.21	1.10 ± 0.16	1.37 ± 0.16	1.01 ± 0.18	0.99 ± 0.11*	0.88 ± 0.07**
Week 13	1.58 ± 0.19	1.62 ± 0.14	1.64 ± 0.30	1.74 ± 0.29	1.41 ± 0.21	0.95 ± 0.10*
Lymphocytes (10^3/μL)						
Day 3	7.53 ± 0.30	7.64 ± 0.48	7.52 ± 0.36	7.51 ± 0.22	7.17 ± 0.43	8.55 ± 0.29
Day 21	7.40 ± 0.40	8.11 ± 0.31	7.97 ± 0.33	7.61 ± 0.31	8.38 ± 0.34	6.42 ± 0.25
Week 13	9.00 ± 0.44	8.54 ± 0.25	8.94 ± 0.24	8.11 ± 0.49	7.87 ± 0.33	6.21 ± 0.23**
Monocytes (10^3/μL)						
Day 3	0.17 ± 0.03	0.12 ± 0.04	0.07 ± 0.03	0.12 ± 0.04	0.14 ± 0.04	0.11 ± 0.03
Day 21	0.25 ± 0.04	0.24 ± 0.07	0.23 ± 0.07	0.18 ± 0.05	0.25 ± 0.04	0.13 ± 0.02
Week 13	0.13 ± 0.06	0.31 ± 0.07	0.27 ± 0.08	0.26 ± 0.05	0.15 ± 0.06	0.06 ± 0.02
Eosinophils (10^3/μL)						
Day 3	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.03
Day 21	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00
Week 13	0.05 ± 0.03	0.05 ± 0.03	0.11 ± 0.04	0.05 ± 0.02	0.00 ± 0.00	0.02 ± 0.02
Clinical Chemistry						
n						
Day 3	10	10	10	10	10	10
Day 21	10	10	9	9	10	10
Week 13	10	10	10	10	10	10
Urea nitrogen (mg/dL)						
Day 3	21.1 ± 0.8	19.5 ± 0.6	18.5 ± 0.5*	20.2 ± 0.8	19.9 ± 0.6	18.7 ± 0.4
Day 21	20.2 ± 0.7	18.6 ± 0.5	19.1 ± 0.5	20.0 ± 0.5	19.1 ± 0.3	19.2 ± 0.4
Week 13	18.5 ± 0.6	17.7 ± 0.4	19.0 ± 0.6	19.8 ± 0.7	18.4 ± 0.5	20.0 ± 0.8
Creatinine (mg/dL)						
Day 3	0.59 ± 0.02	0.56 ± 0.02	0.54 ± 0.02	0.59 ± 0.01	0.56 ± 0.02	0.58 ± 0.04
Day 21	0.61 ± 0.01	0.60 ± 0.02	0.60 ± 0.02	0.62 ± 0.02	0.62 ± 0.01	0.63 ± 0.02
Week 13	0.59 ± 0.03	0.63 ± 0.02	0.60 ± 0.02	0.67 ± 0.02*	0.63 ± 0.02	0.65 ± 0.02
Total protein (g/dL)						
Day 3	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.5 ± 0.1	5.4 ± 0.1	5.4 ± 0.1
Day 21	6.5 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.7 ± 0.1	6.8 ± 0.1**	6.9 ± 0.1**
Week 13	6.9 ± 0.1	7.0 ± 0.1	7.2 ± 0.1	7.4 ± 0.1**	7.3 ± 0.0**	7.3 ± 0.1*
Albumin (g/dL)						
Day 3	4.0 ± 0.1	3.9 ± 0.1	3.9 ± 0.0	4.1 ± 0.0	4.0 ± 0.1	4.0 ± 0.0
Day 21	4.7 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.9 ± 0.1*	5.0 ± 0.1**
Week 13	5.0 ± 0.1	5.0 ± 0.0	5.1 ± 0.1	5.2 ± 0.0	5.2 ± 0.0*	5.3 ± 0.0**

TABLE B1
Hematology and Clinical Chemistry Data for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Male (continued)						
Clinical Chemistry (continued)						
n						
Day 3	10	10	10	10	10	10
Day 21	10	10	9	9	10	10
Week 13	10	10	10	10	10	10
Alanine aminotransferase (IU/L)						
Day 3	49 ± 2	48 ± 2	47 ± 1	50 ± 1	47 ± 2	44 ± 2
Day 21	55 ± 2	57 ± 1	52 ± 1	53 ± 1	51 ± 1	48 ± 1**
Week 13	47 ± 2	49 ± 2	46 ± 3	46 ± 2	42 ± 1	42 ± 1
Alkaline phosphatase (IU/L)						
Day 3	747 ± 31	771 ± 16	822 ± 22	858 ± 17**	849 ± 26*	831 ± 21*
Day 21	536 ± 13	532 ± 8	480 ± 7**	480 ± 9**	474 ± 12**	476 ± 12**
Week 13	269 ± 7	301 ± 9	278 ± 12	305 ± 7*	316 ± 6**	321 ± 6**
Sorbitol dehydrogenase (IU/L)						
Day 3	19 ± 2	18 ± 1	16 ± 1	21 ± 1	19 ± 1	18 ± 2
Day 21	17 ± 2	17 ± 1	15 ± 1	18 ± 1	18 ± 1	17 ± 1
Week 13	16 ± 1	20 ± 1*	20 ± 1*	20 ± 1*	21 ± 1**	19 ± 1*
Bile acids (μmol/L)						
Day 3	33.1 ± 4.8[b]	33.8 ± 4.2[b]	54.2 ± 4.5*	52.6 ± 4.7	46.9 ± 4.0	43.9 ± 7.2
Day 21	37.1 ± 4.6	42.3 ± 6.3	30.5 ± 3.7	35.1 ± 3.5	28.9 ± 2.7	25.5 ± 2.3
Week 13	37.5 ± 4.1	38.3 ± 4.7	33.7 ± 3.0	37.5 ± 3.1	32.0 ± 2.6	40.1 ± 3.8
Thyroid-stimulating hormone (ng/mL)						
Day 21	1.8 ± 0.3	1.4 ± 0.3	1.8 ± 0.2	3.4 ± 0.5*	3.2 ± 0.4*	3.0 ± 0.4*
Week 13	2.0 ± 0.3	1.8 ± 0.2	1.9 ± 0.2	2.3 ± 0.3	2.7 ± 0.3	3.4 ± 0.5*
Total triiodothyronine (ng/dL)						
Day 21	117 ± 6	95 ± 8	90 ± 4*[b]	90 ± 7*	107 ± 6[b]	94 ± 8[c]
Week 13	140 ± 8	113 ± 3*	121 ± 6*	116 ± 6*	119 ± 4*	102 ± 6**
Total thyroxine (μg/dL)						
Day 21	4.2 ± 0.1	3.0 ± 0.2**	1.8 ± 0.2**	1.3 ± 0.2**[b]	1.8 ± 0.1**	1.3 ± 0.2**[b]
Week 13	3.4 ± 0.2	2.1 ± 0.2**	0.6 ± 0.2**	0.5 ± 0.1**	0.5 ± 0.1**	0.1 ± 0.1**

TABLE B1
Hematology and Clinical Chemistry Data for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Female						
Hematology						
n						
Day 3	10	10	10	10	10	10
Day 21	10	10	9	10	10	9
Week 13	10	10	10	10	10	10
Automated hematocrit (%)						
Day 3	41.0 ± 0.6	40.6 ± 0.8	42.2 ± 0.3	42.1 ± 0.5	42.1 ± 0.6	40.1 ± 0.5
Day 21	42.6 ± 0.4	43.6 ± 0.3	43.2 ± 0.4	43.2 ± 0.6	43.9 ± 0.4	43.0 ± 0.4
Week 13	42.5 ± 0.6	42.2 ± 0.3	42.3 ± 0.5	41.8 ± 0.2	40.5 ± 0.4**	38.5 ± 0.2**
Manual hematocrit (%)						
Day 3	46.1 ± 0.7	45.3 ± 1.0	46.7 ± 0.5	45.0 ± 0.4	46.3 ± 0.8	45.3 ± 0.4
Day 21	46.1 ± 0.5	46.5 ± 0.4	45.1 ± 0.4	46.3 ± 0.6	45.6 ± 0.3	44.4 ± 0.4
Week 13	45.4 ± 0.4	45.4 ± 0.4	45.1 ± 0.4	44.4 ± 0.2	43.3 ± 0.6**	40.8 ± 0.4**
Hemoglobin (g/dL)						
Day 3	15.0 ± 0.2	14.9 ± 0.3	15.4 ± 0.1	15.2 ± 0.1	15.4 ± 0.2	14.6 ± 0.2
Day 21	15.4 ± 0.1	15.8 ± 0.2	15.6 ± 0.1	15.9 ± 0.2	15.6 ± 0.1	15.1 ± 0.1
Week 13	15.3 ± 0.2	15.2 ± 0.1	15.1 ± 0.1	15.0 ± 0.1	14.3 ± 0.1**	13.4 ± 0.1**
Erythrocytes (10^6/μL)						
Day 3	6.90 ± 0.13	6.82 ± 0.16	7.14 ± 0.09	7.13 ± 0.10	7.10 ± 0.11	6.73 ± 0.09
Day 21	6.99 ± 0.09	7.20 ± 0.04	7.08 ± 0.07	7.24 ± 0.10	7.29 ± 0.08*	7.15 ± 0.08
Week 13	7.69 ± 0.11	7.60 ± 0.06	7.59 ± 0.09	7.49 ± 0.04	7.14 ± 0.07**	6.48 ± 0.04**
Reticulocytes (10^6/μL)						
Day 3	0.15 ± 0.01	0.18 ± 0.02	0.15 ± 0.01	0.22 ± 0.03	0.17 ± 0.01	0.19 ± 0.02
Day 21	0.11 ± 0.02	0.12 ± 0.01	0.14 ± 0.02	0.16 ± 0.01**	0.14 ± 0.02	0.18 ± 0.01**
Week 13	0.06 ± 0.01	0.10 ± 0.01**	0.10 ± 0.01**	0.09 ± 0.02*	0.12 ± 0.02**	0.13 ± 0.01**
Nucleated erythrocytes (10^3/μL)						
Day 3	0.12 ± 0.05	0.10 ± 0.04	0.06 ± 0.02	0.10 ± 0.04	0.10 ± 0.04	0.08 ± 0.03
Day 21	0.08 ± 0.04	0.11 ± 0.04	0.09 ± 0.04	0.08 ± 0.04	0.05 ± 0.03	0.09 ± 0.03
Week 13	0.02 ± 0.01	0.04 ± 0.03	0.11 ± 0.03*	0.05 ± 0.02	0.11 ± 0.04	0.10 ± 0.03
Mean cell volume (fL)						
Day 3	59.4 ± 0.4	59.6 ± 0.2	59.2 ± 0.3	59.1 ± 0.3	59.3 ± 0.3	59.7 ± 0.3
Day 21	60.9 ± 0.3	60.6 ± 0.2	61.0 ± 0.2	59.7 ± 0.2*	60.2 ± 0.2	60.2 ± 0.2
Week 13	55.2 ± 0.2	55.5 ± 0.1*	55.7 ± 0.2*	55.7 ± 0.1**	56.7 ± 0.1**	59.5 ± 0.1**
Mean cell hemoglobin (pg)						
Day 3	21.7 ± 0.2	21.9 ± 0.2	21.5 ± 0.1	21.4 ± 0.2	21.6 ± 0.1	21.7 ± 0.1
Day 21	22.0 ± 0.1	22.0 ± 0.1	22.0 ± 0.1	21.9 ± 0.2	21.4 ± 0.1**	21.2 ± 0.2**
Week 13	19.9 ± 0.1	20.0 ± 0.1	19.9 ± 0.1	20.0 ± 0.1	20.0 ± 0.1	20.7 ± 0.1**
Mean cell hemoglobin concentration (g/dL)						
Day 3	36.5 ± 0.3	36.8 ± 0.2	36.4 ± 0.2	36.2 ± 0.2	36.5 ± 0.1	36.3 ± 0.2
Day 21	36.2 ± 0.2	36.3 ± 0.2	36.1 ± 0.2	36.7 ± 0.2	35.5 ± 0.2	35.2 ± 0.2*
Week 13	36.1 ± 0.2	35.9 ± 0.1	35.7 ± 0.2	35.9 ± 0.1	35.3 ± 0.1**	34.7 ± 0.2**
Platelets (10^3/μL)						
Day 3	927.9 ± 18.3	920.2 ± 39.6	918.5 ± 21.7	910.2 ± 31.6	866.0 ± 20.2	896.7 ± 14.1
Day 21	803.3 ± 16.1	775.8 ± 16.2	836.7 ± 15.5	771.4 ± 23.0	683.3 ± 25.0**	686.8 ± 19.3**
Week 13	801.4 ± 66.8	801.4 ± 45.4	741.3 ± 34.4	697.9 ± 23.3	687.9 ± 28.4*	641.0 ± 10.9**

TABLE B1
Hematology and Clinical Chemistry Data for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Female (continued)						
Hematology (continued)						
n						
Day 3	10	10	10	10	10	10
Day 21	10	10	9	10	10	9
Week 13	10	10	10	10	10	10
Leukocytes (10^3/μL)						
Day 3	10.09 ± 0.40	10.12 ± 0.41	9.87 ± 0.68	10.86 ± 0.22	10.77 ± 0.32	10.86 ± 0.30
Day 21	9.82 ± 0.52	9.69 ± 0.46	11.29 ± 0.39	10.58 ± 0.33	10.67 ± 0.38	9.84 ± 0.43
Week 13	9.52 ± 0.77	10.08 ± 1.05	10.14 ± 0.39	9.17 ± 0.54	9.67 ± 0.50	8.48 ± 0.45
Segmented neutrophils (10^3/μL)						
Day 3	0.91 ± 0.14	0.73 ± 0.09	1.00 ± 0.19	1.12 ± 0.12	1.34 ± 0.14	1.26 ± 0.08
Day 21	1.47 ± 0.19	1.03 ± 0.16	1.38 ± 0.17	1.22 ± 0.19	1.46 ± 0.13	1.33 ± 0.14
Week 13	1.46 ± 0.14	2.24 ± 0.55	1.70 ± 0.19	1.54 ± 0.20	1.56 ± 0.19	1.14 ± 0.10
Lymphocytes (10^3/μL)						
Day 3	9.09 ± 0.31	9.24 ± 0.35	8.60 ± 0.62	9.40 ± 0.21	9.19 ± 0.31	9.39 ± 0.29
Day 21	8.09 ± 0.47	8.41 ± 0.31	9.66 ± 0.33	9.13 ± 0.31	9.04 ± 0.31	8.31 ± 0.32
Week 13	7.67 ± 0.62	7.67 ± 0.50	8.24 ± 0.28	7.43 ± 0.48	8.04 ± 0.42	7.19 ± 0.37
Monocytes (10^3/μL)						
Day 3	0.16 ± 0.06	0.19 ± 0.05	0.16 ± 0.05	0.29 ± 0.05	0.27 ± 0.06	0.20 ± 0.05
Day 21	0.14 ± 0.03	0.21 ± 0.08	0.21 ± 0.07	0.20 ± 0.05	0.19 ± 0.05	0.21 ± 0.06
Week 13	0.26 ± 0.06	0.17 ± 0.05	0.14 ± 0.04	0.16 ± 0.03	0.05 ± 0.02*	0.14 ± 0.03
Eosinophils (10^3/μL)						
Day 3	0.04 ± 0.02	0.04 ± 0.02	0.12 ± 0.06	0.09 ± 0.02	0.03 ± 0.02	0.01 ± 0.01
Day 21	0.15 ± 0.05	0.11 ± 0.04	0.07 ± 0.03	0.06 ± 0.02	0.01 ± 0.01**	0.04 ± 0.03*
Week 13	0.14 ± 0.05	0.01 ± 0.01*	0.06 ± 0.03	0.04 ± 0.02	0.03 ± 0.02*	0.01 ± 0.01*
Clinical Chemistry						
n						
Day 3	10	10	9	10	10	10
Day 21	10	10	9	10	10	9
Week 13	10	10	10	10	10	10
Urea nitrogen (mg/dL)						
Day 3	21.1 ± 0.9	19.5 ± 0.7	20.0 ± 0.7	20.2 ± 0.8	18.7 ± 0.8	18.1 ± 0.8
Day 21	20.4 ± 0.5	21.5 ± 0.5	21.9 ± 0.9	20.6 ± 0.7	19.3 ± 0.8	19.8 ± 1.0
Week 13	19.4 ± 0.6	19.5 ± 0.7	20.0 ± 0.8	20.8 ± 0.8	18.9 ± 0.9	20.0 ± 0.8
Creatinine (mg/dL)						
Day 3	0.56 ± 0.02	0.54 ± 0.02	0.58 ± 0.02	0.54 ± 0.02	0.57 ± 0.02	0.57 ± 0.02
Day 21	0.59 ± 0.01	0.61 ± 0.01	0.62 ± 0.02	0.60 ± 0.02	0.60 ± 0.02	0.60 ± 0.00
Week 13	0.79 ± 0.02	0.79 ± 0.02	0.79 ± 0.02	0.76 ± 0.02	0.78 ± 0.01	0.79 ± 0.02
Total protein (g/dL)						
Day 3	5.5 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	5.7 ± 0.1	5.6 ± 0.1
Day 21	6.1 ± 0.1	6.3 ± 0.1*	6.3 ± 0.1*	6.3 ± 0.1*	6.4 ± 0.1*	6.7 ± 0.1**
Week 13	7.0 ± 0.1	6.9 ± 0.3	7.1 ± 0.1	7.2 ± 0.1	7.3 ± 0.1*	7.3 ± 0.1*
Albumin (g/dL)						
Day 3	4.2 ± 0.1	4.1 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.3 ± 0.0	4.2 ± 0.1
Day 21	4.6 ± 0.1	4.8 ± 0.1*	4.8 ± 0.1*	4.7 ± 0.1	5.0 ± 0.1**	5.1 ± 0.1**
Week 13	4.9 ± 0.1	4.8 ± 0.2	5.1 ± 0.1	5.1 ± 0.1	5.3 ± 0.1*	5.5 ± 0.1**

TABLE B1
Hematology and Clinical Chemistry Data for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Female (continued)						
Clinical Chemistry (continued)						
n						
Day 3	10	10	9	10	10	10
Day 21	10	10	9	10	10	9
Week 13	10	10	10	10	10	10
Alanine aminotransferase (IU/L)						
Day 3	47 ± 3	51 ± 3	45 ± 3	45 ± 2	45 ± 3	43 ± 3
Day 21	48 ± 1	48 ± 1	45 ± 1	45 ± 1	43 ± 1**	41 ± 2**
Week 13	54 ± 7	50 ± 5	49 ± 3	45 ± 5	39 ± 2	31 ± 1**
Alkaline phosphatase (IU/L)						
Day 3	672 ± 13	680 ± 21	686 ± 19	710 ± 14	712 ± 20	652 ± 20
Day 21	402 ± 9[b]	419 ± 8	408 ± 10	430 ± 11	429 ± 7	349 ± 8
Week 13	253 ± 10	256 ± 14	283 ± 5*	270 ± 8	264 ± 10	256 ± 5
Sorbitol dehydrogenase (IU/L)						
Day 3	19 ± 1	22 ± 1	21 ± 2	20 ± 1	22 ± 1	18 ± 2
Day 21	17 ± 1	18 ± 1	21 ± 2	21 ± 1*	23 ± 2**	32 ± 2**
Week 13	22 ± 2	23 ± 2	25 ± 2	24 ± 2	24 ± 2	27 ± 2
Bile acids (µmol/L)						
Day 3	43.0 ± 4.5	50.0 ± 9.8	42.4 ± 4.6	40.0 ± 4.1	40.1 ± 5.0	47.0 ± 5.1
Day 21	36.2 ± 6.0	33.6 ± 2.7	31.0 ± 3.3	35.5 ± 4.5	38.7 ± 4.3	50.5 ± 4.4
Week 13	36.2 ± 5.4	43.8 ± 9.2	28.7 ± 3.3	27.6 ± 4.2	39.5 ± 6.4	34.0 ± 3.0
Thyroid-stimulating hormone (ng/mL)						
Day 21	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.2	1.7 ± 0.2**	1.3 ± 0.2*
Week 13	1.0 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.4 ± 0.2
Total triiodothyronine (ng/dL)						
Day 21	117 ± 6	110 ± 9	85 ± 6**	92 ± 6*	111 ± 5	89 ± 4*
Week 13	131 ± 5	112 ± 5*	107 ± 6**	102 ± 5**	101 ± 5**	99 ± 6**
Total thyroxine (µg/dL)						
Day 21	3.2 ± 0.2	2.3 ± 0.2*	0.8 ± 0.1**	0.6 ± 0.1**	0.7 ± 0.1**	0.2 ± 0.1**
Week 13	2.3 ± 0.2	1.7 ± 0.2*	0.5 ± 0.1**	0.2 ± 0.0**	0.1 ± 0.0**	0.0 ± 0.0**

* Significantly different (P 0.05) from the vehicle control group by Dunn's or Shirley's test
** P 0.01
Trend is significantly increased (P 0.01) by Jonckheere's test.
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n = 9
[c] n = 8

TABLE B2
Clinical Chemistry Data for Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
n	10	10	10	10	10	10
Male						
Urea nitrogen (mg/dL)	27.5 ± 1.9	25.4 ± 1.3	28.7 ± 1.4	25.6 ± 1.0	26.2 ± 1.7	26.6 ± 1.5
Creatinine (mg/dL)	0.42 ± 0.02	0.40 ± 0.02	0.47 ± 0.02	0.39 ± 0.02	0.43 ± 0.02	0.40 ± 0.03
Total protein (g/dL)	5.5 ± 0.2	5.6 ± 0.1	5.5 ± 0.1	5.7 ± 0.1	5.6 ± 0.1	5.5 ± 0.1
Albumin (g/dL)	3.8 ± 0.1	3.9 ± 0.1	4.0 ± 0.1	4.1 ± 0.1*	4.1 ± 0.1	4.0 ± 0.0
Alanine aminotransferase (IU/L)	29 ± 3[b]	30 ± 2	43 ± 9	30 ± 5	30 ± 4	29 ± 2
Alkaline phosphatase (IU/L)	82 ± 3	84 ± 2	83 ± 2	83 ± 2	86 ± 5	83 ± 2
Sorbitol dehydrogenase (IU/L)	49 ± 2	47 ± 1	49 ± 2	47 ± 1	50 ± 2	52 ± 1
Bile acids (µmol/L)	16.8 ± 0.8	18.0 ± 0.6	20.0 ± 0.7*	18.3 ± 0.8	19.5 ± 1.0	18.6 ± 0.6
Female						
Urea nitrogen (mg/dL)	18.2 ± 1.1	19.5 ± 1.1	17.9 ± 1.1	19.2 ± 0.9	18.3 ± 1.0	16.5 ± 0.7
Creatinine (mg/dL)	0.41 ± 0.01	0.39 ± 0.02	0.40 ± 0.02	0.45 ± 0.02	0.42 ± 0.03	0.39 ± 0.02
Total protein (g/dL)	5.3 ± 0.1	5.6 ± 0.0*	5.5 ± 0.1	5.5 ± 0.1	5.4 ± 0.0	5.4 ± 0.1
Albumin (g/dL)	4.1 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	4.3 ± 0.0	4.3 ± 0.0**	4.3 ± 0.0**
Alanine aminotransferase (IU/L)	27 ± 2	28 ± 3	24 ± 2	26 ± 3	33 ± 5	24 ± 2
Alkaline phosphatase (IU/L)	137 ± 4	121 ± 5	125 ± 6	135 ± 4[b]	118 ± 10	141 ± 11
Sorbitol dehydrogenase (IU/L)	37 ± 2	39 ± 2	37 ± 1	38 ± 2	38 ± 3	38 ± 1
Bile acids (µmol/L)	17.2 ± 0.9	16.3 ± 0.4	17.4 ± 0.8	18.2 ± 1.1	18.3 ± 0.6	17.9 ± 0.7

* Significantly different (P 0.05) from the vehicle control group by Dunn's test
** Significantly different (P 0.01) from the vehicle control group by Shirley's test
Trend is significantly increased (P 0.01) by Jonckheere's test.
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n = 9

APPENDIX C
ORGAN WEIGHTS
AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE C1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 16-Day Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	12.5 mg/kg	32 mg/kg	80 mg/kg	200 mg/kg	500 mg/kg
Male						
n	5	5	5	5	5	5
Necropsy body wt	196 ± 8	191 ± 7	186 ± 9	195 ± 6	190 ± 5	198 ± 3
Heart						
Absolute	0.738 ± 0.020	0.717 ± 0.019	0.707 ± 0.029	0.757 ± 0.023	0.745 ± 0.035	0.734 ± 0.011
Relative	3.80 ± 0.20	3.76 ± 0.08	3.82 ± 0.09	3.88 ± 0.05	3.92 ± 0.09	3.72 ± 0.03
R. Kidney						
Absolute	0.867 ± 0.029	0.890 ± 0.045	0.873 ± 0.045	0.991 ± 0.031	0.959 ± 0.040	0.994 ± 0.031
Relative	4.44 ± 0.09	4.65 ± 0.09	4.70 ± 0.07	5.09 ± 0.11**	5.04 ± 0.11**	5.03 ± 0.13**
Liver						
Absolute	9.970 ± 0.198	10.828 ± 0.777	10.723 ± 0.507	11.751 ± 0.484*	11.950 ± 0.459**	12.462 ± 0.282**
Relative	51.13 ± 1.24	56.39 ± 1.86*	57.79 ± 0.49**	60.20 ± 1.07**	62.97 ± 1.70**	63.07 ± 1.19**
Lung						
Absolute	1.140 ± 0.038	1.145 ± 0.082	1.395 ± 0.072*	1.365 ± 0.076*	1.368 ± 0.081*	1.420 ± 0.076*
Relative	5.84 ± 0.09	5.97 ± 0.23	7.52 ± 0.10**	7.02 ± 0.41**	7.21 ± 0.38**	7.19 ± 0.40**
Spleen						
Absolute	0.586 ± 0.011	0.593 ± 0.027	0.607 ± 0.024	0.656 ± 0.034	0.645 ± 0.023	0.662 ± 0.016*
Relative	3.01 ± 0.11	3.11 ± 0.13	3.28 ± 0.07	3.36 ± 0.10*	3.40 ± 0.11*	3.35 ± 0.05*
R. Testis						
Absolute	1.145 ± 0.066	1.113 ± 0.022	1.008 ± 0.093	1.197 ± 0.020	1.125 ± 0.061	1.176 ± 0.006
Relative	5.84 ± 0.17	5.85 ± 0.22	5.39 ± 0.27	6.16 ± 0.20	5.91 ± 0.18	5.95 ± 0.05
Thymus						
Absolute	0.518 ± 0.015	0.396 ± 0.012**	0.353 ± 0.008**	0.351 ± 0.011**	0.329 ± 0.006**	0.345 ± 0.005**
Relative	2.67 ± 0.14	2.08 ± 0.08**	1.92 ± 0.12**	1.80 ± 0.05**	1.74 ± 0.05**	1.74 ± 0.03**

TABLE C1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 16-Day Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	12.5 mg/kg	32 mg/kg	80 mg/kg	200 mg/kg	500 mg/kg
Female						
n	5	5	5	5	5	5
Necropsy body wt	137 ± 4	132 ± 2	131 ± 2	130 ± 2	131 ± 4	134 ± 2
Heart						
Absolute	0.508 ± 0.015	0.526 ± 0.010	0.530 ± 0.015	0.519 ± 0.010	0.522 ± 0.017	0.549 ± 0.013
Relative	3.70 ± 0.06	4.00 ± 0.06*	4.05 ± 0.12*	3.98 ± 0.06*	3.98 ± 0.05*	4.10 ± 0.09**
R. Kidney						
Absolute	0.635 ± 0.012	0.648 ± 0.006	0.646 ± 0.013	0.649 ± 0.019	0.665 ± 0.025	0.680 ± 0.013
Relative	4.63 ± 0.09	4.93 ± 0.07*	4.94 ± 0.11*	4.98 ± 0.08*	5.07 ± 0.12**	5.08 ± 0.03**
Liver						
Absolute	6.370 ± 0.224	6.651 ± 0.121	6.958 ± 0.148	7.217 ± 0.175*	7.783 ± 0.374**	8.043 ± 0.241**
Relative	46.40 ± 1.12	50.61 ± 1.10*	53.18 ± 0.91**	55.39 ± 1.05**	59.21 ± 1.54**	59.99 ± 1.01**
Lung						
Absolute	0.987 ± 0.055	0.870 ± 0.023	0.940 ± 0.014	0.963 ± 0.021	1.103 ± 0.079	1.159 ± 0.096*[b]
Relative	7.20 ± 0.41	6.61 ± 0.16	7.19 ± 0.12	7.40 ± 0.26	8.41 ± 0.57	8.66 ± 0.78*[b]
Spleen						
Absolute	0.414 ± 0.011	0.397 ± 0.011	0.436 ± 0.008	0.432 ± 0.013	0.445 ± 0.017	0.458 ± 0.013*
Relative	3.02 ± 0.05	3.02 ± 0.08	3.34 ± 0.08*	3.32 ± 0.14*	3.39 ± 0.07**	3.42 ± 0.08**
Thymus						
Absolute	0.380 ± 0.007	0.307 ± 0.007**	0.279 ± 0.006**	0.259 ± 0.014**	0.271 ± 0.012**	0.222 ± 0.013**
Relative	2.77 ± 0.05	2.34 ± 0.08**	2.13 ± 0.06**	1.99 ± 0.13**	2.07 ± 0.11**	1.66 ± 0.10**
Uterus						
Absolute	0.395 ± 0.066	0.332 ± 0.060	0.393 ± 0.072	0.271 ± 0.023	0.283 ± 0.025	0.334 ± 0.039
Relative	2.87 ± 0.46	2.54 ± 0.47	3.02 ± 0.57	2.07 ± 0.15	2.14 ± 0.15	2.51 ± 0.32

* Significantly different (P 0.05) from the vehicle control group by Williams' test
** P 0.01
Trend is significantly increased (P 0.01) by Jonckheere's test.
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] n = 4

TABLE C2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Male						
n	10	10	10	10	10	10
Necropsy body wt	347 ± 7	345 ± 7	355 ± 7	354 ± 8	346 ± 6	315 ± 5**
Heart						
Absolute	1.047 ± 0.018	1.029 ± 0.026	1.053 ± 0.023	1.059 ± 0.016	1.062 ± 0.025	1.142 ± 0.018**
Relative	3.02 ± 0.03	2.99 ± 0.04	2.97 ± 0.04	3.00 ± 0.06	3.07 ± 0.04	3.62 ± 0.04**
R. Kidney						
Absolute	1.264 ± 0.034	1.253 ± 0.043	1.304 ± 0.027	1.293 ± 0.022	1.411 ± 0.037*	1.325 ± 0.030
Relative	3.64 ± 0.04	3.63 ± 0.07	3.68 ± 0.05	3.65 ± 0.03	4.08 ± 0.07**	4.20 ± 0.06**
Liver						
Absolute	12.756 ± 0.293	12.660 ± 0.482	13.760 ± 0.377	14.045 ± 0.407*	14.948 ± 0.350**	15.221 ± 0.466**
Relative	36.77 ± 0.26	36.65 ± 0.77	38.82 ± 0.75*	39.62 ± 0.57**	43.19 ± 0.44**	48.17 ± 0.87**
Lung						
Absolute	1.593 ± 0.044	1.572 ± 0.035	1.764 ± 0.081	1.769 ± 0.112	1.681 ± 0.068	1.725 ± 0.056
Relative	4.59 ± 0.09	4.57 ± 0.10	4.97 ± 0.20	4.98 ± 0.26	4.85 ± 0.13	5.46 ± 0.12**
Spleen						
Absolute	0.700 ± 0.015	0.728 ± 0.024	0.749 ± 0.021	0.757 ± 0.025	0.808 ± 0.027**	0.807 ± 0.016**
Relative	2.02 ± 0.03	2.11 ± 0.05	2.12 ± 0.06	2.14 ± 0.05	2.33 ± 0.05**	2.56 ± 0.04**
R. Testis						
Absolute	1.456 ± 0.044	1.443 ± 0.031	1.462 ± 0.025	1.493 ± 0.025	1.504 ± 0.035	1.459 ± 0.025
Relative	4.20 ± 0.08	4.19 ± 0.07	4.13 ± 0.10	4.23 ± 0.09	4.36 ± 0.11	4.63 ± 0.07**
Thymus						
Absolute	0.348 ± 0.021	0.344 ± 0.012	0.362 ± 0.016	0.325 ± 0.028	0.288 ± 0.009*	0.221 ± 0.014**
Relative	1.00 ± 0.04	1.00 ± 0.05	1.02 ± 0.05	0.92 ± 0.07	0.83 ± 0.02*	0.70 ± 0.04**

TABLE C2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Female						
n	10	10	10	10	10	10
Necropsy body wt	196 ± 3	200 ± 3	192 ± 3	191 ± 2	189 ± 3	182 ± 4**
Heart						
Absolute	0.684 ± 0.015	0.684 ± 0.012	0.689 ± 0.012	0.676 ± 0.011	0.696 ± 0.011	0.710 ± 0.024
Relative	3.49 ± 0.05	3.42 ± 0.05	3.60 ± 0.06	3.54 ± 0.05	3.68 ± 0.04*	3.90 ± 0.07**
R. Kidney						
Absolute	0.710 ± 0.012	0.739 ± 0.019	0.705 ± 0.018	0.717 ± 0.012	0.747 ± 0.012	0.743 ± 0.022
Relative	3.62 ± 0.03	3.69 ± 0.07	3.68 ± 0.06	3.75 ± 0.04	3.95 ± 0.03**	4.08 ± 0.05**
Liver						
Absolute	7.050 ± 0.163	7.238 ± 0.252	6.885 ± 0.149	7.119 ± 0.183	7.643 ± 0.160*	8.177 ± 0.215**
Relative	36.01 ± 0.71	36.10 ± 1.05	35.92 ± 0.60	37.21 ± 0.74	40.39 ± 0.62**	44.97 ± 0.63**
Lung						
Absolute	1.198 ± 0.027	1.304 ± 0.045	1.210 ± 0.036	1.165 ± 0.032	1.288 ± 0.049	1.187 ± 0.023
Relative	6.12 ± 0.12	6.52 ± 0.22	6.31 ± 0.15	6.09 ± 0.16	6.80 ± 0.22*	6.55 ± 0.15
Spleen						
Absolute	0.472 ± 0.010	0.528 ± 0.012*	0.503 ± 0.008	0.491 ± 0.013	0.507 ± 0.015	0.529 ± 0.016*
Relative	2.41 ± 0.05	2.64 ± 0.06*	2.62 ± 0.03	2.57 ± 0.06	2.68 ± 0.07**	2.92 ± 0.11**
Thymus						
Absolute	0.291 ± 0.015	0.284 ± 0.014	0.255 ± 0.014	0.251 ± 0.016	0.229 ± 0.016**	0.163 ± 0.015**
Relative	1.49 ± 0.09	1.42 ± 0.07	1.33 ± 0.07	1.31 ± 0.08	1.21 ± 0.07*	0.90 ± 0.08**
Uterus						
Absolute	0.563 ± 0.041	0.645 ± 0.045	0.532 ± 0.025	0.640 ± 0.055	0.660 ± 0.058	0.628 ± 0.043
Relative	2.88 ± 0.21	3.22 ± 0.21	2.78 ± 0.14	3.35 ± 0.29	3.48 ± 0.30	3.46 ± 0.22

* Significantly different (P 0.05) from the vehicle control group by Williams' or Dunnett's test
** P 0.01
Trend is significantly increased (P 0.01) by Jonckheere's test.
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE C3
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 16-Day Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	1 mg/kg	3.2 mg/kg	10 mg/kg	32 mg/kg	100 mg/kg
Male						
n	5	5	5	5	5	5
Necropsy body wt	26.3 ± 0.3	25.8 ± 0.6	26.3 ± 0.4	26.4 ± 0.7	26.4 ± 0.6	26.6 ± 1.0
Heart						
Absolute	0.130 ± 0.002	0.132 ± 0.004	0.138 ± 0.003	0.136 ± 0.004	0.142 ± 0.005*	0.144 ± 0.006*
Relative	4.92 ± 0.05	5.13 ± 0.10	5.23 ± 0.15	5.17 ± 0.04	5.37 ± 0.06**	5.43 ± 0.13**
R. Kidney						
Absolute	0.278 ± 0.015	0.264 ± 0.002	0.269 ± 0.007	0.278 ± 0.014	0.286 ± 0.008	0.286 ± 0.017
Relative	10.56 ± 0.54	10.26 ± 0.17	10.24 ± 0.30	10.50 ± 0.27	10.81 ± 0.16	10.73 ± 0.23
Liver						
Absolute	1.627 ± 0.043	1.659 ± 0.055	1.691 ± 0.036	1.808 ± 0.054*	1.893 ± 0.030**	2.034 ± 0.066**
Relative	61.85 ± 1.68	64.38 ± 1.32	64.25 ± 0.79	68.55 ± 0.91**	71.70 ± 1.37**	76.45 ± 0.91**
Lung						
Absolute	0.169 ± 0.006	0.170 ± 0.006	0.176 ± 0.009	0.168 ± 0.008	0.176 ± 0.003	0.172 ± 0.007
Relative	6.42 ± 0.29	6.62 ± 0.22	6.69 ± 0.26	6.36 ± 0.16	6.67 ± 0.15	6.45 ± 0.16
Spleen						
Absolute	0.066 ± 0.002	0.064 ± 0.002	0.067 ± 0.003	0.066 ± 0.002	0.065 ± 0.003	0.074 ± 0.001
Relative	2.51 ± 0.07	2.47 ± 0.03	2.54 ± 0.12	2.51 ± 0.06	2.45 ± 0.08	2.80 ± 0.12
R. Testis						
Absolute	0.107 ± 0.002	0.104 ± 0.002	0.107 ± 0.005	0.103 ± 0.005	0.109 ± 0.003	0.101 ± 0.005
Relative	4.07 ± 0.06	4.03 ± 0.06	4.08 ± 0.20	3.88 ± 0.08	4.12 ± 0.09	3.81 ± 0.15
Thymus						
Absolute	0.059 ± 0.003	0.052 ± 0.004	0.053 ± 0.002	0.040 ± 0.005**	0.040 ± 0.002**	0.035 ± 0.001**
Relative	2.25 ± 0.15	2.04 ± 0.20	2.00 ± 0.05	1.52 ± 0.19**	1.51 ± 0.06**	1.31 ± 0.04**

TABLE C3
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 16-Day Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	1 mg/kg	3.2 mg/kg	10 mg/kg	32 mg/kg	100 mg/kg
Female						
n	5	5	5	5	5	5
Necropsy body wt	20.1 ± 0.3	18.7 ± 0.6	20.2 ± 0.4	19.5 ± 0.3	19.7 ± 0.4	20.3 ± 0.4
Heart						
Absolute	0.106 ± 0.002	0.100 ± 0.003	0.108 ± 0.002	0.107 ± 0.004	0.107 ± 0.004	0.113 ± 0.003
Relative	5.27 ± 0.05	5.34 ± 0.13	5.38 ± 0.08	5.48 ± 0.13	5.42 ± 0.08	5.57 ± 0.11
R. Kidney						
Absolute	0.162 ± 0.005	0.163 ± 0.006	0.170 ± 0.006	0.163 ± 0.006	0.178 ± 0.011	0.184 ± 0.007
Relative	8.07 ± 0.22	8.68 ± 0.10	8.41 ± 0.16	8.37 ± 0.21	9.01 ± 0.35*	9.09 ± 0.26*
Liver						
Absolute	1.208 ± 0.036	1.132 ± 0.025	1.259 ± 0.020	1.261 ± 0.038	1.337 ± 0.065*	1.594 ± 0.038**
Relative	60.17 ± 1.42	60.65 ± 1.93	62.51 ± 0.88	64.75 ± 1.41*	67.81 ± 1.93**	78.60 ± 0.99**
Lung						
Absolute	0.146 ± 0.002	0.140 ± 0.006	0.146 ± 0.003	0.151 ± 0.004	0.158 ± 0.006	0.156 ± 0.005
Relative	7.28 ± 0.04	7.48 ± 0.18	7.22 ± 0.08	7.79 ± 0.24	8.04 ± 0.20*	7.71 ± 0.15
Spleen						
Absolute	0.071 ± 0.002	0.067 ± 0.002	0.072 ± 0.003	0.069 ± 0.004	0.069 ± 0.003	0.082 ± 0.002
Relative	3.55 ± 0.08	3.56 ± 0.06	3.59 ± 0.11	3.54 ± 0.20	3.49 ± 0.12	4.03 ± 0.09*
Thymus						
Absolute	0.078 ± 0.005	0.062 ± 0.004*	0.070 ± 0.005	0.059 ± 0.002**	0.051 ± 0.005**	0.052 ± 0.002**
Relative	3.87 ± 0.20	3.32 ± 0.18	3.47 ± 0.19	3.06 ± 0.13**	2.59 ± 0.20**	2.57 ± 0.10**
Uterus						
Absolute	0.107 ± 0.011	0.121 ± 0.021	0.096 ± 0.008	0.108 ± 0.013	0.118 ± 0.028	0.100 ± 0.012
Relative	5.36 ± 0.61	6.48 ± 1.12	4.78 ± 0.43	5.57 ± 0.69	6.07 ± 1.50	4.89 ± 0.55

* Significantly different (P 0.05) from the vehicle control group by Williams' or Dunnett's test
** Significantly different (P 0.01) from the vehicle control group by Williams' test
Trend is significantly increased (P 0.01) by Jonckheere's test.
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE C4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Male						
n	10	10	10	10	10	10
Necropsy body wt	37.3 ± 1.1	35.3 ± 0.7	37.4 ± 1.2	37.2 ± 1.3	38.1 ± 1.1	37.7 ± 1.6
Heart						
Absolute	0.163 ± 0.006	0.151 ± 0.005	0.162 ± 0.003	0.161 ± 0.005	0.174 ± 0.003	0.174 ± 0.004
Relative	4.40 ± 0.22	4.28 ± 0.10	4.36 ± 0.15	4.37 ± 0.16	4.60 ± 0.14	4.67 ± 0.16
R. Kidney						
Absolute	0.304 ± 0.010 [b]	0.303 ± 0.009	0.309 ± 0.008	0.326 ± 0.010	0.328 ± 0.009	0.325 ± 0.005
Relative	8.09 ± 0.28[b]	8.61 ± 0.26	8.31 ± 0.24	8.85 ± 0.38	8.64 ± 0.20	8.71 ± 0.28
Liver						
Absolute	1.661 ± 0.052	1.609 ± 0.053	1.715 ± 0.058	1.830 ± 0.064	1.929 ± 0.075*[b]	2.038 ± 0.104**
Relative	44.64 ± 1.15	45.50 ± 0.86	46.15 ± 1.75	49.62 ± 2.17*	50.77 ± 1.90**[b]	53.91 ± 0.73**
Lung						
Absolute	0.239 ± 0.014	0.250 ± 0.012	0.261 ± 0.016	0.236 ± 0.014	0.236 ± 0.018	0.236 ± 0.012
Relative	6.44 ± 0.39	7.10 ± 0.35	7.04 ± 0.49	6.37 ± 0.33	6.23 ± 0.49	6.35 ± 0.42
Spleen						
Absolute	0.075 ± 0.002	0.071 ± 0.002	0.076 ± 0.003	0.076 ± 0.004	0.083 ± 0.002*	0.087 ± 0.002**
Relative	2.02 ± 0.07	2.03 ± 0.05	2.06 ± 0.09	2.04 ± 0.10	2.19 ± 0.06	2.32 ± 0.08**
R. Testis						
Absolute	0.125 ± 0.002	0.118 ± 0.002	0.122 ± 0.004	0.122 ± 0.003	0.125 ± 0.004	0.118 ± 0.002
Relative	3.38 ± 0.09	3.34 ± 0.08	3.29 ± 0.11	3.31 ± 0.13	3.30 ± 0.09	3.18 ± 0.12
Thymus						
Absolute	0.043 ± 0.003	0.043 ± 0.004	0.042 ± 0.003[b]	0.034 ± 0.002	0.037 ± 0.003[b]	0.029 ± 0.002**
Relative	1.15 ± 0.08	1.22 ± 0.11	1.13 ± 0.09[b]	0.92 ± 0.05	0.96 ± 0.07[b]	0.77 ± 0.06**

TABLE C4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Female						
n	10	10	10	10	10	10
Necropsy body wt	26.0 ± 0.7	27.8 ± 0.6	28.6 ± 0.7*	26.4 ± 0.5	26.4 ± 0.9	26.2 ± 0.5
Heart						
Absolute	0.121 ± 0.003	0.124 ± 0.002	0.124 ± 0.003	0.122 ± 0.003	0.123 ± 0.002	0.136 ± 0.003**
Relative	4.66 ± 0.07	4.48 ± 0.14	4.37 ± 0.14	4.62 ± 0.07	4.71 ± 0.13	5.18 ± 0.07**
R. Kidney						
Absolute	0.176 ± 0.005	0.176 ± 0.003	0.174 ± 0.003	0.172 ± 0.005	0.181 ± 0.004	0.183 ± 0.005
Relative	6.80 ± 0.12	6.34 ± 0.11	6.13 ± 0.20*	6.50 ± 0.15	6.92 ± 0.18	6.98 ± 0.10
Liver						
Absolute	1.083 ± 0.034	1.139 ± 0.030	1.198 ± 0.024	1.168 ± 0.037	1.275 ± 0.048**	1.380 ± 0.041**
Relative	41.79 ± 0.97	41.03 ± 0.67	42.03 ± 0.88	44.17 ± 0.87	48.44 ± 1.41**	52.67 ± 0.97**
Lung						
Absolute	0.204 ± 0.008	0.187 ± 0.005	0.177 ± 0.006*	0.186 ± 0.007	0.201 ± 0.007	0.195 ± 0.006
Relative	7.89 ± 0.30	6.74 ± 0.19*	6.24 ± 0.26**	7.05 ± 0.26	7.69 ± 0.36	7.45 ± 0.18
Spleen						
Absolute	0.083 ± 0.004	0.081 ± 0.002	0.092 ± 0.003	0.081 ± 0.002	0.094 ± 0.003*	0.106 ± 0.003**
Relative	3.17 ± 0.08	2.94 ± 0.10	3.22 ± 0.12	3.07 ± 0.05	3.56 ± 0.09**	4.06 ± 0.13**
Thymus						
Absolute	0.042 ± 0.004	0.046 ± 0.004	0.042 ± 0.002	0.037 ± 0.002	0.039 ± 0.003	0.037 ± 0.002
Relative	1.62 ± 0.12	1.66 ± 0.10	1.48 ± 0.10	1.41 ± 0.07	1.47 ± 0.13	1.41 ± 0.07
Uterus						
Absolute	0.128 ± 0.013	0.144 ± 0.016	0.144 ± 0.011	0.127 ± 0.013	0.130 ± 0.014	0.170 ± 0.011
Relative	4.88 ± 0.42	5.18 ± 0.55	5.06 ± 0.40	4.79 ± 0.45	5.02 ± 0.59	6.48 ± 0.40

* Significantly different (P 0.05) from the vehicle control group by Williams' or Dunnett's test
** P 0.01
Trend is significantly increased (P 0.01) by Jonckheere's test.
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] n = 9

APPENDIX D
REPRODUCTIVE TISSUE EVALUATIONS AND ESTROUS CYCLE CHARACTERIZATION

TABLE D1
Summary of Reproductive Tissue Evaluations for Male Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	3 mg/kg	10 mg/kg	30 mg/kg
n	10	10	10	9
Weights (g)				
Necropsy body wt	347 ± 7	354 ± 8	346 ± 6	313 ± 5**
L. cauda epididymis	0.1645 ± 0.0090	0.1785 ± 0.0030	0.1820 ± 0.0069	0.1872 ± 0.0070
L. epididymis	0.4704 ± 0.0077	0.4993 ± 0.0089*	0.4874 ± 0.0089	0.4598 ± 0.0068
L. testis	1.4742 ± 0.0355	1.5255 ± 0.0178	1.5651 ± 0.0331	1.4936 ± 0.0242
Spermatid measurements				
Spermatid heads (10^7/g testis)	9.54 ± 0.37	9.02 ± 0.44	9.00 ± 0.19	9.63 ± 0.27
Spermatid heads (10^7/testis)	13.96 ± 0.30	13.72 ± 0.57	14.07 ± 0.37	14.35 ± 0.31
Spermatid count (mean/10^{-4} mL suspension)	69.78 ± 1.48	68.58 ± 2.83	70.33 ± 1.85	71.75 ± 1.55
Epididymal spermatozoal measurements				
Motility (%)	78.78 ± 3.30	84.23 ± 1.28	81.62 ± 0.93	81.32 ± 1.97
Concentration (10^6/g cauda epididymal tissue)	412 ± 32	469 ± 100	433 ± 29	402 ± 26

* Significantly different (P 0.05) from the vehicle control group by Dunnett's test
** Significantly different (P 0.01) from the vehicle control group by Williams' test
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Data are presented as mean ± standard error. Differences from the vehicle control group for spermatid parameters and epididymal spermatozoal measurements are not significant by Dunn's test.

TABLE D2
Estrous Cycle Characterization for Female Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	3 mg/kg	10 mg/kg	30 mg/kg
n	10	10	10	10
Necropsy body wt (g)	196 ± 3	191 ± 2	189 ± 3	182 ± 4**
Estrous cycle length (days)	4.95 ± 0.05	5.05 ± 0.05	5.15 ± 0.11	5.05 ± 0.05
Estrous stages (% of cycle)				
Diestrus	35.8	42.5	40.8	41.7
Proestrus	15.0	12.5	20.0	15.8
Estrus	29.2	25.8	20.0	21.7
Metestrus	20.0	19.2	19.2	20.8

** Significantly different (P 0.01) from the vehicle control group by Williams' test
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Weights and estrous cycle lengths are presented as mean ± standard error. Differences from the vehicle control group for estrous cycle lengths are not significant by Dunn's test. By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages.

TABLE D3
Summary of Reproductive Tissue Evaluations for Male Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	3 mg/kg	10 mg/kg	30 mg/kg
n	8	6	9	10
Weights (g)				
Necropsy body wt	37.7 ± 1.3	38.5 ± 1.3	38.6 ± 1.1	37.7 ± 1.6
L. cauda epididymis	0.0218 ± 0.0019	0.0218 ± 0.0020	0.0210 ± 0.0010	0.0212 ± 0.0006
L. epididymis	0.0585 ± 0.0020	0.0650 ± 0.0041	0.0588 ± 0.0024	0.0547 ± 0.0015
L. testis	0.1201 ± 0.0016	0.1138 ± 0.0030	0.1187 ± 0.0040	0.1159 ± 0.0024
Spermatid measurements				
Spermatid heads (10^7/g testis)	19.48 ± 0.54	19.22 ± 0.70	20.02 ± 0.74	19.69 ± 0.39
Spermatid heads (10^7/testis)	2.34 ± 0.05	2.18 ± 0.05	2.36 ± 0.05	2.28 ± 0.04
Spermatid count (mean/10^{-4} mL suspension)	73.00 ± 1.47	68.08 ± 1.44	73.64 ± 1.47	71.18 ± 1.34
Epididymal spermatozoal measurements				
Motility (%)	79.07 ± 2.17	78.65 ± 1.76[b]	81.54 ± 1.07[c]	80.56 ± 1.12[d]
Concentration (10^6/g cauda epididymal tissue)	514 ± 83	219 ± 24*[b]	336 ± 77[c]	223 ± 33**[d]

* Significantly different (P 0.05) from the vehicle control group by Shirley's test
** P 0.01
Trend is significantly decreased (P 0.01) by Jonckheere's test.
[a] Data are presented as mean ± standard error. Differences from the vehicle control group are not significant by Dunn's test (spermatid parameters, epididymal spermatozoal motility) or Dunnett's test (weights).
[b] n = 4
[c] n = 7
[d] n = 9

TABLE D4
Estrous Cycle Characterization for Female Mice in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene[a]

	Vehicle Control	3 mg/kg	10 mg/kg	30 mg/kg
n	10	10	10	10
Necropsy body wt (g)	26.0 ± 0.7	26.4 ± 0.5	26.4 ± 0.8	26.2 ± 0.5
Estrous cycle length (days)	4.75 ± 0.62[b]	4.45 ± 0.19	4.85 ± 0.46	4.05 ± 0.05
Estrous stages (% of cycle)				
Diestrus	45.8	31.7	41.7	25.8
Proestrus	15.8	13.3	13.3	15.8
Estrus	23.3	35.8	28.3	35.0
Metestrus	15.0	19.2	16.7	23.3

[a] Weights and estrous cycle lengths are presented as mean ± standard error. Differences from the vehicle control group for estrous cycle lengths are not significant by Dunn's test. By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages.
[b] Estrous cycle was longer than 12 days or was unclear in 2 of 10 animals.

APPENDIX E
HEPATIC CYTOCHROME P_{450} RESULTS

TABLE E1
Summary of Hepatic Cytochrome P_{450}1A Staining Presence and Intensity in Rats in the 13-Week Gavage Study of 3,3′,4,4′-Tetrachloroazobenzene

Parameter	Vehicle Control	0.1 mg/kg	1 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Male						
Day 21						
Number examined	10	10	9	9	10	10
Number with staining	0	0	0	0	0	0
Week 13						
Number examined	10	10	10	10	10	10
Number with staining	0	0	0	0	2 (0.2)[a]	10** (1.3)
Female						
Day 21						
Number examined	10	10	9	10	10	9
Number with staining	0	0	0	0	0	3 (0.3)
Week 13						
Number examined	10	10	10	10	10	10
Number with staining	0	0	0	0	9** (0.9)	10** (1.2)

** Significantly different (P 0.01) from the vehicle control group by the Fisher exact test
[a] Intensity scale: 1 = minimal, 2 = mild

APPENDIX F
GENETIC TOXICOLOGY

TABLE F1
Mutagenicity of 3,3′,4,4′-Tetrachloroazobenzene in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Revertants/Plate[b]					
		−S9		+30% hamster S9		+30% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
TA100	0	101 ± 5.3	97 ± 3.8	93 ± 4.2	113 ± 3.5	114 ± 11.7	100 ± 3.5
	1		104 ± 4.1		108 ± 5.5		93 ± 4.8
	3		90 ± 3.2		97 ± 5.1		94 ± 5.0
	10		93 ± 4.7		99 ± 5.8		91 ± 6.1
	33		93 ± 3.5		98 ± 4.0		112 ± 6.4
	100	80 ± 5.8	103 ± 3.7[c]	110 ± 5.0[c]	109 ± 4.0[c]	147 ± 9.5[c]	104 ± 2.0[c]
	333	69 ± 2.5		98 ± 6.2[c]		139 ± 16.3[c]	
	1,000	74 ± 0.7		87 ± 4.5[c]		101 ± 7.4[c]	
	3,333	69 ± 3.8[c]		95 ± 2.8[c]		108 ± 6.8[c]	
	10,000	95 ± 10.1[c]		106 ± 1.8[c]		127 ± 11.3[c]	
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control[d]		456 ± 26.4	635 ± 32.7	779 ± 4.5	843 ± 8.1	520 ± 4.4	594 ± 7.8

		−S9	+30% hamster S9	+30% rat S9
TA1535	0	14 ± 2.8	20 ± 6.5	18 ± 2.3
	100	13 ± 1.2	15 ± 1.8	27 ± 2.3
	333	9 ± 1.3	15 ± 3.7	21 ± 2.0
	1,000	13 ± 0.9	20 ± 3.7	21 ± 1.5
	3,333	9 ± 0.6[c]	18 ± 0.3[c]	16 ± 2.0[c]
	10,000	8 ± 0.3[c]	16 ± 1.8[c]	21 ± 0.3[c]
Trial summary		Negative	Negative	Negative
Positive control		538 ± 6.1	396 ± 45.1	52 ± 0.9

		+30% rat S9
TA1537	0	6 ± 0.9
	10	3 ± 0.9
	25	5 ± 0.6
	50	4 ± 1.5
	75	5 ± 1.2[c]
	100	3 ± 0.3[c]
Trial summary		Negative
Positive control		407 ± 9.2

TABLE F1
Mutagenicity of 3,3′,4,4′-Tetrachloroazobenzene in *Salmonella typhimurium*

Strain	Dose (μg/plate)	Revertants/Plate				
		−S9	+30% hamster S9	+30% rat S9		
				Trial 1	Trial 2	Trial 3
TA97	0	89 ± 4.7	150 ± 11.7	200 ± 3.4	131 ± 7.1	153 ± 3.5
	10					144 ± 4.8
	25					172 ± 3.3
	50				248 ± 14.5	217 ± 14.6
	75			268 ± 23.1	268 ± 23.1	304 ± 22.9
	100	81 ± 4.5	186 ± 5.0	368 ± 12.3	206 ± 3.2	290 ± 15.1
	200				120 ± 9.4	
	300				170 ± 13.5	
	333	92 ± 6.4	169 ± 8.2	155 ± 9.1		
	1,000	83 ± 10.3	180 ± 7.7	232 ± 38.9		
	3,333	85 ± 4.7[c]	159 ± 5.7[c]	177 ± 17.0[c]		
	10,000	79 ± 3.5[c]	160 ± 3.9[c]	182 ± 10.6[c]		
Trial summary		Negative	Negative	Equivocal	Positive	Positive
Positive control		253 ± 11.3	815 ± 48.5	520 ± 31.0	410 ± 7.0	458 ± 35.3

		−S9		+30% hamster S9		+30% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
TA98	0	21 ± 2.9	32 ± 2.1	41 ± 4.7	28 ± 3.4	31 ± 2.6	34 ± 4.1
	1		22 ± 2.1		32 ± 2.6		33 ± 2.6
	3		33 ± 2.6		28 ± 3.8		35 ± 3.2
	10		19 ± 3.0		31 ± 3.8		31 ± 4.3
	33		26 ± 3.7		31 ± 3.8		35 ± 3.2
	100	18 ± 3.8[c]	33 ± 5.1[c]	40 ± 1.5[c]	28 ± 3.3[c]	39 ± 2.7[c]	25 ± 3.2[c]
	333	22 ± 0.9[c]		40 ± 1.5[c]		37 ± 2.1[c]	
	1,000	19 ± 0.3[c]		36 ± 2.5[c]		40 ± 3.4[c]	
	3,333	21 ± 2.3[c]		40 ± 3.2[c]		43 ± 1.8[c]	
	10,000	18 ± 2.0[c]		39 ± 1.8[c]		29 ± 3.8[c]	
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		148 ± 24.8	211 ± 7.3	887 ± 3.9	805 ± 16.2	224 ± 5.0	349 ± 10.1

[a] Study was performed at Environmental Health Research and Testing, Inc. The detailed protocol is presented in Mortelmans *et al*. (1986). 0 μg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] Precipitate on plate
[d] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA97 and TA1537), and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE F2
Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Mice Treated with 3,3′,4,4′-Tetrachloroazobenzene by Intraperitoneal Injection[a]

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	P Value[c]
Corn oil[d]		5	0.3 ± 0.2	
Cyclophosphamide[e]	25	5	2.3 ± 0.7	0.001
3,3′,4,4′-Tetrachloroazobenzene	50	5	0.4 ± 0.2	0.386
	100	5	0.6 ± 0.5	0.222
	150	5	0.6 ± 0.3	0.222
	200	5	0.1 ± 0.1	0.778
			P = 0.596[f]	

[a] Study was performed at Integrated Laboratory Systems. The detailed protocol is presented in Shelby *et al*. (1993). PCE = polychromatic erythrocyte.
[b] Mean ± standard error
[c] Pairwise comparison of treated group with solvent control micronuclei frequency; significant at P < 0.006
[d] Solvent control
[e] Positive control
[f] Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test; significant at P 0.025 (ILS, 1990)

TABLE F3
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Mice Following Treatment with 3,3′,4,4′-Tetrachloroazobenzene by Gavage for 13 Weeks[a]

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs[b]	P Value[c]
Male				
Corn oil[d]		5	2.3 ± 0.3	
3,3′,4,4′-Tetrachloroazobenzene	0.1	5	2.9 ± 0.2	0.202
	1	5	2.5 ± 0.2	0.386
	3	5	3.4 ± 0.2	0.072
	10	5	4.5 ± 0.4*	0.004
	30	5	4.6 ± 0.4*	0.003
			P = 0.001[e]	
Female				
Corn oil		5	2.3 ± 0.3	
3,3′,4,4′-Tetrachloroazobenzene	0.1	5	2.5 ± 0.3	0.386
	1	5	2.3 ± 0.3	0.500
	3	5	2.5 ± 0.2	0.386
	10	5	4.0 ± 0.2	0.016
	30	5	3.8 ± 0.3	0.027
			P = 0.005	

* Significantly different (P 0.005) from the control group
[a] Study was performed at Environmental Health Research and Testing, Inc. The detailed protocol is presented in MacGregor *et al*. (1990). NCE = normochromatic erythrocyte.
[b] Mean ± standard error
[c] Pairwise compairison of treated groups with solvent control micronuclei frequency; significant at P < 0.005
[d] Solvent control
[e] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test; significant at P 0.025 (ILS, 1990)