National Toxicology Program
Toxicity Report Series
Number 28

NTP Technical Report
on Toxicity Studies of

Tetrachlorophthalic Anhydride

(CAS No. 117-08-8)

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

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Post Office Box 12233
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NIH Publication 93-3351
January 1993

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

Foreword

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

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

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This NTP report on the toxicity studies of tetrachlorophthalic anhydride is based primarily on 13-week studies that began in May 1987 and ended in September 1987 at EG&G Mason Research Institute, Worcester, MA.

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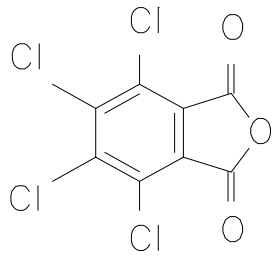
ABSTRACT

Tetrachlorophthalic Anhydride



Molecular Formula	$C_8Cl_4O_3$
CAS Number	117-08-8
Molecular Weight	285.90
Synonyms	4,5,6,7-Tetrachloro-1,3-isobenzofurandione; 1,3-Dioxy-4,5,6,7-tetrachloroisobenzofuran; 3,4,5,6-Tetrachloro-1,2-benzenedicarboxylic anhydride; Niagathal; Tetrathal

Tetrachlorophthalic anhydride (TCPA) is primarily used as a flame retardant in plastics. Toxicology studies were conducted by administering TCPA by oral gavage to F344/N rats and B6C3F$_1$ mice for 13 weeks. Evaluations included histopathology, clinical pathology, and analyses of reproductive system parameters. The genetic toxicity of TCPA was assessed with *in vitro* tests of mutagenicity in *Salmonella typhimurium* and induction of sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells; sister chromatid exchanges and chromosomal aberrations were also determined in mouse bone marrow cells following *in vivo* exposure. The ability of TCPA to induce sex-linked recessive lethal mutations was also studied *in vivo* in *Drosophila melanogaster*.

Groups of 10 rats and 10 mice of each sex received TCPA in corn oil vehicle by oral gavage (5 days/week) at doses of 0, 94, 187, 375, 750, and 1500 mg/kg. The deaths of 5 male rats and 1 female rat in the 1500 mg/kg dose group and 1 female rat in the 750 mg/kg dose group were considered due to chemical toxicity. Mean final body weights and body weight gains were depressed in male rats in the 375, 750, and 1500 mg/kg groups and in all groups of female rats receiving TCPA. Relative liver weights were slightly increased in males and females at doses of 187 mg/kg and higher, although a dose relationship was not apparent. Heart weights of surviving male rats in the high-dose group were also increased. Male and female rats exhibited dose-dependent increases in kidney weights and in the incidence and severity of renal tubule necrosis and/or dilation. No clinical pathology changes were clearly associated with chemical exposure.

There were no chemical-related effects on survival, body weights, or organ weights in dosed mice. No chemical-related lesions were identified in organs examined microscopically. Decreases in red blood cell parameters consistent with a mild, poorly regenerative anemia were the only evidence of possible compound toxicity in dosed mice.

Sperm morphology and vaginal cytology evaluations in rats and mice revealed no adverse changes related to TCPA exposure. In genetic toxicology studies, TCPA, tested with and without exogenous metabolic activation (S9), was not mutagenic in *Salmonella typhimurium* and did not induce sister chromatid exchanges or chromosomal aberrations in Chinese hamster ovary cells. In the *Drosophila melanogaster* sex-linked recessive lethal test, TCPA gave equivocal results when administered by feeding and negative results when administered by injection. No induction of chromosomal aberrations was observed in bone marrow cells of mice 17 hours after intraperitoneal injection of TCPA, although an increase in sister chromatid exchanges was detected in these cells 23 hours after injection.

In summary, clear evidence of organ toxicity following administration of TCPA in corn oil by gavage for 13 weeks was limited to the kidney of rats. The no-observed-adverse-effect level for histopathololgic lesions in this tissue was not achieved with doses as low as 94 mg/kg per day. No significant adverse effects were seen in mice given doses as high as 1500 mg/kg per day for 13 weeks.

PEER REVIEW PANEL

The members of the Peer Review Panel who evaluated the draft report on the toxicity studies of tetrachlorophthalic anhydride on June 24, 1992 are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, panel members determine if the design and conditions of these NTP studies are appropriate and ensure that this toxicity study report presents the experimental results and conclusions fully and clearly.

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SUMMARY OF PEER REVIEW COMMENTS

On June 24, 1992, the Technical Reports Review Subcommittee of the Board of Scientific Counselors for the National Toxicology Program met in Research Triangle Park, NC, to review the draft technical report on the toxicity studies of tetrachlorophthalic anhydride (TCPA).

Dr, J. Mahler, NIEHS, introduced the toxicity studies of tetrachlorophthalic anhydride by reviewing the uses of the chemical, the experimental design, and the results.

Dr. van Zwieten, a principal reviewer, said the report was generally well written and reflected the results obtained. He said the discussion of the organ weight changes could be improved, in particular the discussion of the increases in heart weight in high-dose male rats and kidney weight in female rats. Dr. Mahler said that there were no histopathologic changes in the hearts, and that the relative weight changes likely were secondary to markedly lower body weights. He agreed to include more information in the report.

Dr. Davis, a second principal reviewer, questioned why TCPA was studied, and then, why the gavage route was used. He pointed out the downward trend in the use of TCPA, the limited clear-cut toxicity reported for TCPA in the literature, and inhalation as the apparent potential route for human exposure. Dr. Mahler replied that the nominating agency, the National Cancer Institute, was interested in studying TCPA as a representative aromatic anhydride because of structural similarity to carcinogenic aromatic halides. Regarding the route of exposure, Dr. Mahler noted that preliminary findings from an industry-sponsored inhalation toxicity study did not show evidence of significant systemic exposure, and thus gavage was chosen to provide systemic exposure.

There were no other comments, and the panel agreed to accept the report with the suggested changes.

INTRODUCTION

Physical Properties, Production, Use, and Exposure

Tetrachlorophthalic anhydride (TCPA) is a white, odorless, free-flowing powder with a melting point of 255°C, a boiling point of 371°C, and a vapor pressure of 0.14 x 10^{-7} mm Hg at 20° C. It is nonhygroscopic and only slightly soluble in water (*Condensed Chemical Dictionary*, 1981).

TCPA is used primarily as a flame retardant in plastics (epoxy resins, polyesters, and polyurethanes) that must conform to fire performance standards. The chlorine in the molecule contributes to fire retardancy, although the relatively low chlorine content of TCPA (49% by weight) limits its use to formulations that do not require a high degree of flame retardancy (*Kirk-Othmer*, 1980). It is considered a ``reactive type'' flame retardant, indicating that the anhydride moiety reacts to become chemically bound to the polymer backbone of the plastic. TCPA is also used as an intermediate in the production of some pigments.

TCPA is produced in the United States by only 1 manufacturer, Monsanto Chemical Company (St. Louis, MO). Monsanto has reported a 1981 production of 3 to 4 million pounds of TCPA, approximately 72% of which was exported. This volume represents approximately 59% of the company's 1977 production of 5 to 7 million pounds; this downward trend in production is due to the availability of alternative fire retardants to the plastics industry. Monsanto markets TCPA under the trade name Tetrathal®, a white, flaked solid available in 50-pound bags.

In the workplace, human exposure to TCPA occurs via dust inhalation; a survey found that 2067 workers (787 females) were potentially exposed to TCPA during 1981 through 1983 (NIOSH, 1990). Neither the Occupational Safety and Health Administration (OSHA) nor the American Conference of Governmental Industrial Hygienists, Incorporated (ACGIH) has set exposure limits for this compound. Monsanto has estimated that workers are exposed to 1 to 5 mg/m^3 airborne TCPA and the manufacturer has recommended a

threshold limit value of 12 mg/m^3 (Monsanto, 1972). TCPA dust collected during the manufacturing process is recycled or allocated to landfills. Monsanto has estimated airborne losses to be 0.05 pounds per 1000 pounds of product, with similar amounts lost during customer use operations.

TCPA has been identified by the U.S. Environmental Protection Agency as an air pollutant (CIS, 1979) and it is expected to react photochemically only to a minimal extent (USEPA, 1982). The agency has anticipated that the majority of airborne TCPA would precipitate in the soil in the form of its parent compound and would undergo rapid hydrolysis in the soil environment, minimizing the probability of persistence and bioaccumulation. However, the chlorinated ring structure of tetrachlorophthalic acid is believed to resist degradation, and significant persistence and bioaccumulation of that compound is a possibility. Because TCPA incorporated into plastics is either immobilized within or chemically bonded to a polymer matrix, exposure to the general public from this source is believed to be negligible.

Absorption, Disposition, Metabolism, and Excretion

There are no data available in the literature regarding absorption, distribution, and excretion of TCPA in humans or animals.

The effect of TCPA administration on hepatic microsomal metabolism has been studied in rats and mice (Ridley *et al.*, 1988). Male Sprague-Dawley rats were administered 25, 100, 250, or 500 mg TCPA in corn oil/kg body weight by gavage for 7 days. Following treatment, a dose-dependent reduction in zoxazolamine paralysis time was noted in rats given 100, 250, or 500 mg/kg; hexobarbital sleep time was not affected. In rats in the 500 mg/kg dose group, TCPA also induced statistically significant increases in hepatic aminopyrine N-demethylase and aniline hydroxylase activities and in total cytochrome P_{450}. Male CD-1 mice given 250, 500, or 1000 mg/kg TCPA in corn oil by gavage for 7 days exhibited no changes in zoxazolamine paralysis time or hexobarbital sleep time; microsomal enzyme levels were not measured in mice.

Toxicity

HUMAN TOXICITY

Several cases of occupational asthma attributed to TCPA exposure have been reported in the literature. Schlueter *et al.* (1978) reported that 5 workers involved in the production of epoxy resins developed respiratory symptoms and physiologic abnormalities following the introduction of TCPA into the product formulation. Inhalation challenge with TCPA in 3 of the workers reproduced their symptoms and resulted in both immediate and delayed responses. Immunologic studies did not demonstrate specific antibodies; however, clinical signs suggested a hypersensitivity reaction rather than an irritant effect. The presence of IgE antibody specific to TCPA was later reported in 7 female workers exposed to the chemical via an epoxy resin (Howe *et al.*, 1983); the antibody was identified following skin prick and radioallergosorbent tests. These findings seem to indicate that TCPA causes occupational asthma via an allergic rather than an irritant mechanism. Subsequent reports have further documented the use of immunologic assays to identify workers at risk for allergic lung disease due to acid anhydrides (Grammer *et al.*, 1987; Flaherty *et al.*, 1988).

ANIMAL TOXICITY

In an acute toxicity screen of TCPA, nonfasted albino rats of each sex were given a 33.3% corn oil suspension of TCPA; the acute oral LD_{50} was found to be greater than 15.8 g/kg. The dermal LD_{50} was estimated to be greater than 5 g/kg when albino rabbits received a continuous 24-hour skin application of a 33.3% TCPA suspension. Slight topical irritation was reported when 100 mg of powdered TCPA was applied in the conjunctival sac of the eye of rabbits and when rabbits were exposed continuously for 24 hours to topical applications of a 10% suspension of TCPA in corn oil. No deaths or adverse effects were reported in rats during a 14-day observation period following a 4-hour inhalation exposure to TCPA dust (particle size unspecified) at a concentration of 3.6 mg/L air (USEPA, 1982).

Subchronic inhalation studies with TCPA have been conducted in rats (USEPA, 1982). Groups of 5 male and 5 female rats were exposed to TCPA dust (67% of the particles reportedly ranging in size from 1 to 10 microns) at concentrations of 0.64 and 1.2 mg/L air, or to TCPA fumes at concentrations of 0.04 and 0.13 mg/L air. Animals were exposed

for 6 hours per day, 5 days per week, for 4 weeks, for a total of 20 exposures. Body weight gains of exposed animals were lower than those of controls and serum alanine aminotransferase activity of rats exposed to either concentration of TCPA dust was greater than that of controls. Absolute liver weights of all TCPA-exposed groups were greater than those of controls and corresponded to the liver hypertrophy that was observed microscopically. Histopathologic adverse pulmonary changes were also found in all treatment groups, and lung weights in rats treated with the highest fume concentration were greater than those of controls. Hematologic and urinary parameters were not affected. Groups of 15 male and 15 female Sprague-Dawley rats were exposed for 13 weeks (6 hours per day, 5 days per week) to TCPA fines fumes (1.7 to 2.6 microns in diameter), fines dust (2.7 to 3.4 microns in diameter), or dust (3.4 microns in diameter); target concentrations were 0.5, 5.0, and 50 mg/m^3. There were no effects on survival, body weight gain, or hematology or urinalysis parameters. Exposure-related findings included hepatocellular hypertrophy and increased lung weights associated with multifocal accumulations of alveolar macrophages and alveolar hemorrhages.

REPRODUCTIVE TOXICITY

A teratology study with TCPA was conducted by Monsanto in 1982. Groups of 24 pregnant female Sprague-Dawley rats were administered 250, 1000, or 2000 mg/kg TCPA in corn oil by gavage on days 6 through 19 of gestation; the animals were killed on gestation day 20. Litters from the 2000 mg/kg group showed a slightly increased incidence of skeletal malformations compared to controls. No other evidence of maternal toxicity, embryotoxicity, fetotoxicity, or teratogenic effects was observed (USEPA, 1982).

CARCINOGENICITY

Chronic toxicity/carcinogenicity studies of TCPA have not been conducted. A chronic bioassay of the nonchlorinated analog of TCPA, phthalic anhydride, was performed in F344 rats and B6C3F$_1$ mice (NCI, 1979). Phthalic anhydride administered in feed at concentrations of up to 15,000 ppm for rats, 25,000 ppm for male mice, and 12,500 ppm for female mice did not affect survival or tumor incidence.

GENETIC TOXICITY

Mutagenicity tests indicate that TCPA is not mutagenic. When tested with and without exogenous metabolic activation (S9), TCPA was not mutagenic in *Salmonella typhimurium* strains TA100, TA1535, TA1537 or TA98 (Zeiger *et al.*, 1985) and did not induce sister chromatid exchanges or chromosomal aberrations in Chinese hamster ovary cells (Galloway *et al.*, 1987). In germ cells of adult male *Drosophila melanogaster*, no induction of sex-linked recessive lethal (SLRL) mutations was observed in flies treated by abdominal injection of 500 ppm TCPA in saline (Valencia *et al.*, 1985). However, oral administration of TCPA by feeding (1000 ppm) to adult males produced a slight increase in mutations; these feeding test results were considered equivocal (Valencia *et al.*, 1985).

Study Rationale and Design

TCPA was nominated by the National Cancer Institute for carcinogenicity testing as a representative of the multi-ring anhydride class of chemicals. Supporting factors were the relatively large production volume and structural similarity to carcinogenic aromatic halides such as hexachlorobenzene and 2,4,6-trichlorophenol (NCI, 1979). Acute toxicity data from rat studies supplied by the manufacturer were used in dose setting for the 13-week studies in F344/N rats and B6C3F$_1$ mice described in this report. Gavage was chosen as the route of administration in order to achieve maximal systemic body burdens of the chemical. The studies performed included reproductive system, clinical pathology, and histopathologic evaluations. TCPA was also evaluated for mutagenicity in *S. typhimurium*, induction of sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells and in mouse bone marrow cells, and for induction of sex-linked recessive lethal mutations in *D. melanogaster*.

MATERIALS AND METHODS

Procurement and Characterization of Tetrachlorophthalic Anhydride

Tetrachlorophthalic anhydride (TCPA; CAS number 117-08-8) was obtained in 1 lot (Lot EL2327DL) from Aldrich Chemical Company (Milwaukee, WI). Chemical analyses performed by Midwest Research Institute (Kansas City, MO) identified the chemical, a white powder, as TCPA. Infrared, ultraviolet/visible, and nuclear magnetic resonance spectra were consistent with the structure of TCPA and with the literature reference. Cumulative analytical data, based on elemental analyses, Karl Fischer water analysis, titration, and 2 gas chromatographic systems, indicated a purity of 99%. Elemental analysis results for carbon and chlorine agreed with theoretical values; analysis for hydrogen indicated approximately 0.02%. Karl Fischer water analysis indicated less than 0.8% water in the sample. Differential potentiometric titrations with 0.1N sodium methoxide and 0.1N sodium hydroxide to determine the anhydride content indicated a purity of 99% ± 1%. Two gas chromatographic systems resolved 1 impurity with an area of approximately 0.4% relative to the major peak. The bulk chemical was also analyzed by high-resolution gas chromatography/mass spectroscopy/selected ion monitoring for the presence of 16 chlorinated dibenzodioxins and dibenzofurans. None of the 16 compounds were detected (detection limits less than 1 ppm for each compound monitored).

Stability studies performed at Midwest Research Institute indicated that TCPA was stable as a bulk chemical for 2 weeks at temperatures up to 60°C when stored protected from light. At the study laboratory, the bulk chemical was stored in the dark at 4° ± 3°C. Bulk chemical reanalyses performed by the study laboratory with infrared spectroscopy, gas chromatography, and ultraviolet extinction analysis showed consistent purity levels throughout the studies.

Dose Formulations

Because studies indicated that TCPA is unstable when mixed with rodent feed, gavage was selected as the route of administration. Corn oil was selected as the vehicle because of the limited solubility of TCPA in water. Dose formulations were prepared weekly by mixing TCPA in corn oil (w/w). Before mixing, TCPA was ground and sieved (USS No. 100), then weighed and added to a beaker containing the required amount of corn oil; the corn oil was magnetically stirred during the addition. The resulting suspension was aliquoted into labeled dosing vials containing magnetic stir bars. Formulations were stored at approximately 4° ± 3°C and were discarded 7 days after the date of preparation.

Results of one 21-day stability study of 2 mg/mL TCPA in corn oil indicated significant losses at ambient temperature and at 5°C. Samples stored in sealed bottles in the dark at room temperature lost approximately 10% TCPA after 7 days and 53% after 21 days. At 5°C, losses were approximately 4% of the chemical after 7 days and approximately 16% after 21 days. No significant loss of chemical was seen in a 3-hour stability test in which samples were open to air and light.

A second 21-day stability study of TCPA in corn oil demonstrated that 2 mg/mL solutions were unstable at -20°C as well as at 5°C. The losses of chemical stored sealed in the dark at 5°C were approximately 15% after 7 days and 40% after 21 days, and solutions stored at -20°C exhibited losses of approximately 13% after 7 days and 34% after 21 days. However, suspensions of TCPA in corn oil at a higher concentration, 20 mg/mL, showed no significant loss of chemical after 7 days of storage at 5°C or after 14 days of storage at -20°C. Losses after 21 days of storage at 5°C and -20°C were approximately 4%.

The study laboratory evaluated the homogeneity of the dose formulations. Samples from the highest and lowest concentrations of the TCPA dose formulations were taken from the top, middle, and bottom of a dosing container under simulated animal room conditions; the coefficient of variation for both dose levels was under 5%.

Analyses of the TCPA dose formulations were conducted at the study laboratory using high-performance liquid chromatography. Samples of all concentrations were taken from

the initial, middle, and final mixes of the studies for analysis. Residual dosing mixtures (animal room samples) were also submitted from each interval. Results of all analyses were within 10% of theoretical values, with the exception of 1 animal room sample (the 78.6 mg/g sample submitted at the midpoint of the study) which was 11.2% higher than the theoretical value. Results of referee analyses performed by the analytical chemistry laboratory at the start of the studies and once during dosing agreed with the results from the study laboratory.

The Mazola® corn oil (Best Foods, Union, NJ) that was used as the vehicle in these gavage studies was analyzed monthly for peroxide level using the Official Method Cd 8-53 of the American Oil Chemists Society. All peroxide levels were within acceptable limits.

Toxicity Study Designs

Male and female F344/N rats and B6C3F$_1$ mice used in these studies were obtained from Simonsen Laboratories (Gilroy, CA). Rats and mice were shipped to the study laboratory at approximately 3 to 4 weeks of age, quarantined for 12 to 16 days, and placed on study at approximately 5 to 6 weeks of age. Blood samples for determination of viral and/or bacterial antibody titers were collected 3 times for rats and once for mice during the studies. Rat sera were screened for viral antibody titers at the beginning of the study, at 4 weeks, and at the end of the study (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b). Sera taken at the start of the study from 6 female rats tested positive for RCV/SDA (rat corona virus/sialodacryoadenitis virus); sera from 8 female rats showed positive results when tested 4 weeks into the study for RCV/SDA, as did sera taken at the end of the study from 5 male and 5 female rats. Sera obtained at the end of the study from 4 male and 4 female mice tested negative in the antibody screenings. Additional details concerning study design and performance are listed in Table 1.

Groups of 10 rats and 10 mice of each sex were administered TCPA in corn oil by gavage at doses of 0, 94, 187, 375, 750, and 1500 mg/kg 5 days per week, excluding weekends and holidays, for 13 weeks. Fourteen-day studies were not performed because of the reported high acute LD$_{50}$ of TCPA. Doses selected for the studies were based in part on information supplied by the manufacturer. Rats were housed 5 per cage, and mice were

housed in individual cages. City water (Worcester, MA) and NIH-07 Open Formula mash diet (Zeigler Brothers, Gardners, PA) were available *ad libitum*. Animal rooms were maintained at 67° to 77°F and 30% to 70% relative humidity, with at least 10 room air changes per hour and 12 hours of fluorescent light daily.

Complete necropsies were performed on all animals. The brain, heart, right kidney, liver, lung, spleen, right testis, and thymus were weighed prior to fixation. Organs and tissues were examined for gross lesions and fixed in 10% neutral buffered formalin. Tissues to be examined microscopically were trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed on all control animals, on all animals in the highest dose group with at least 60% survivors, and on all animals, including those that died or were killed moribund before the end of the studies, in the higher dose groups. Target tissues were identified; these tissues, as well as gross lesions, were examined in all animals from the lower dose groups until a no-effect level was determined. In rats, the target organ was the kidney, which was evaluated for males and females at all dose levels. In mice, no target organs were identified by microscopic examination. All tissues examined microscopically are listed in Table 1.

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

SUPPLEMENTAL EVALUATIONS

Clinical Pathology

Clinical pathology studies were performed on rats and mice in the studies. Clinical chemistry evaluations were conducted for male and female rats in the 0, 94, 375, and 1500 mg/kg groups on days 6 and 20 and at the end of the study. Hematology

parameters were evaluated for rats and mice in all dose groups at the end of the study. At all time points, rats and mice were anesthetized with CO_2 and bled from the retro-orbital sinus. Blood for hematology was collected in containers with EDTA as the anticoagulant. Details are presented in Table 1.

Sperm Morphology and Vaginal Cytology Evaluations

Sperm morphology evaluations were performed on male rats from the 0, 94, 375, and 750 mg/kg dose groups. Vaginal cytology evaluations were performed on female rats from the 0, 94, 375, and 1500 mg/kg dose groups. Sperm morphology and vaginal cytology evaluations were performed on mice from the 0, 94, 375, and 1500 mg/kg dose groups. Methods were those described by Morrissey *et al.* (1988). Briefly, for the 7 days prior to sacrifice, the vaginal vaults of the females of each species and dose group were lavaged, and the aspirated lavage fluid and cells were stained with Toluidine Blue. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and were used to ascertain estrous cycle stage (*i.e.*, diestrus, proestrus, estrus, or metestrus).

Sperm morphology was evaluated at necropsy in the following manner. The right epididymis was 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 Tyrode's buffer (mice) was applied to slides, and a small incision was made at the distal border of the epididymal tail. 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 5 fields per slide.

Following completion of sperm motility estimates, each right cauda epididymis was placed in buffered 0.9% saline solution. Cauda were gently 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.

TABLE 1 Experimental Design and Materials and Methods in 13-Week Gavage Studies of Tetrachlorophthalic Anhydride

EXPERIMENTAL DESIGN

Study Laboratory	EG&G Mason Research Institute, Worcester, MA	
Size of Study Groups	10 males and 10 females of each species per dose group	
Route of Administration	Gavage	
Dose Volume	Rats:	5 mL/kg body weight
	Mice:	10 mL/kg body weight
Concentration of Dose Formulations	Rats:	0, 20.3, 40.1, 78.6, 151.2, or 280.9 mg/g in corn oil
	Mice:	0, 10.2, 20.3, 40.1, 78.6, or 151.2 mg/g in corn oil
Doses/Duration of Dosing	Rats:	0, 94, 187, 375, 750, or 1500 mg/kg 5 days per week for 13 weeks
	Mice:	0, 94, 187, 375, 750, or 1500 mg/kg 5 days per week for 13 weeks
Date of First Dose	Rats:	Males, 3 June 1987 or 4 June 1987
		Females, 5 June 1987 or 6 June 1987
	Mice:	Males, 26 May 1987
		Females, 27 May 1987
Date of Last Dose	Rats:	Males, 1 September 1987 or 2 September 1987
		Females, 3 September 1987 or 4 September 1987
	Mice:	Males, 25 August 1987 or 26 August 1987
		Females 27 August 1987 or 28 August 1987
Necropsy Date	Rats:	Males, 1 September 1987 or 2 September 1987
		Females, 3 September 1987 or 4 September 1987
	Mice:	Males, 25 August 1987 or 26 August 1987
		Females 27 August 1987 or 28 August 1987
Type and Frequency of Observation	Animals were observed twice daily. Clinical signs of toxicity were recorded weekly. Food consumption was recorded weekly by cage. Individual body weights were recorded at study initiation, weekly thereafter, and at necropsy.	
Necropsy and Histologic Examinations	All animals received a complete necropsy. The protocol required that tissues be examined microscopically in all control animals, all animals that died or were sacrificed in a moribund condition before group sacrifice, all animals in the highest dose group with at least 60% survivors, and all surviving animals in the higher dose groups. In addition to any gross lesions, tissues to be examined were: adrenal glands, bone (femur, sternebrae, or vertebrae with marrow) brain (3 sections), clitoral and preputial glands (rats), esophagus, gallbladder (mice), heart, intestine (large: cecum, colon, rectum; small: duodenum, jejunum, ileum), kidneys, liver, lungs and mainstem bronchi, lymph nodes (mandibular and mesenteric), mammary glands (including surface skin), nasal cavity and turbinates (3 sections), ovaries, pancreas, parathyroid glands, pituitary gland, prostate gland, salivary glands, seminal vesicles, spinal cord and sciatic nerve (if neurologic signs were present), spleen, stomach (forestomach and glandular stomach), testes with epididymis, thymus, thyroid gland, trachea, urinary bladder, and uterus. The kidney was identified as a target organ in rats and was examined microscopically for males and females in all dose groups.	

TABLE 1 Experimental Design and Materials and Methods in 13-Week Gavage Studies of Tetrachlorophthalic Anhydride (continued)

Supplemental Evaluations	Hematology: Hematology evaluations were conducted at the end of the study for rats and mice in all dose groups. The following hematology parameters were measured with a Series 7000 Whole Blood Analyzer (Baker Instruments, Allentown, PA): erythrocyte count (RBC), hematocrit (HCT), hemoglobin concentration (HGB), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), mean cell volume (MCV), and leukocyte count (WBC). Leukocyte differential/morphologic assessment and reticulocyte counts were determined by microscopic examination of blood smears stained with modified Romanowsky and new methylene blue stains, respectively. Platelet counts were performed with a Series 810 Whole Blood Platelet Analyzer (Baker Instruments, Allentown, PA). Clinical Chemistry (rats only): Clinical chemistry studies were conducted on days 6, 20, and at study termination for rats in the 0, 94, 375, and 1500 mg/kg groups. The following clinical chemistry parameters were measured with a Gemini Miniature Centrifugal Analyzer (Electronucleonics Inc., Fairfield, NJ): alanine aminotransferase (ALT), albumin, creatinine, γ-glutamyltransferase (GGT), glucose, and urea nitrogen (UN). Sperm Morphology/Vaginal Cytology: Male rats dosed with 0, 94, 375, or 750 mg/kg TCPA and male mice dosed with 0, 94, 375, or 1500 mg/kg TCPA were evaluated for necropsy body and reproductive tissue weights and spermatozoal data. Female rats and mice dosed with 0, 94, 375, or 1500 mg/kg TCPA were evaluated for necropsy body weight, estrous cycle length, and the percent of cycle spent in the various stages.
ANIMALS AND ANIMAL MAINTENANCE	
Strain and Species	F344/N Rats B6C3F$_1$ Mice
Animal Source	Simonsen Laboratories, Gilroy, CA
Time Held Before Study	Rats: Males, 13 to 14 days Females, 15 to 16 days Mice: Males, 12 days Females, 13 days
Age When Placed on Study	Rats: 6 weeks Mice: 5 weeks
Age When Killed	Rats: 19 to 20 weeks Mice: 18 weeks
Method of Animal Distribution	Animals were weighed and assigned to dose groups using random number tables.
Diet	NIH-07 Open Formula Mash (Zeigler Brothers, Inc., Gardners, PA) available *ad libitum*
Animal Room Environment	Rats were housed 5 per cage and mice were housed in individual cages. Temperature was maintained at 67° to 77°F and relative humidity at 30% to 70%, with at least 10 air changes per hour. Fluorescent light was provided for 12 hours per day.

Genetic Toxicity Studies

SALMONELLA MUTAGENICITY TEST PROTOCOL

Testing was performed as reported by Zeiger *et al.* (1985). TCPA was sent to 2 testing laboratories as a coded aliquot. It was incubated with the *Salmonella typhimurium* tester strains (TA98, TA100, TA1535, and TA1537) either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with *l*-histidine and *d*-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37°C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and of at least 5 doses of TCPA. The high dose was limited by toxicity. All assays were repeated.

CHINESE HAMSTER OVARY CELL CYTOGENETICS PROTOCOLS

Testing was performed as reported by Galloway *et al.* (1987). TCPA was supplied as a coded aliquot. It was tested in cultured Chinese hamster ovary (CHO) cells for induction of sister chromatid exchanges (SCEs) and chromosomal aberrations (Abs) both in the presence and the absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 and cofactor mix. Cultures were handled under gold lights to prevent photolysis of bromodeoxyuridine-substituted DNA. Each test consisted of concurrent solvent and positive controls and of at least 3 doses of TCPA; the high dose was limited to 750 µg/mL. A single flask per dose was used, and trials yielding equivocal or positive results were repeated.

In the SCE test without S9, CHO cells were incubated for 26 hours with TCPA in McCoy's 5A medium supplemented with fet al bovine serum, *l*-glutamine, and antibiotics. Bromodeoxyuridine (BrdU) was added 2 hours after culture initiation. After 26 hours, the medium containing TCPA was removed and replaced with fresh medium plus BrdU and Colcemid, and incubation was continued for 2 hours. Cells were then harvested by mitotic shake-off, fixed, and stained with Hoechst 33258 and Giemsa. In the SCE test with S9,

cells were incubated with TCPA, serum-free medium, and S9 for 2 hours. The medium was then removed and replaced with medium containing serum and BrdU and no TCPA, and incubation proceeded for an additional 26 hours, with Colcemid present for the final 2 hours. Harvesting and staining were the same as for cells treated without S9. All slides were scored blind and those from a single test were read by the same person. Fifty second-division metaphase cells were scored for frequency of SCEs/cell from each dose level.

In the Abs test without S9, cells were incubated in McCoy's 5A medium with TCPA for 12 hours; Colcemid was added and incubation continued for 2 hours. The cells were then harvested by mitotic shake-off, fixed, and stained with Giemsa. For the Abs test with S9, cells were treated with TCPA and S9 for 2 hours, after which the treatment medium was removed and the cells were incubated for 12 hours in fresh medium, with Colcemid present for the final 2 hours. Cells were harvested in the same manner as for the treatment without S9.

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

DROSOPHILA MELANOGASTER TEST PROTOCOL

The assays for induction of sex-linked recessive lethal (SLRL) mutations were performed with adult flies as described by Valencia *et al.* (1985). TCPA was supplied as a coded aliquot. It was assayed in the SLRL test by feeding for 3 days to adult Canton-S wild-type males no more than 24 hours old at the beginning of treatment. Because an equivocal response was obtained, it was retested by injection into adult males.

To administer the chemical by injection, a glass Pasteur pipette was drawn out in a flame to a microfine filament and the tip was broken off to allow delivery of the test solution.

Injection was performed either manually, by attaching a rubber bulb to the other end of the pipette and forcing through sufficient solution (0.2 to 0.3 µL) to slightly distend the abdomen of the fly, or by attaching the pipette to a microinjector, which automatically delivered a calibrated volume. Flies were anaesthetized with ether and immobilized on a strip of tape. Injection into the thorax, under the wing, was performed with the aid of a dissecting microscope.

Toxicity tests were performed to set concentrations of TCPA at a level that would induce 30% mortality after 72 hours of feeding or 24 hours after injection, while keeping induced sterility at an acceptable level. Oral exposure was achieved by allowing Canton-S males to feed for 72 hours on a solution of TCPA in 5% sucrose. In the injection experiments, 24- to 72-hour-old Canton-S males were treated with a solution of TCPA dissolved in saline or peanut oil and were allowed to recover for 24 hours. In the adult exposures, treated males were mated to 3 *Basc* females for 3 days and were given fresh females at 2-day intervals to produce 3 matings of 3, 2, and 2 days (in each case, sample sperm from successive matings were treated at successively earlier postmeiotic stages). F_1 heterozygous females were mated with their siblings and then placed in individual vials. F_1 daughters from the same parental male were kept together to identify clusters. (A cluster occurs when a number of mutants from a given male result from a single spontaneous premeiotic mutation event, and is identified when the number of mutants from that male exceeds the number predicted by a Poisson distribution). A cluster was identified and all data from the male in question were discarded. Presumptive lethal mutations were identified as vials containing fewer than 5% of the expected number of wild-type males after 17 days; these were retested to confirm the response.

MOUSE BONE MARROW CYTOGENETICS PROTOCOLS

Dose range-finding studies were performed in the absence of adequate toxicity information from the literature. The highest dose was limited by toxicity. TCPA was tested for induction of SCEs in mouse bone marrow using the standard harvest time of 23 hours and for induction of Abs in mouse bone marrow using the standard harvest time of 17 hours. Because the frequency of SCEs and Abs in bone marrow cells was not determined at the

extended harvest times of 42 and 36 hours, respectively, the results of these tests are considered to be incomplete.

For the *in vivo* SCE test, 5 male B6C3F$_1$ mice per dose group were injected intraperitoneally with TCPA dissolved in dimethylsulfoxide (injection volume = 0.1 mL). Solvent control mice received equivalent injections of dimethylsulfoxide only. The positive control was dimethylbenzanthracene. The bone marrow cells were collected 23 hours after treatment. The mice were implanted subcutaneously with a BrdU tablet (McFee *et al.*, 1992) 24 hours before harvest (1 hour before TCPA treatment). The use of BrdU allowed selection of the appropriate cell population (cells in the second metaphase following TCPA treatment) for scoring. Two hours before sacrifice, the mice received an intraperitoneal injection of colchicine in saline. The animals were killed by cervical dislocation 23 hours after treatment. One or both femurs were removed and the marrow was flushed out with phosphate-buffered saline (pH 7.0). The cells were treated with a hypotonic salt solution, fixed, and dropped onto chilled slides. After a 24-hour drying period, the slides were stained using fluorescence-plus-Giemsa and scored. Twenty-five second-division metaphase cells were scored from each of 4 animals per treatment. Responses were evaluated as SCEs per cell.

For the Abs test, 10 male B6C3F$_1$ mice per dose group were injected intraperitoneally with TCPA dissolved in dimethylsulfoxide (injection volume = 0.1 mL). Solvent control mice received equivalent injections of dimethylsulfoxide only. The positive control was dimethylbenzanthracene. The mice were subcutaneously implanted with a BrdU tablet (McFee *et al.*, 1992) 18 hours before the scheduled harvest (1 hour before injection with TCPA). The use of BrdU allowed selection of the appropriate cell population for scoring. (Abs induced by chemical administration are present in maximum number at the first metaphase following treatment; they decline in number during subsequent nuclear divisions due to cell death.) Two hours before sacrifice, the mice received an intraperitoneal injection of colchicine in saline. The animals were killed by cervical dislocation 17 hours after TCPA injection (18 hours after BrdU dosing). One or both femurs were removed and the marrow was flushed out with phosphate-buffered saline (pH 7.0). Cells were treated with a hypotonic salt solution, fixed, and dropped onto chilled slides. After a 24-hour drying period, the slides were stained with Giemsa and scored.

Fifty first-division metaphase cells were scored from each of 8 animals per treatment. Responses were evaluated as the percentage of aberrant metaphase cells, excluding gaps.

Statistical Methods

ANALYSIS OF CONTINUOUS VARIABLES

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which are approximately normally distributed, were analyzed using the parametric multiple comparisons procedures of Williams (1971, 1972) or Dunnett (1955). Hematology, clinical chemistry, and urinalysis data, which typically have skewed distributions, were analyzed using the nonparametric multiple comparisons methods of Shirley (1977) or Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of dose-response trends and to determine whether a trend-sensitive test (Williams, Shirley) was more appropriate for pairwise comparisons than a test capable of detecting departures from monotonic dose response (Dunnett, Dunn). If the P-value from Jonckheere's test was greater than or equal to 0.10, Dunn's or Dunnett's test was used rather than Shirley's or Williams' test.

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

ANALYSIS OF VAGINAL CYTOLOGY DATA

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

ANALYSIS OF MUTAGENICITY IN *SALMONELLA TYPHIMURIUM*

A positive response in the *Salmonella typhimurium* assay was defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any 1 strain/activation combination. An equivocal response was defined as an increase in revertants that was not dose related, not reproducible, or not of sufficient magnitude to support a determination of mutagenicity. A negative response was obtained when no increase in revertant colonies was observed following chemical treatment. There was no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

ANALYSIS OF CHINESE HAMSTER OVARY CELL CYTOGENETICS DATA

For the SCE data, statistical analyses were conducted on the slopes of the dose-response curves (Galloway *et al.*, 1987). An SCE frequency 20% above the concurrent solvent control value was chosen as a statistically conservative positive response. The probability of this level of difference occurring by chance at 1 dose point is less than 0.01; the probability for such a chance occurrence at 2 dose points is less than 0.001. An increase of 20% or greater at any single dose in conjunction with a trend P-value less than 0.025 was considered weak evidence of activity; increases at 2 or more doses indicated that the trial was positive. A statistically significant trend ($P<0.05$), in the absence of any responses reaching 20% above background, led to a call of equivocal.

Chromosomal aberration data are presented as percentage of cells with aberrations. Statistical analyses were conducted on both the dose-response curve and individual dose points (Galloway *et al.*, 1987). For a single trial, a statistically significant ($P<0.05$) difference for 1 dose point and a significant trend ($P<0.015$) were considered weak evidence for a positive response; significant differences for 2 or more doses indicated the trial was positive. A positive trend, in the absence of a statistically significant increase at any 1 dose point, led to a conclusion of equivocal activity.

ANALYSIS OF *DROSOPHILA MELANOGASTER* DATA

Sex-linked recessive lethal data were analyzed by simultaneous comparison with the concurrent and historical controls using a normal approximation to the binomial test (Margolin *et al.*, 1983). A test result was considered positive if the P-value was less than

or equal to 0.01 and the mutation frequency in the tested group was greater than 0.10% or if the P-value was less than or equal to 0.05 and the frequency in the treatment group was greater than 0.15%. A test was considered to be inconclusive if the P-value was between 0.05 and 0.01 but the frequency in the treatment group was between 0.10% and 0.15% or if the P-value was between 0.10 and 0.05 but the frequency in the treatment group was greater than 0.10%. A test was considered negative if the P-value was greater than or equal to 0.10 or if the frequency in the treatment group was less than 0.10%.

ANALYSIS OF MOUSE BONE MARROW CYTOGENETICS DATA

The SCE and Abs data were analyzed by a one-tailed trend test (Margolin *et al.*, 1986).

Quality Assurance

The studies of TCPA were performed in compliance with the United States Food and Drug Administration's Good Laboratory Practices regulations (21 CFR 58). The Quality Assurance Unit of EG&G Mason Research Institute performed audits and inspections of protocols, procedures, data, and reports throughout the course of these studies. The operations of the Quality Assurance Unit were monitored by the NTP.

RESULTS

13-Week Gavage Study in F344/N Rats

Seven male and 3 female rats died prior to the end of the study (Table 2). Five male rats in the high-dose (1500 mg/kg) group died during weeks 5 through 8, with death attributed to chemical toxicity. The death of another male in this dose group was considered a gavage accident. One male rat in the 187 mg/kg group died early from unknown causes. Two female rats, 1 each from the 750 and 1500 mg/kg groups, died during weeks 6 through 10, with both deaths considered to be due to chemical toxicity. One female control rat died early as the result of a gavage accident. Mean final body weights and body weight gains of male rats in the 375, 750, and 1500 mg/kg groups and of female rats in all dosed groups were notably less than those of controls (Table 2 and Figure 1).

Rats in all dosed groups had decreased feed consumption compared to that of the controls; in males, these decreases were dose related, and the average feed consumption of the 1500 mg/kg group was 12% lower than that of the controls. There were no compound-related clinical findings of toxicity in male rats. Urine staining in most females in the 750 and 1500 mg/kg groups and diarrhea in all females in the 1500 mg/kg group were attributed to treatment with tetrachlorophthalic anhydride (TCPA).

Changes were observed in some absolute and relative organ weights in dosed males and females (Table 3). Absolute and relative kidney weights were increased in a dose-dependent manner in female rats; in males, statistically significant increases in relative kidney weight were found at doses of 187 mg/kg and greater. An increase in relative heart weights of male rats in the 1500 mg/kg group was attributed to the markedly decreased final body weights of that group relative to controls. Liver weights showed inconsistent changes with increasing dose in each sex, but the trend was toward a mild increase in relative weight. Spleen and thymus weights appeared somewhat decreased with increasing TCPA dose, even though the usual pattern for these organs is toward an increase in relative weight in lighter animals. Complete organ weight data for rats are presented in Appendix A (Table A1).

Clinical chemistry evaluations were performed on days 6 and 20 and at the end of the study for male and female rats in the 0, 94, 375, and 1500 mg/kg groups. Hematology studies were conducted at the end of the study for rats in all dose groups. Data summaries are presented in Appendix B.

TABLE 2 Survival and Weight Gain of F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride

Dose (mg/kg)	Survival[1]	Mean Body Weight (grams)			Final Weight Relative to Vehicle Controls (%)[4]
		Initial[2]	Final	Change[3]	
MALE					
0	10/10	126	338	212	
94	10/10	124	319	195	94
187	9/10[5]	124	328	204	97
375	10/10	123	301	178	89
750	10/10	124	306	181	90
1500	4/10[6]	128	290	167	86
FEMALE					
0	9/10[7]	108	196	85	
94	10/10	108	178	70	91
187	10/10	104	179	75	92
375	10/10	108	175	67	90
750	9/10[8]	108	175	66	90
1500	9/10[9]	108	175	70	90

[1] Number surviving at 13 weeks/number of animals per dose group.
[2] Body weight was measured before the first dose. Subsequent calculations were based on animals surviving to the end of the study.
[3] Mean weight change of the animals in each group.
[4] (Treated group mean/vehicle control group mean) x 100.
[5] Week of death = 12.
[6] Week of death = 5, 5, 5, 5, 8, 11 (gavage accident).
[7] Week of death = 1 (gavage accident).
[8] Week of death = 10.
[9] Week of death = 6.

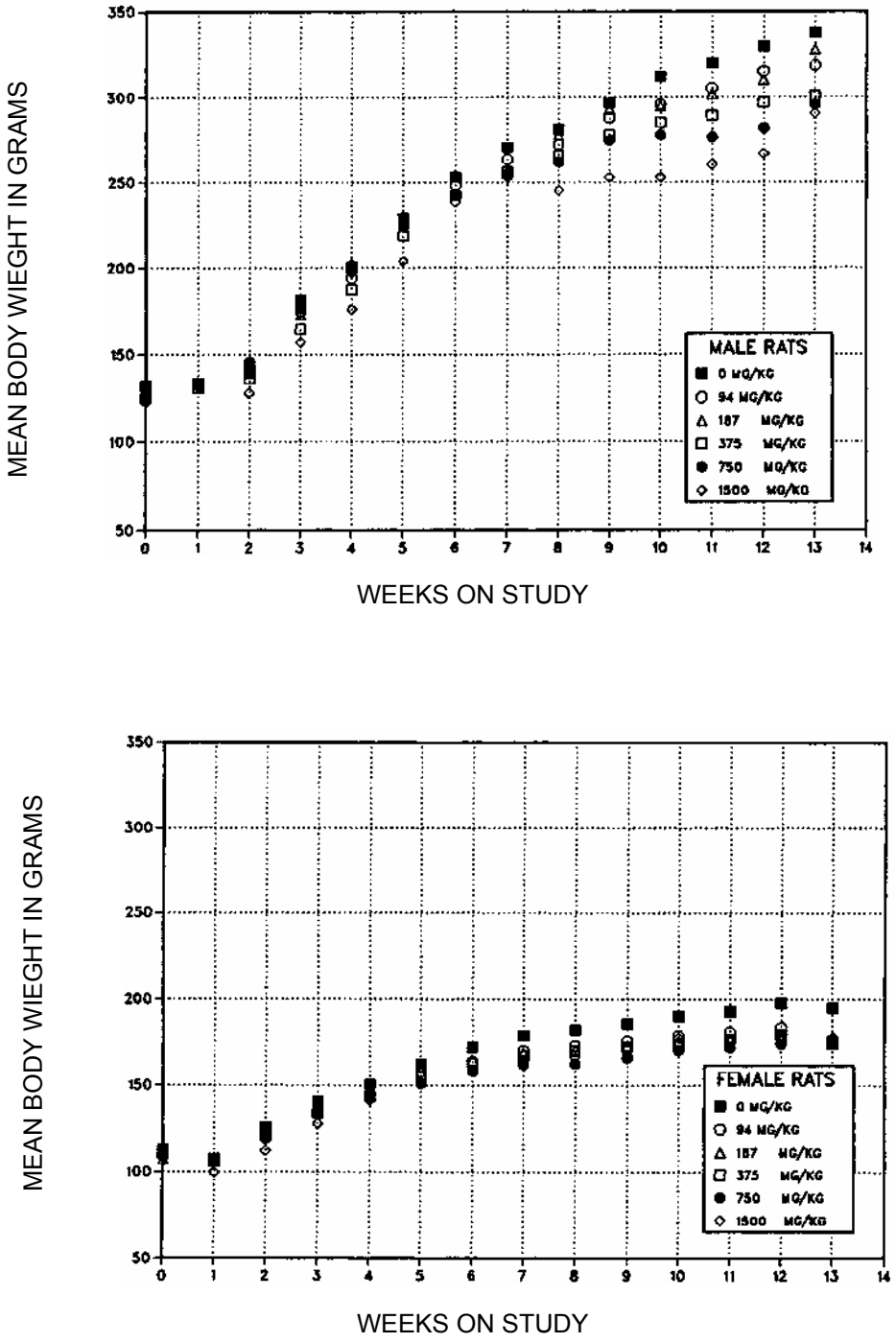

FIGURE 1 Body Weights of F344/N Rats Administered Tetrachlorophthalic Anhydride by Gavage for 13 Weeks

TABLE 3 Selected Organ Weights of F344/N Rats Administered Tetrachlorophthalic Anhydride by Gavage for 13 Weeks[1]

	Dose (mg/kg)					
	0	94	187	375	750	1500
MALE						
n	10	10	9	10	10	4
Necropsy body wt (g)	320 ± 5	301 ± 10	317 ± 10	283 ± 10**	285 ± 8**	247 ± 14**
Heart						
Absolute	1.17 ± 0.06	1.08 ± 0.05[2]	0.97 ± 0.04*[3]	1.08 ± 0.04*	0.88 ± 0.03**	1.22 ± 0.11*
Relative	3.67 ± 0.15	3.58 ± 0.18[2]	2.98 ± 0.13[3]	3.83 ± 0.13	3.09 ± 0.05	5.06 ± 0.76**
Right Kidney						
Absolute	1.149 ± 0.038	1.186 ± 0.042	1.279 ± 0.032	1.142 ± 0.054	1.155 ± 0.055	1.097 ± 0.070
Relative	3.59 ± 0.08	3.96 ± 0.12	4.04 ± 0.08*	4.02 ± 0.10*	4.04 ± 0.11*	4.54 ± 0.57**
Liver						
Absolute	10.87 ± 0.27	10.39 ± 0.44	12.80 ± 0.47**	9.69 ± 0.45	11.11 ± 0.33	9.35 ± 0.30
Relative	33.99 ± 0.38	34.51 ± 0.97	40.38 ± 1.03*	34.13 ± 0.70**	39.02 ± 0.68**	38.17 ± 1.74**
Spleen						
Absolute	0.68 ± 0.02	0.62 ± 0.02*	0.63 ± 0.01*	0.54 ± 0.02**	0.54 ± 0.01**	0.46 ± 0.04**[4]
Relative	2.11 ± 0.04	2.06 ± 0.04	1.99 ± 0.07	1.92 ± 0.03*	1.89 ± 0.03**	1.97 ± 0.28[4]
Thymus						
Absolute	0.36 ± 0.03	0.32 ± 0.03	0.36 ± 0.02	0.31 ± 0.02	0.28 ± 0.02*	0.23 ± 0.04**
Relative	1.12 ± 0.09	1.04 ± 0.07	1.13 ± 0.06	1.11 ± 0.05	0.97 ± 0.06	0.94 ± 0.21
FEMALE						
n	9	10	10	10	8	9
Necropsy body wt (g)	184 ± 3	166 ± 3**	174 ± 4**	164 ± 4**	172 ± 4**	160 ± 3**
Heart						
Absolute	0.70 ± 0.03	0.63 ± 0.05	0.60 ± 0.01	0.63 ± 0.03	0.59 ± 0.02	0.67 ± 0.05
Relative	3.81 ± 0.18	3.79 ± 0.22	3.47 ± 0.06	3.84 ± 0.20	3.47 ± 0.08	4.16 ± 0.30
Right Kidney						
Absolute	0.68 ± 0.02	0.64 ± 0.03	0.72 ± 0.02	0.74 ± 0.03	0.85 ± 0.02**	0.85 ± 0.03**
Relative	3.71 ± 0.08	3.87 ± 0.12	4.16 ± 0.06*	4.50 ± 0.08**	5.07 ± 0.10**	5.36 ± 0.23**
Liver						
Absolute	5.56 ± 0.17	5.13 ± 0.12	6.28 ± 0.21	5.22 ± 0.22	6.45 ± 0.32*	5.41 ± 0.16
Relative	30.26 ± 0.93	30.90 ± 0.46	36.11 ± 0.78*	31.83 ± 0.87*	37.90 ± 1.45**	33.99 ± 1.37**
Thymus						
Absolute	0.24 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.21 ± 0.01	0.24 ± 0.02	0.18 ± 0.01**
Relative	1.30 ± 0.05	1.34 ± 0.07	1.24 ± 0.04	1.27 ± 0.06	1.42 ± 0.09	1.13 ± 0.10

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

[2] n = 9.

[3] n = 8.

[4] n = 3.

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

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

Numbers of neutrophils were increased in female rats in the 750 and 1500 mg/kg groups. Neutrophil numbers were also increased in female rats in the 187 and 375 mg/kg groups and in male rats in the 1500 mg/kg group, but these increases were not statistically significant. Lymphocytes were decreased in male rats in the 375 and 1500 mg/kg groups. Decreases in lymphocytes also occurred in males in the 750 mg/kg group and in females in the 1500 mg/kg group, but the decreases were not statistically significant. These lymphocyte changes were not accompanied by changes in leukocyte counts. Increases in platelet count and γ-glutamyltransferase activity were seen in male rats in the 1500 mg/kg group at the end of the study. The concentration of urea nitrogen was decreased in female rats in the 1500 mg/kg group at all time points, though at the end of the study the decrease was not statistically significant. In male rats, urea nitrogen concentration was decreased at day 20 (1500 mg/kg group) and at the end of the study (94, 375, and 1500 mg/kg groups). Changes in these and other hematology and clinical chemistry parameters in male and female rats were minor and sporadic and were not considered clinically significant (Appendix B).

No gross lesions were associated with TCPA treatment. Treatment-related microscopic lesions were identified in the kidney and consisted of renal tubule degenerative changes, characterized in general by tubule epithelial necrosis at higher dose levels and tubule dilation at lower dose levels (Table 4). Necrosis of the tubule epithelium was seen in the majority of male and female rats in the 1500 mg/kg groups and in most male rats in the 750 mg/kg group. In the 1500 mg/kg group, moderate to marked necrosis was present in rats that died early and involved both cortical and medullary tubules. In these cases, the tubules were filled with casts of granular cell debris or protein. Tubules affected by acute necrosis, in which the lining epithelium was completely denuded, were intermixed with tubules in which attempts at regeneration were evidenced by attenuated epithelium or epithelial cells with increased cytoplasmic basophilia and nuclear/cytoplasmic ratio. Minimal to mild necrosis, characterized by single cell necrosis and sloughing, which was localized primarily to the outer stripe of the outer medulla, was present in high-dose rats that survived to the end of the study and in most males in the 750 mg/kg group. Dilation of tubules was seen in all dosed groups. This finding was also localized to the outer stripe of the outer medulla and was generally mild in degree. In affected tubules, lumens were more open than in controls and typically contained bridging proteinaceous strands. The

lining epithelial cells had a less prominent brush border and reduced height. The incidence and severity of this lesion increased with dose in all but the highest dose group of males, where dilation may have been obscured by associated sloughing of necrotic epithelial cells. Lesions consistent with spontaneous nephropathy of Fischer rats were present in both control and dosed animals; no increases in incidence or severity were associated with TCPA treatment.

Sperm morphology and vaginal cytology evaluations in rats revealed no changes between exposed animals and controls that were considered of biological significance for any of the parameters evaluated (Appendix C).

TABLE 4 Incidence and Severity of Renal Tubule Lesions in F344/N Rats Administered Tetrachlorophthalic Anhydride by Gavage for 13 Weeks[1]

	Dose (mg/kg)					
	0	94	187	375	750	1500
MALE						
Renal tubules						
Necrosis	0/10	0/10	0/10	0/10	7/10 (1.0)	7/7[2] (2.1)
Dilation	0/10	5/10 (1.0)	9/10 (1.0)	10/10 (1.6)	10/10 (2.0)	1/7[2] (2.0)
FEMALE						
Renal tubules						
Necrosis	0/10	0/10	0/10	0/10	0/10	8/10 (1.6)
Dilation	0/10	6/10 (1.0)	6/10 (1.0)	10/10 (1.4)	9/10 (1.8)	10/10 (2.0)

[1] Incidence is the number of animals with lesions from groups of 10. Average severity score () was based on the number of animals with lesions; 1=minimal, 2=mild, 3=moderate, 4=marked.

[2] Autolysis precluded morphologic evaluation in 3 animals in this group.

13-Week Gavage Study in B6C3F$_1$ Mice

One female mouse in the 750 mg/kg group died during Week 2. No other deaths or moribund sacrifices occurred during this study (Table 5). There were no differences in feed consumption between dosed and control mice, and no clinical signs of toxicity were noted. In all dose groups of females and most dose groups of males, mean final body weights and body weight gains were similar to those of the controls (Table 5, Figure 2); mean final body weights and body weight gains of male mice receiving 375 or 750 mg/kg TCPA were notably greater than those of the controls.

TABLE 5 Survival and Weight Gain of B6C3F$_1$ Mice in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride

Dose (mg/kg)	Survival[1]	Mean Body Weight (grams) Initial[2]	Final	Change[3]	Final Weight Relative to Controls (%)[4]
MALE					
0	10/10	21.2	28.3	7.1	
94	10/10	22.1	29.0	6.9	102
187	10/10	21.7	29.5	7.8	104
375	10/10	21.3	30.3	9.0	107
750	10/10	22.0	30.2	8.2	107
1500	10/10	21.5	28.2	6.7	99
FEMALE					
0	10/10	17.7	26.0	8.3	
94	10/10	17.4	25.3	7.9	97
187	10/10	17.9	24.9	7.1	96
375	10/10	17.9	26.3	8.5	101
750	9/10[5]	18.1	26.1	8.0	100
1500	10/10	18.1	25.6	7.5	98

[1] Number surviving at 13 weeks/number of animals per dose group.
[2] Body weight was measured before the first dose. Subsequent calculations were based on animals surviving to the end of the study.
[3] Mean weight change of the animals in each group.
[4] (Treated group mean/control group mean) x 100.
[5] Week of death = 2.

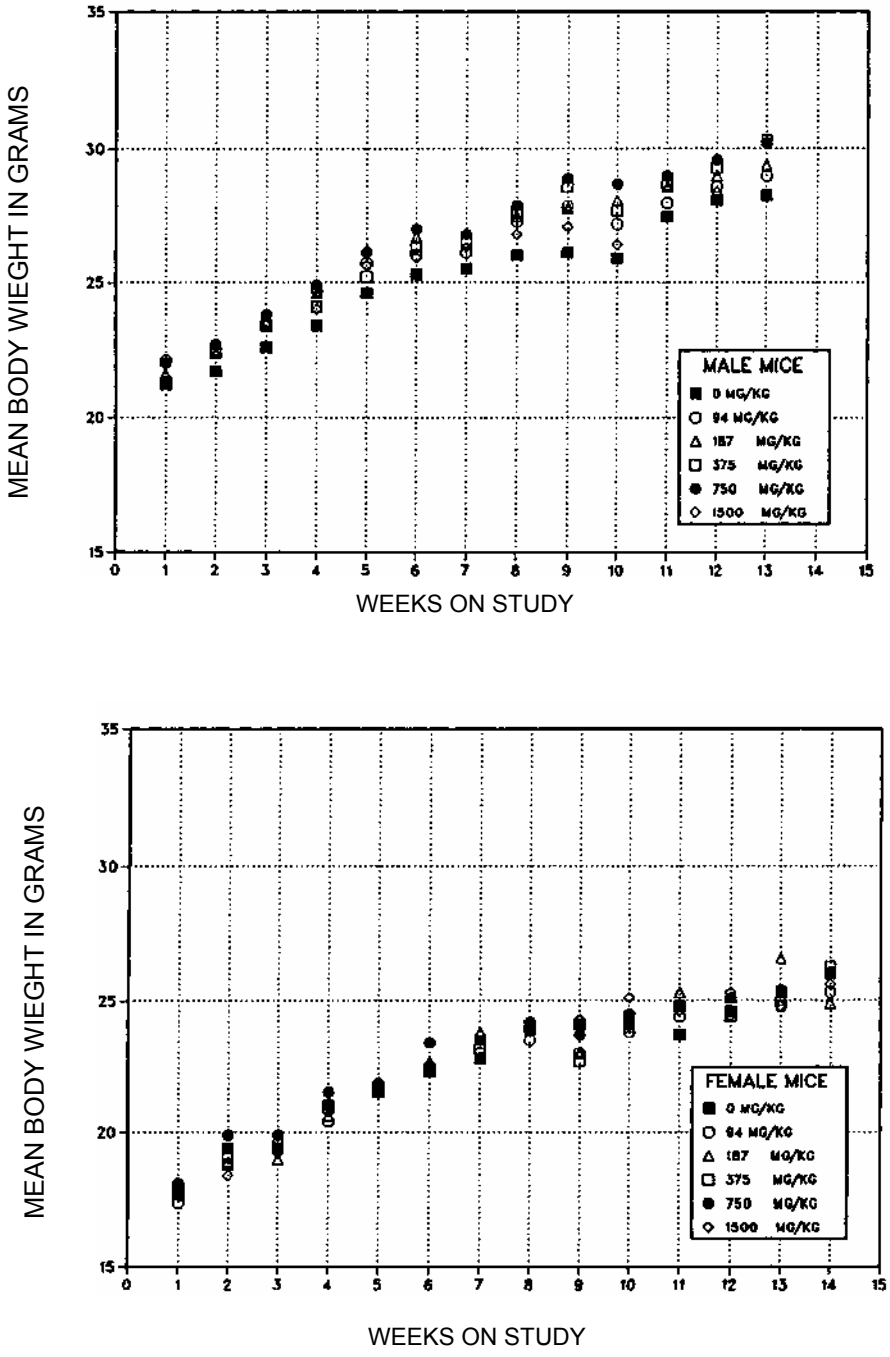

FIGURE 2 Body Weights of B6C3F$_1$ Mice Administered Tetrachlorophthalic Anhydride by Gavage for 13 Weeks

In general, there were no clearly treatment-related changes in organ weights of male or female mice (Table 6). The decreased lung and kidney weights of males in the high-dose (1500 mg/kg) group were of questionable biological significance. Increased heart weights were noted in males receiving doses of 187 or 750 mg/kg, but not in other dose groups. There did not appear to be any biologically significant changes in organ weights of dosed females. Complete organ weight data for mice are presented in Appendix A (Table A2).

TABLE 6 Selected Organ Weights of B6C3F$_1$ Mice Administered Tetrachlorophthalic Anhydride by Gavage for 13 Weeks[1]

	Dose (mg/kg)					
	0	94	187	375	750	1500
MALE						
n	10	10	10	10	10	10
Necropsy body wt	26.8 ± 0.6	27.4 ± 0.5	28.3 ± 0.7	28.0 ± 0.3	28.6 ± 0.3*	26.3 ± 0.4
Heart						
Absolute	0.139 ± 0.004	0.140 ± 0.007	0.169 ± 0.011*	0.141 ± 0.005	0.184 ± 0.010**	0.126 ± 0.003
Relative	5.20 ± 0.14	5.12 ± 0.26	6.00 ± 0.38	5.06 ± 0.22	6.41 ± 0.30**	4.79 ± 0.12
Right Kidney						
Absolute	0.250 ± 0.007	0.253 ± 0.007	0.256 ± 0.006	0.243 ± 0.007	0.248 ± 0.005	0.217 ± 0.006**
Relative	9.35 ± 0.20	9.21 ± 0.17	9.06 ± 0.22	8.68 ± 0.22*	8.68 ± 0.18*	8.27 ± 0.17**
Lung						
Absolute	0.259 ± 0.011	0.255 ± 0.015	0.259 ± 0.013	0.220 ± 0.013	0.244 ± 0.012	0.215 ± 0.008*
Relative	9.69 ± 0.38	9.35 ± 0.62	9.17 ± 0.48	7.84 ± 0.42*	8.53 ± 0.45*	8.17 ± 0.29*

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

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

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

Hematology evaluations were conducted for male and female mice in all dose groups at the end of the study. Data summaries are presented in Appendix B. A dose-related decrease in hemoglobin concentration occurred in males (375, 750, and 1500 mg/kg groups) and females (94, 187, 750, and 1500 mg/kg groups). Decreases in hematocrit and erythrocyte count occurred in high-dose males. Numbers of lymphocytes were decreased in males (187, 375, 750, and 1500 mg/kg groups) and females (94 mg/kg group). In males in the

375 mg/kg group, the change in lymphocyte count was accompanied by a decreased leukocyte count.

No treatment-related gross lesions were noted at necropsy and no treatment-related microscopic lesions were identified in dosed mice.

Sperm morphology evaluations revealed a statistically significant decrease in sperm motility in male mice in the 1500 mg/kg group ($P \leq 0.01$); values were not decreased, however, relative to historical control data. TCPA administration did not affect any other parameters measured in sperm morphology or vaginal cytology evaluations in mice (Appendix C).

Genetic Toxicity Studies

TCPA was tested in 2 laboratories for induction of mutations in *Salmonella typhimurium* strains TA100, TA1535, TA1537, and TA98, with and without Aroclor 1254-induced rat and hamster liver S9; all results were negative (Table D1; Zeiger *et al.*, 1985). TCPA did not induce sister chromatid exchanges (SCEs) (Table D2) or chromosomal aberrations (Abs) (Table D3) in Chinese hamster ovary cells with or without S9 (Galloway *et al.*, 1987). In the SCE test with S9, a single dose was judged positive in the first trial; however, the response could not be reproduced in a subsequent trial, and the SCE test was considered negative. TCPA did not induce sex-linked recessive lethal mutations in germ cells of adult male *Drosophila melanogaster* (Table D4) when administered by injection at a dose of 500 ppm, but when it was administered by feeding (1000 ppm) to adult males, a slight increase in recessive lethal mutations was detected and the response was judged to be equivocal (Valencia *et al.*, 1985).

TCPA was tested for induction of SCEs and Abs in mouse bone marrow cells in vivo. A dose-related increase in SCEs occurred in a single SCE trial with bone marrow sampling performed 23 hours after intraperitoneal injection of TCPA, and results of this trial were considered to be positive (Table D5). No increase in the frequency of Abs was observed in bone marrow cells of male mice sampled 17 hours after injection with TCPA (Table D6).

DISCUSSION

Thirteen-week studies were conducted in rats and mice to characterize the toxicity of tetrachlorophthalic anhydride (TCPA) by oral gavage exposure. In rats, chemical-related changes were observed in the kidney. At equivalent doses, no adverse effects were found in mice.

Treatment-related lesions were present in the kidney of male and female rats. Increases in kidney weights were associated with microscopic changes in the renal tubules ranging from minimal to mild dilation at lower doses to moderate to marked tubule necrosis at the higher doses. Early deaths of rats receiving 1500 mg/kg were attributed to tubule necrosis. The fact that deaths occurred 5 to 8 weeks after initiation of dosing and were associated histologically with acute tubule necrosis admixed with regenerative attempts suggests an ongoing, cumulative toxic effect of the compound. Necrosis was also present in the high-dose rats that survived to study termination as well as in most male rats in the 750 mg/kg group. Tubule necrosis in these animals was much less severe and more localized to the outer stripe of the outer medulla.

Predilection of TCPA nephrotoxicity for the outer stripe of the outer medulla is consistent with the general sensitivity of the proximal tubule to toxic injury, and in particular the P3 segment, which comprises most of the outer stripe (Montgomery and Seely, 1990; Alden and Frith, 1991). Relevant to the current studies, it is noteworthy that organic halides are a well-recognized class of tubule toxins, and TCPA and its derivatives are structurally similar to these compounds. Site specific toxicity of organohalides and other tubule toxicants to the P3 segment has been attributed to the presence of mixed function oxidases in this region of the nephron, resulting in localized formation of reactive metabolites (*e.g.*, chloroform), or the presence of other bioactivating intracellular enzymes within P3 cells (*e.g.*, activation of halogenated alkenes through the β-lyase pathway) (Alden and Frith, 1991). Whether these or other mechanisms are involved in TCPA nephrotoxicity is unknown.

At lower doses of TCPA, the primary histopathologic lesion was tubule dilation within the outer stripe of the outer medulla. In most cases, tubule dilation was not associated with tubule epithelial cell necrosis; however, because it occurred at the same site as tubule necrosis, and occurred simultaneously with tubule necrosis at higher doses, a pathogenetic relationship between the 2 lesions is suggested. It could be speculated that dilation is a sequela to tubule necrosis, since some features of the involved tubules (*e.g.*, reduced height of epithelial cells and poorly defined brush border) (Wallin *et al.*, 1992) were suggestive of postnecrotic regeneration. Dilation may have been due to increased intratubular fluid, perhaps an expression of impaired function (resorption) following sublethal damage to cells of the P3 segment.

Serological screening of sentinel rats for this study demonstrated positive titers to rat corona virus/sialodacryoadenitis virus (RCV/SDA). Because there was little clinical or histologic evidence of an infection in the study rats, and because the target organ of TCPA was a site not primarily affected by infection with this virus, interpretation of the study results was not considered compromised by these positive serologic findings.

With the exception of slight decreases in red blood cell parameters suggestive of a poorly regenerative anemia in males, no adverse effects of TCPA were observed in mice in these studies. Results of genotoxicity tests with TCPA suggest little potential for genetic effects. The only positive results occurred in an unreplicated mouse bone marrow sister chromatid exchange test; the observed increase in sister chromatid exchange frequency, although dose related, was small, and no frequency in a single dose group was significantly elevated over that in the control group (Appendix D).

In summary, clear evidence of organ toxicity following administration of TCPA in corn oil by gavage for 13 weeks was limited to the kidney of rats. The no-observed-adverse-effect level for histopathologic lesions in this tissue was not achieved with doses as low as 94 mg/kg per day. No significant adverse effects were seen in mice given doses as high as 1500 mg/kg per day for 13 weeks.

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APPENDIX A

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

Table A1 Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride A-2

Table A2 Organ Weights and Organ-Weight-to-Body-Weight Ratios for B6C3F1 Mice in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride A-4

TABLE A1 Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride[1]

	Vehicle Control	94 mg/kg	187 mg/kg	375 mg/kg	750 mg/kg	1500 mg/kg
MALE						
n	10	10	9	10	10	4
Necropsy body wt	320 ±5	301 ± 10	317±10	283 ±10**	285 ± 8**	247 ±14**
Brain						
Absolute	1.931 ± 0.021	1.926 ± 0.025	1.900 ± 0.009	1.858 ± 0.025*	1.842 ± 0.023**	1.821 ± 0.017**
Relative	6.05 ± 0.10	6.45 ± 0.1 7	6.03 ± 0.20	6.61 ± 0.17*	6.49 ± 0.1 4*	7.45 ± 0.41**
Heart						
Absolute	1.173 + 0.056	1.075 ± 0.049[2]	0.968 ± 0.038*[3]	1.081 ± 0.044*	0.881 ± 0.028**	1.217 ± 0.106*
Relative	3.67 ± 0.15	3.58 ± 0.18[2]	2.98 + 0.13[3]	3.83 ± 0.13	3.09 ± 0.05	5.06 ± 0.76 **
Right Kidney						
Absolute	1.149 + 0.038	1.186 ± 0.042	1.279 ±0.032	1.142 ± 0.054	1.1 55 ±0.055	1.097 ± 0.070
Relative	3.59 ± 0.08	3.96 ± 0.1 2	4.04 ± 0.08*	4.02 ± 0.10*	4.04 ±0.11*	4.54 ± 0.57**
Liver						
Absolute	10.873 ± 0.273	10.386 ± 0.442	12.804 ± 0.468**	9.690 ± 0.445	11.109 ± 0.331	9.353 ± 0.301
Relative	33.99 ± 0.38	34.51 ± 0.97	40.38 ±1.03*	34.13 ± 0.70**	39.02 ± 0.68**	38.17 ± 1.74**
Lung						
Absolute	1.893 ± 0.073	1.745 ± 0.050	1.958 ± 0.073	1.694 ± 0.072*	1.667 ± 0.033*	1.454 ± 0.048**
Relative	5.92 ± 0.21	5.83 ± 0.1 5	6.22 ± 0.31	5.98 ± 0.14	5.87 ± 0.10	5.99 ± 0.53
Spleen						
Absolute	0.675 ± 0.016	0.620 ± 0.024*	0.626 ±0.012*	0.541 ± 0.017**	0.536 ±0.011**	0.460 ± 0.037**[4]
Relative	2.11 ± 0.04	2.06 ± 0.04	1.99 ±0.07	1.92 ± 0.03*	1.89 ±0.03**	1 .97 ± 0.28[4]
Right Testis						
Absolute	1.414 ± 0.036[2]	1 .392 ± 0.029	1.41 8 ± 0.022	1.396 ± 0.026	1.391 ±0.029	1.136 ± 0.203**
Relative	4.44 ± 0.15[2]	4.65 ± 0.1 2	4.49 ± 0.11	4.97 ± 0.13	4.90 ± 0.09	4.75 ± 1.00
Thymus						
Absolute	0.357 ± 0.029	0.317 ±0.028	0.360 ± 0.022	0.313 ± 0.017	0.281 ± 0.023*	0.225 ± 0.037**
Relative	1.12 ± 0.09	1.04 ± 0.07	1.13 ± 0.06	1.11 ± 0.05	0.97 ± 0.06	0.94 ± 0.21
FEMALE						
n	9	10	10	10	8	9
Necropsy body wt	184 ± 3	166 ± 3**	174 ± 4**	164 ± 4**	172 ± 4**	160 ± 3**
Brain						
Absolute	1.707 ± 0.051	1.744 ± 0.020	1.739 ± 0.0 19	1.750 ± 0.045	1.730 ± 0.022	1.670 ± 0.021
Relative	9.27 ± 0.21	10.53 ± 0.1 4**	10.03 ± 0.21**	10.71 ± 0.23**	10.17 ± 0.17**	10.49 ± 0.24**
Heart						
Absolute	0.700 ± 0.031	0.631 ± 0.045	0.603 ±0.01 2	0.626 ± 0.029	0.593 ± 0.016	0.665 ± 0.050
Relative	3.81 ± 0.18	3.79 ± 0.22	3.47 ± 0.06	3.84 ± 0.20	3.47 ± 0.08	4.16 ± 0.30
Right Kidney						
Absolute	0.683 ± 0.018	0.644 ± 0.028	0.723 ± 0.021	0.737 ± 0.026	0.850 ± 0.023**	0.853 ± 0.034**
Relative	3.71 ± 0.08	3.87 ± 0.12	4.1 6 ± 0.06*	4.50 ± 0.08**	5.07 ± 0.10**	5.36 ± 0.23**
Liver						
Absolute	5.564 ± 0.1 69	5.130 ± 0.122	6.283 ± 0.208	5.219 ± 0.222	6.445 ± 0.322*	5.405 ± 0.1 57
Relative	30.26 ± 0.93	30.90 ± 0.46	36.11 ± 0.78*	31. 83 ± 0.87*	37.90 ±1.45**	33.99 ±1.37**

TABLE A1 Organ Weights and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride (continued)

	Vehicle Control	94 mg/kg	187 mg/kg	375 mg/kg	750 mg/kg	1500 mg/kg
FEMALE (continued)						
Lung						
Absolute	1.262 ± 0.036	1.151 ± 0.034	1.325 ± 0.044	1.188 ± 0.071	1.262 ± 0.046	1.145 ± 0.028
Relative	6.86 ± 0.18	6.94 ± 0.1 7	7.63 ± 0.22	7.23 ± 0.32	7.49 ± 0.20	7.19 ± 0.22
Spleen						
Absolute	0.430 ± 0.010	0.384 ± 0.013	0.424 ± 0.0 13	0.411 ± 0.021	0.408 ± 0.014	0.388 ± 0.014
Relative	2.34 ± 0.07	2.31 ± 0.07	2.44 ± 0.05	2.51 ± 0.10	2.41 ± 0.05	2.43 ± 0.08
Thymus						
Absolute	0.238 ± 0.010	0.223 ± 0.01 2	0.216 ± 0.006	0.208 ± 0.010	0.236 ± 0.01 8	0.179 ± 0.014**
Relative	1.30 ± 0.05	1.34 ± 0.07	1.24 ± 0.04	1.27 ± 0.06	1.42 ± 0.09	1.13 ± 0.10

[1] Organ weights and body weights are given in grams; relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight (mean ± standard error).
[2] n=9.
[3] n=8.
[4] n=3.
* Significantly different ($P \leq 0.05$) from the control group by Williams' test or Dunnett's test.
** Significantly different ($P \leq 0.01$) from the control group by Williams' test or Dunnett's test.

TABLE A2 Organ Weights and Organ-Weight-to-Body-Weight Ratios for B6C3F$_1$ Mice in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride[1]

	Vehicle Control	94 mg/kg	187 mg/kg	375 mg/kg	750 mg/kg	1500 mg/kg
MALE						
n	10	10	10	10	10	10
Necropsy body wt	26.8 ± 0.6	27.4 ± 0.5	28.3 ± 0.7	28.0 ± 0.3	28.6 ± 0.3*	26.3 ± 0.4
Brain						
Absolute	0.447 ± 0.007	0.447 ± 0.006	0.449 ± 0.005	0.443 ± 0.007	0.455 ± 0.006	0.441 ± 0.004
Relative	16.76 ± 0.39	16.39 ± 0.43	15.89 ± 0.31	15.87 ± 0.34	15.90 ± 0.18	16.82 ± 0.23
Heart						
Absolute	0.139 ± 0.004	0.140 ± 0.007	0.169 ± 0.011*	0.141 ± 0.005	0.184 ± 0.010**	0.126 ± 0.003
Relative	5.20 ± 0.14	5.12 ± 0.26	6.00 ± 0.38	5.06 ± 0.22	6.41 ± 0.30**	4.79 ± 0.12
Right Kidney						
Absolute	0.250 ± 0.007	0.253 ± 0.007	0.256 ± 0.006	0.243 ± 0.007	0.248 ± 0.005	0.217 ± 0.006**
Relative	9.35 ± 0.20	9.21 ± 0.17	9.06 ± 0.22	8.68 ± 0.22*	8.68 ±0.1 8*	8.27 ± 0.17**
Liver						
Absolute	1.396 ± 0.049	1.384 ± 0.036	1.433 ± 0.046	1.293 ± 0.028	1.441 ± 0.025	1.310 ± 0.034
Relative	52.05 ±1.05	50.49 ± 0.78	50.67 ±1.56	46.21 ± 0.85*	50.32 ± 0.61*	49.87 ± 1.00*
Lung						
Absolute	0.259 ± 0.011	0.255 ± 0.015	0.259 ± 0.013	0.220 ± 0.013	0.244 ±0.01 2	0.215 ± 0.008*
Relative	9.69 ± 0.38	9.35 ± 0.62	9.17 ± 0.48	7.84 ± 0.42*	8.53 ± 0.45*	8.17 ± 0.29*
Spleen						
Absolute	0.061 ± 0.002	0.065 ± 0.001	0.064 ± 0.003	0.064 ± 0.001	0.064 ± 0.002	0.057 ± 0.002
Relative	2.29 ± 0.06	2.36 ± 0.04	2.26 ± 0.10	2.28 ± 0.07	2.23 ± 0.07	2.19 ± 0.08
Right Testis						
Absolute	0.118 ± 0.003	0.120 ± 0.001	0.122 ± 0.002	0.120 ± 0.003	0.122 ± 0.001	0.117 ± 0.003
Relative	4.44 ± 0.1 2	4.40 ± 0.09	4.31 ± 0.07	4.28 ± 0.09	4.25 ± 0.06	4.46 ± 0.09
Thymus						
Absolute	0.039 ± 0.005	0.037 ± 0.003	0.038 ± 0.005	0.036 ± 0.002	0.036 ± 0.003	0.032 ± 0.003
Relative	1.46 ± 0.15	1.36 ± 0.11	1.31 ± 0.14	1.30 ± 0.08	1.24 ± 0.1 2	1.22 ± 0.09
FEMALE						
n	10	10	10	10	9	10
Necropsy body wt	24.0 ± 0.5	23.2 ± 0.3	25.2 ± 0.7	24.0 ± 0.5	24. 7 ± 0.3	24.1 ± 0.4
Brain						
Absolute	0.470 ± 0.006	0.459 ± 0.004	0.442 ± 0.007**	0.454 ± 0.004	0.461 ± 0.005	0.467 ± 0.007
Relative	19.63 ± 0.44	19.81 ± 0.35	17.67 ± 0.58*	18.98 ± 0.33	18.68 ± 0.25	19.47 ± 0.53
Heart						
Absolute	0.126 ± 0.005	0.113 ± 0.004	0.118 ± 0.003	0.118 ± 0.006	0.128 ± 0.005	0.121 ± 0.005
Relative	5.25 ± 0.1 9	4.86 ± 0.1 6	4.70 ± 0.1 5	4.92 ± 0.29	5.20 ± 0.21	5.03 ± 0.23
Right Kidney						
Absolute	0.183 ± 0.003	0.169 ± 0.005*	0.177 ± 0.003	0.171 ± 0.003	0.173 ± 0.003	0.176 ± 0.004
Relative	7.64 ± 0.1 8	7.28 ± 0.23	7.07 ± 0.21	7.13 ± 0.19	6.99 ± 0.1 2	7.31 ± 0.12
Liver						
Absolute	1.301 ± 0.035	1.165 ± 0.021	1.324 ± 0.045	1.165 ± 0.033	1.268 ± 0.032	1.286 ± 0.053
Relative	54.17 ± 0.99	50.20 ± 0.80	52.76 ± 2.08	48.57 ± 1.04*	51.30 ± 0.93	53.30 ± 1.40

TABLE A2 Organ Weights and Organ-Weight-to-Body-Weight Ratios for B6C3F$_1$ Mice in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride (continued)

	Vehicle Control	94 mg/kg	187 mg/kg	375 mg/kg	750 mg/kg	1500 mg/kg
FEMALE (continued)						
Lung						
Absolute	0.231 ± 0.008	0.221 ± 0.013	0.220 ± 0.007	0.221 ± 0.012	0.231 ± 0.009	0.218 ± 0.008
Relative	9.67 ± 0.39	9.48 ± 0.44	8.78 ± 0.40	9.23 ± 0.45	9.39 ± 0.40	9.07 ± 0.38
Spleen						
Absolute	0.085 ± 0.003	0.082 ± 0.003	0.074 ± 0.004	0.078 ± 0.002	0.083 ± 0.004	0.084 ± 0.003
Relative	3.53 ± 0.11	3.52 ±0.11	2.91 ±0.13**	3.26 ±0.11	3.36 ±0.1 5	3.48 ± 0.1 2
Thymus						
Absolute	0.055 ± 0.003	0.048 ± 0.003	0.048 ± 0.006	0.056 ± 0.004	0.048 ± 0.006	0.055 ± 0.004
Relative	2.30 ± 0.12	2.08 ± 0.11	1.88 ± 0.22	2.31 ± 0.16	1.92 ± 0.22	2.29 ± 0.15

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

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

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

APPENDIX B

Hematology and Clinical Chemistry Results

Table B1 Hematology Data for F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride B-2

Table B2 Clinical Chemistry Data for F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride B-3

Table B3 Hematology Data for B6C3F$_1$ Mice in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride B-5

TABLE B1 Hematology Data for F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride[1]

Analysis	Vehicle Control	94 mg/kg	187 mg/kg	375 mg/kg	750 mg/kg	1500 mg/kg
MALE						
n	10	10	9	10	9	3
Hematocrit (%)	45.2 ± 0.6	45.6 ± 0.4	43.9 ± 0.5	46.1 ± 0.8	44.7 ± 0.4	46.2 ± 1.3
Hemoglobin (g/dL)	16.2 ± 0.2	16.3 ± 0.2	15.8 ± 0.1	16.5 ± 0.2	15.7 ± 0.0**	16.5 ± 0.5
Erythrocytes (10^6/μL)	8.73 ± 0.11	8.83 ± 0.08	8.55 ± 0.09	8.95 ± 0.12	8.61 ± 0.05	8.87 ± 0.25
Reticulocytes (10^6/μL)	0.20 ± 0.03	0.25 ± 0.05	0.27 ± 0.02	0.25 ± 0.05	0.23 ± 0.03	0.29 ± 0.07
Mean cell volume (fL)	51.9 ± 0.2	51.7 ± 0.3	51.2 ± 0.2	51.6 ± 0.4	51.8 ± 0.2	52.3 ± 0.3
Mean cell hemoglobin (pg)	18.6 ± 0.1	18.4 ± 0.1	18.5 ± 0.2	18.4 ± 0.1	18.2 ± 0.1	18.6 ± 0.0
Mean cell hemoglobin concentration (g/dL)	35.9 ± 0.3	35.7 ± 0.3	36.0 ± 0.2	35.8 ± 0.3	35.1 ± 0.3	35.7 ± 0.2
Platelets (10^3/μL)	566.1 ± 7.3	587.5 ± 7.8	563.3 ± 11.6	598.7 ± 13.3	583.8 ± 10.7	709.3 ± 29.9**
Leukocytes (10^3/μL)	8.45 ± 0.56	8.06 ± 0.44	7.89 ± 0.58	7.52 ± 0.29	7.31 ± 0.54	7.20 ± 0.61
Segmented neutrophils (10^3/μL)	1.52 ± 0.08	1.89 ± 0.20	1.40 ± 0.14	1.93 ± 0.15	1.77 ± 0.21	2.57 ± 0.35
Lymphocytes (10^3/μL)	6.43 ± 0.49	5.68 ± 0.31	6.12 ± 0.51	5.08 ± 0.34*	5.14 ± 0.46	4.28 ± 0.41*
Monocytes (10^3/μL)	0.38 ± 0.08	0.36 ± 0.06	0.27 ± 0.05	0.38 ± 0.05	0.30 ± 0.08	0.32 ± 0.09
Eosinophils (10^3/μL)	0.10 ± 0.04	0.12 ± 0.03	0.10 ± 0.03	0.13 ± 0.04	0.10 ± 0.04	0.03 ± 0.03
FEMALE						
n	7	9	6	10	5	9
Hematocrit (%)	43.1 ± 0.6	43.6 ± 0.5	43.9 ± 0.6	42.6 ± 0.6	43.5 ± 1.4	45.7 ± 0.8
Hemoglobin (g/dL)	15.5 ± 0.1	15.5 ± 0.1	15.7 ± 0.2	15.4 ± 0.2	15.6 ± 0.3	16.4 ± 0.3
Erythrocytes (10^6/μL)	7.90 ± 0.08	7.99 ± 0.09	8.08 ± 0.08	7.88 ± 0.16	8.07 ± 0.21	8.47 ± 0.16*
Reticulocytes (10^6/μL)	0.17 ± 0.03	0.16 ± 0.02	0.16 ± 0.02	0.18 ± 0.03	0.19 ± 0.06	0.22 ± 0.03
Mean cell volume (fL)	54.6 ± 0.4	54.4 ± 0.2	54.2 ± 0.5	54.1 ± 0.7	54.0 ± 0.6	54.0 ± 0.7
Mean cell hemoglobin (pg)	19.7 ± 0.1	19.4 ± 0.1	19.4 ± 0.1	19.6 ± 0.3	19.3 ± 0.1	19.3 ± 0.2
Mean cell hemoglobin concentration (g/dL)	36.1 ± 0.5	35.6 ± 0.2	35.8 ± 0.4	36.2 ± 0.2	35.8 ± 0.5	35.8 ± 0.5
Platelets (10^3/μL)	621.3 ± 17.2	620.6 ± 10.5	565.0 ± 20.9	676.6 ± 13.0	615.6 ± 19.0	647.9 ± 23.8
Leukocytes (10^3/μL)	6.53 ± 0.40	6.22 ± 0.59	6.87 ± 0.64	6.75 ± 0.41	6.58 ± 0.66	5.92 ± 0.49
Segmented neutrophils (10^3/μL)	1.07 ± 0.12	1.04 ± 0.13	1.68 ± 0.38	1.27 ± 0.17	1.53 ± 0.09*	1.87 ± 0.18**
Lymphocytes (10^3/μL)	4.97 ± 0.36	4.80 ± 0.52	4.68 ± 0.57	5.09 ± 0.32	4.56 ± 0.60	3.66 ± 0.37
Monocytes (10^3/μL)	0.42 ± 0.07	0.32 ± 0.08	0.40 ± 0.06	0.32 ± 0.06	0.36 ± 0.10	0.33 ± 0.07
Eosinophils (10^3/μL)	0.07 ± 0.02	0.05 ± 0.02	0.10 ± 0.03	0.08 ± 0.03	0.09 ± 0.03	0.06 ± 0.02

1 Mean ± standard error.
* Siginificantly different (P≤0.05) from the control group by Shirley's test.
** Significantly different (P≤0.01) from the control group by Shirley's test.

TABLE B2 Clinical Chemistry Data for F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride[1]

Analysis	Vehicle Control	94 mg/kg	375 mg/kg	1500 mg/kg
MALE				
n	10	10	10	10
Alanine aminotransferase (IU/L)				
Day 6	35 ± 2	32 ± 1	30 ± 2*	35 ± 2
Day 20	31 ± 1	35 ± 2	31 ± 2	31 ± 2
Day 91	43 ± 2	41 ± 2[2]	41 ± 3	42 ± 4[3]
Albumin (g/dL)				
Day 6	4.3 ± 0.2	4.2 ± 0.2	4.0 ± 0.1	4.2 ± 0.2
Day 20	4.3 ± 0.1	4.3 ± 0.1	4.4 ± 0.1[2]	4.3 ± 0.1
Day 91	5.4 ± 0.2	5.4 ± 0.2[2]	5.5 ± 0.2	6.0 ± 0.2[3]
Creatinine (mg/dL)				
Day 6	0.36 ± 0.03	0.33 ± 0.02	0.36 ± 0.03	0.43 ± 0.03
Day 20	0.41 ± 0.02	0.41 ± 0.02	0.39 ± 0.03	0.41 ± 0.02
Day 91	0.59 ± 0.04	0.56 ± 0.02[2]	0.57 ± 0.02	0.65 ± 0.09[3]
γ-glutamyltransferase (IU/L)				
Day 6	1.0 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	1.0 ± 0.3
Day 20	1.6 ± 0.5	3.2 ± 0.8	2.0 ± 0.7	2.6 ± 0.5
Day 91	1.0 ± 0.3	2.1 ± 0.6	1.7 ± 0.2	4.8 ± 1.6**[3]
Glucose (mg/dL)				
Day 6	76 ± 4	79 ± 4	78 ± 3	77 ± 5
Day 20	72 ± 4	79 ± 3	74 ± 4	73 ± 4
Day 91	126 ± 6	128 ± 9[2]	143 ± 10	121 ± 9[3]
Urea nitrogen (mg/dL)				
Day 6	14.4 ± 0.9	13.5 ± 1.2	13.9 ± 1.3	12.5 ± 0.9
Day 20	13.9 ± 0.7[2]	13.1 ± 0.8	12.0 ± 1.0	11.6 ± 0.4*
Day 91	16.4 ± 0.7	13.8 ± 0.6*[2]	13.6 ± 0.7*	13.8 ± 1.8*[3]
FEMALE				
n	9	10	10	10
Alanine aminotransferase (IU/L)				
Day 6	38 ± 4	37 ± 3	40 ± 4	48 ± 4
Day 20	26 ± 1	26 ± 1	27 ± 1	27 ± 2
Day 91	34 ± 2	28 ± 1	29 ± 1	34 ± 2[2]
Albumin (g/dL)				
Day 6	4.3 ± 0.1	4.2 ± 0.2	4.3 ± 0.2	4.1 ± 0.1
Day 20	4.8 ± 0.2	4.8 ± 0.1	4.9 ± 0.1	4.9 ± 0.2
Day 91	5.3 ± 0.1	5.4 ± 0.1	5.2 ± 0.1	5.4 ± 0.1[2]
Creatinine (mg/dL)				
Day 6	0.30 ± 0.04	0.40 ± 0.04	0.41 ± 0.03	0.35 ± 0.04
Day 20	0.40 ± 0.02	0.40 ± 0.02	0.41 ± 0.02	0.42 ± 0.03
Day 91	0.49 ± 0.04	0.48 ± 0.03	0.51 ± 0.05	0.53 ± 0.06[2]
γ-glutamyltransferase (IU/L)				
Day 6	1.0 ± 0.4	1.8 ± 0.6	1.7 ± 0.6	2.2 ± 0.7
Day 20	1.4 ± 0.4	2.2 ± 0.6	1.4 ± 0.5	1.2 ± 0.4
Day 91	2.6 ± 0.5	1.6 ± 0.4	1.7 ± 0.3	2.9 ± 1.2[2]

TABLE B2 Clinical Chemistry Data for F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride (continued)

Analysis	Vehicle Control	94 mg/kg	375 mg/kg	1500 mg/kg
FEMALE (continued)				
Glucose (mg/dL)				
Day 6	81 ± 3	76 ± 3	77 ± 2	71 ± 3*
Day 20	82 ± 1	83 ± 4[2]	77 ± 4	69 ± 3**
Day 91	114 ± 9	104 ± 4	101 ± 3	110 ± 7[2]
Urea nitrogen (mg/dL)				
Day 6	14.8 ± 1.1	14.5 ± 0.8	12.1 ± 0.6	11.3 ± 0.6**
Day 20	14.2 ± 1.5	13.0 ± 0.3	12.9 ± 1.1	10.1 ± 0.7*
Day 91	15.7 ± 0.8	15.1 ± 1.2	14.0 ± 0.7	13.8 ± 1.1[2]

1 Mean ± standard error.
2 n=9.
3 n=4.
* Significantly different (P≤0.05) from the control group by Dunn's test or Shirley's test.
** Significantly different (P≤0.01) from the control group by Shirley's test.

TABLE B3 Hematology Data for B6C3F$_1$ Mice in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride[1]

Analysis	Vehicle Control	94 mg/kg	187 mg/kg	375 mg/kg	750 mg/kg	1500 mg/kg
MALE						
n	10	10	10	10	10	10
Hematocrit (%)	49.5 ± 0.8	48.3 ± 0.9	48.1 ± 1.0	47.3 ± 0.9	49.7 ± 0.9	45.3 ± 1.1**
Hemoglobin (g/dL)	16.8 ± 0.1	16.6 ± 0.2	16.3 ± 0.2	16.2 ± 0.1*	16.0 ± 0.1**	15.5 ± 0.3**
Erythrocytes (10^6/µL)	9.77 ± 0.15	9.65 ± 0.21	9.49 ± 0.21	9.45 ± 0.13	9.79 ± 0.20	9.09 ± 0.21*
Reticulocytes (10^6/µL)	0.28 ± 0.02	0.24 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	0.24 ± 0.05	0.27 ± 0.02
Mean cell volume (fL)	50.6 ± 0.2	50.1 ± 0.5	50.8 ± 0.3	49.9 ± 0.4	50.9 ± 0.2	50.0 ± 0.4
Mean cell hemoglobin (pg)	17.2 ± 0.3	17.3 ± 0.2	17.3 ± 0.2	17.2 ± 0.3	16.4 ± 0.2**	17.1 ± 0.3
Mean cell hemoglobin concentration (g/dL)	33.9 ± 0.5	34.4 ± 0.4	34.0 ± 0.5	34.3 ± 0.7	32.3 ± 0.4*	34.3 ± 0.6
Platelets (10^3/µL)	740.2 ± 36.8	742.0 ± 32.5	734.2 ± 23.7	764.1 ± 35.4	787.6 ± 24.1	827.6 ± 79.0
Leukocytes (10^3/µL)	3.10 ± 0.29	2.69 ± 0.18	2.39 ± 0.28	1.86 ± 0.16*	2.73 ± 0.36	3.08 ± 0.36
Segmented neutrophils (10^3/µL)	0.45 ± 0.05	0.52 ± 0.06	0.41 ± 0.06	0.39 ± 0.06	0.78 ± 0.31	0.68 ± 0.09
Lymphocytes (10^3/µL)	2.61 ± 0.25	2.12 ± 0.15	1.94 ± 0.24*	1.43 ± 0.15**	1.86 ± 0.29**	2.28 ± 0.32*
Monocytes (10^3/µL)	0.06 ± 0.02	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.07 ± 0.02
Eosinophils (10^3/µL)	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.04 ± 0.02	0.04 ± 0.01*
FEMALE						
n	10	10	10	10	9	10
Hematocrit (%)	48.0 ± 0.5	46.9 ± 0.4	46.1 ± 0.6	48.1 ± 1.0	46.7 ± 0.3	47.2 ± 0.7
Hemoglobin (g/dL)	16.1 ± 0.1	15.7 ± 0.1**	15.6 ± 0.2*	16.3 ± 0.2	15.6 ± 0.1*	15.6 ± 0.2*
Erythrocytes (10^6/µL)	9.42 ± 0.10	9.36 ± 0.09	9.14 ± 0.13	9.60 ± 0.23	9.25 ± 0.09	9.50 ± 0.21
Reticulocytes (10^6/µL)	0.22 ± 0.02	0.20 ± 0.02	0.18 ± 0.03	0.19 ± 0.02	0.19 ± 0.03	0.17 ± 0.02
Mean cell volume (fL)	50.8 ± 0.2	50.2 ± 0.3	50.5 ± 0.2	50.3 ± 0.6	50.4 ± 0.5	49.6 ± 0.5
Mean cell hemoglobin (pg)	17.1 ± 0.2	16.8 ± 0.2	17.1 ± 0.2	17.1 ± 0.3	16.9 ± 0.2	16.5 ± 0.2
Mean cell hemoglobin concentration (g/dL)	33.6 ± 0.3	33.4 ± 0.3	33.8 ± 0.4	34.0 ± 0.5	33.5 ± 0.2	33.2 ± 0.4
Platelets (10^3/µL)	756.0 ± 21.5	716.4 ± 23.5	708.6 ± 50.3	758.7 ± 27.0	695.0 ± 25.2	733.3 ± 19.4
Leukocytes (10^3/µL)	4.67 ± 0.55	3.12 ± 0.32	3.30 ± 0.29	3.73 ± 0.51	3.70 ± 0.35	4.78 ± 0.52
Segmented neutrophils (10^3/µL)	0.90 ± 0.22	0.58 ± 0.13	0.46 ± 0.09	0.50 ± 0.08	0.66 ± 0.10	0.83 ± 0.13
Lymphocytes (10^3/µL)	3.61 ± 0.33	2.48 ± 0.22*	2.78 ± 0.23	3.15 ± 0.41	2.93 ± 0.32	3.77 ± 0.39
Monocytes (10^3/µL)	0.14 ± 0.06	0.05 ± 0.01	0.07 ± 0.02	0.06 ± 0.04	0.07 ± 0.03	0.14 ± 0.04
Eosinophils (10^3/µL)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.01	0.03 ± 0.02

[1] Mean ± standard error.

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

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

APPENDIX C

Reproductive Tissue Evaluations and Estrous Cycle Characterization

Table C1 Summary of Reproductive Tissue Evaluations in Male F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride C-2

Table C2 Summary of Estrous Cycle Characterization in Female F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride C-2

Table C3 Summary of Reproductive Tissue Evaluations in Male B6C3F$_1$ Mice in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride C-3

Table C4 Summary of Estrous Cycle Characterization in Female B6C3F$_1$ Mice in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride C-3

TABLE C1 Summary of Reproductive Tissue Evaluations in Male F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride[1]

Study Parameters	Vehicle Control	94 mg/kg	375 mg/kg	750 mg/kg
Weights (g)				
Necropsy body weight	320 ± 5	301 ± 10	283 ± 10**	285 ± 8**
Right epididymis	0.418 ± 0.006	0.407 ± 0.008	0.388 ± 0.011*[2]	0.371 ± 0.007**
Right cauda epididymis	0.187 ± 0.004	0.183 ± 0.005	0.168 ± 0.005*	0.158 ± 0.003**
Right testis	1.414 ± 0.036[2]	1.392 ± 0.029	1.396 ± 0.026	1.391 ± 0.029
Spermatozoal measurements				
Motility (%)	97.78 ± 0.53	96.07 ± 1.25	96.45 ± 0.76	95.90 ± 0.86
Concentration (10^6/g cauda epididymal tissue)	667.4 ± 15.2	675.2 ± 28.0	641.7 ± 40.4	645.6 ± 34.7

[1] Data are presented as mean ± standard error; n=10. Differences from the control group for spermatozoal measurements are not significant by Dunn's test or Shirley's test; testicular weights are not significant by Dunnett's test.
[2] n=9.
* Significantly different (P≤0.05) from the control group by Shirley's test.
** Significantly different (P≤0.01) from the control group by Shirley's test or WIlliams' test.

TABLE C2 Summary of Estrous Cycle Characterization in Female F344/N Rats in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride[1]

Study Parameters	Vehicle Control	94 mg/kg	375 mg/kg	1500 mg/kg
Necropsy body weight (g)	184 ± 3	166 ± 3**	164 ± 4**	160 ± 3**
Estrous cycle length (days)	4.89 ± 0.20	5.00 ± 0.15	4.56 ± 0.18[2]	4.78 ± 0.15
Estrous stages as % of cycle				
Diestrus	34.9	37.1	37.1	41.3
Proestrus	17.5	21.4	17.1	15.9
Estrus	27.0	27.1	31.4	22.2
Metestrus	20.6	14.3	14.3	20.6

[1] Data are presented as mean ± standard error. For the 94 mg/kg and 375 mg/kg dose groups, n=10. For the control group and the 1500 mg/kg dose group, n=9. Estrous cycle lengths are not significant by Dunn's test. By multivariate analysis of variance (MANOVA), dosed groups do not differ significantly from controls in the relative length of time spent in the estrous stages.
[2] Estrous cycle longer than seven days or unclear in 1 of 10 animals.
** Significantly different (P≤0.01) from the control group by Williams' test.

TABLE C3 Summary of Reproductive Tissue Evaluations in Male B6C3F$_1$ Mice in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride[1]

Study Parameters	Vehicle Control	94 mg/kg	375 mg/kg	1500 mg/kg
Weights (g)				
Necropsy body weight	26.8 ± 0.6	27.4 ± 0.5	28.0 ± 0.3	26.3 ± 0.4
Right epididymis	0.041 ± 0.001	0.041 ± 0.001	0.041 ± 0.001	0.040 ± 0.001
Right cauda epididymis	0.015 ± 0.000	0.015 ± 0.001	0.015 ± 0.001	0.014 ± 0.001
Right testis	0.118 ± 0.003	0.120 ± 0.001	0.120 ± 0.003	0.117 ± 0.003
Spermatozoal measurements				
Motility (%)	96.73 ± 0.72	96.16 ± 0.44	96.19 ± 0.58	78.96 ± 7.59**
Concentration (10^6/g cauda epididymal tissue)	1533 ± 50	1413 ± 46	1413 ± 68	1386 ± 87

[1] Data are presented as mean ± standard error; n=10. Differences from the control group for necropsy body weights are not siginificant by Dunnett's test; epididymal and cauda epididymal weights are not significant by Dunn's test; testicular weights are not significant by Dunnett's test; spermatozoal concentrations are not significant by Shirley's test.

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

TABLE C4 Summary of Estrous Cycle Characterization in Female B6C3F$_1$ Mice in the 13-Week Gavage Study of Tetrachlorophthalic Anhydride[1]

Study Parameters	Vehicle Control	94 mg/kg	375 mg/kg	1500 mg/kg
Necropsy body weight (g)	24.0 ± 0.5	23.2 ± 0.3	24.0 ± 0.5	24.1 ± 0.4
Estrous cycle length (days)	4.10 ± 0.18	4.10 ± 0.10	4.50 ± 0.22	4.30 ± 0.15
Estrous stages as % of cycle				
Diestrus	24.3	25.7	31.4	24.3
Proestrus	15.7	18.6	14.3	14.3
Estrus	37.1	31.4	38.6	42.9
Metestrus	22.9	24.3	15.7	18.6

[1] Data are presented as mean ± standard error; n=10. Necropsy body weights are not significant by Williams' test. Estrous cycle lengths are not significant by Dunn's test. By multivariate analysis of variance (MANOVA), dosed groups do not differ significantly from controls in the relative length of time spent in the estrous stages.

APPENDIX D

Genetic Toxicology

Table D1 Mutagenicity of Tetrachlorophthalic Anhydride in *Salmonella typhimurium* ... D-2

Table D2 Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Tetrachlorophthalic Anhydride ... D-5

Table D3 Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Tetrachlorophthalic Anhydride ... D-7

Table D4 Frequency of Sex-Linked Recessive Lethal Mutations in *Drosophila melanogaster* Treated with Tetrachlorophthalic Anhydride ... D-8

Table D5 Induction of Sister Chromatid Exchanges in Mouse Bone Marrow Cells by Tetrachlorophthalic Anhydride ... D-8

Table D6 Induction of Chromosomal Aberrations in Mouse Bone Marrow Cells by Tetrachlorophthalic Anhydride ... D-9

TABLE D1 Mutagenicity of Tetrachlorophthalic Anhydride in *Salmonella typhimurium*[1]

Strain	Dose (µg/plate)	Revertants/plate[2] -S9 Trial 1	-S9 Trial 2	+10% hamster S9 Trial 1	+10% hamster S9 Trial 2	+10% rat S9 Trial 1	+10% rat S9 Trial 2
Study performed at SRI International							
TA100	0.0	102 ± 5.3	100 ± 2.5	108 ± 9.7	93 ± 2.3	111 ± 8.1	100 ± 5.9
	33.3	90 ± 4.5		90 ± 5.8		123 ± 8.4	
	100.0	96 ± 4.0	103 ± 2.9	94 ± 15.6	66 ± 5.1	104 ± 5.1	87 ± 5.5
	333.3	95 ± 5.3	96 ± 10.2	92 ± 4.9	59 ± 3.5	99 ± 10.9	92 ± 9.5
	1000.0	82 ± 5.5	75 ± 8.0	75 ± 3.6	59 ± 4.5	110 ± 8.2	99 ± 0.9
	3333.3	88 ± 1.2[3]	56 ± 8.4[4]	64 ± 11.8	52 ± 6.9	102 ± 1.0[3]	75 ± 0.7
	6666.7		Toxic		31 ± 4.2		38 ± 3.7[4]
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control[5]		526 ± 12.0	358 ± 15.3	1278 ± 34.7	1953 ± 36.1	627 ± 18.2	823 ± 37.4
TA1535	0.0	25 ± 3.1	15 ± 1.5	15 ± 1.9	7 ± 0.9	9 ± 0.3	8 ± 1.5
	33.3	14 ± 1.2		16 ± 2.3		9 ± 2.5	
	100.0	17 ± 1.0	17 ± 1.2	11 ± 2.0	12 ± 3.3	9 ± 1.7	7 ± 1.3
	333.3	20 ± 0.7	20 ± 4.3	11 ± 1.5	6 ± 1.2	11 ± 2.7	9 ± 0.0
	1000.0	18 ± 3.5	16 ± 2.6	8 ± 1.5	10 ± 1.8	13 ± 4.2	11 ± 2.4
	3333.3	13 ± 5.5[3]	12 ± 1.7[4]	9 ± 2.9	9 ± 1.5	8 ± 2.2[3]	9 ± 0.9
	6666.7		Toxic		7 ± 1.5		6 ± 3.5
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		444 ± 17.0	338 ± 21.3	458 ± 3.3	340 ± 27.4	374 ± 12.8	321 ± 21.4
TA1537	0.0	16 ± 1.5	7 ± 1.7	24 ± 1.7	16 ± 1.3	24 ± 4.3	16 ± 0.9
	33.3	9 ± 0.7		26 ± 1.3		24 ± 4.3	
	100.0	6 ± 1.9	8 ± 0.9	24 ± 1.8	12 ± 2.1	21 ± 2.0	12 ± 2.4
	333.3	9 ± 1.5	8 ± 0.9	28 ± 1.2	12 ± 1.5	29 ± 1.8	12 ± 2.6
	1000.0	7 ± 1.2	7 ± 1.0	20 ± 2.7	10 ± 2.3	22 ± 2.2	12 ± 1.7
	3333.3	6 ± 0.9[3]	7 ± 0.9[4]	14 ± 1.2	9 ± 2.4	27 ± 2.1[3]	8 ± 0.3
	6666.7		0 ± 0.0[4]		4 ± 0.9		6 ± 1.5
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		166 ± 5.6	59 ± 6.9	245 ± 7.5	221 ± 28.0	238 ± 45.9	553 ± 191.4
TA98	0.0	16 ± 1.5	26 ± 4.0	46 ± 4.2	29 ± 2.1	34 ± 3.1	36 ± 2.3
	33.3	13 ± 3.5		35 ± 1.5		29 ± 2.1	
	100.0	13 ± 3.5	25 ± 3.1	33 ± 6.0	38 ± 2.7	33 ± 6.8	34 ± 3.5
	333.3	13 ± 2.6	31 ± 3.5	26 ± 1.9	37 ± 3.2	28 ± 2.3	34 ± 1.5
	1000.0	18 ± 1.5	27 ± 1.3	26 ± 0.3	35 ± 5.0	27 ± 3.5	37 ± 2.1
	3333.3	15 ± 0.3	12 ± 1.7	24 ± 4.5	38 ± 1.9	26 ± 3.8[3]	28 ± 2.6
	6666.7		15 ± 3.1[4]		32 ± 2.9		26 ± 4.3
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		615 ± 9.0	738 ± 59.5	1397 ± 41.3	1596 ± 54.6	798 ± 62.0	847 ± 64.9

TABLE D1 Mutagenicity of Tetrachlorophthalic Anhydride in *Salmonella typhimurium* (continued)

Strain	Dose (µg/plate)	Revertants/plate -S9 Trial 1	-S9 Trial 2	+10% hamster S9 Trial 1	+10% hamster S9 Trial 2	+10% rat S9 Trial 1	+10% rat S9 Trial 2
Study performed at Case Western Reserve University							
TA100	0.0	84 ± 3.3	96 ± 5.8	100 ± 5.4	122 ± 8.9	108 ± 15.5	116 ± 5.2
	1.0	76 ± 4.4		74 ± 4.4		97 ± 8.7	
	3.3	80 ± 1.2		89 ± 12.1		97 ± 5.8	
	10.0	78 ± 1.2	89 ± 6.6	111 ± 1.8	99 ± 19.0	98 ± 6.1	94 ± 5.7
	33.0	90 ± 1.9	99 ± 11.4	104 ± 2.3	101 ± 13.4	104 ± 8.1	92 ± 10.0
	100.0	97± 7.1	93 ± 12.2	109 ± 2.6	108 ± 13.0	94 ± 8.1	89 ± 6.9
	333.0		92 ± 6.7		103 ± 14.4		82 ± 14.2
	1000.0		94 ± 10.1		95 ± 17.7		81 ± 13.0
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		348 ± 78.7	292 ± 7.7	1631 ± 49.2	1272 ± 15.5	1822 ± 84.6	1239 ± 45.7
TA1535	0.0	4 ± 0.9	5 ± 0.6	4 ± 1.2	7 ± 2.5	5 ± 1.5	7 ± 0.9
	1.0	2 ± 0.9		3 ± 0.7		6 ± 1.9	
	3.3	1 ± 0.0		4 ± 0.7		1 ± 0.6	
	10.0	3 ± 1.0	6 ± 1.2	2 ± 0.3	8 ± 3.4	3 ± 0.3	11 ± 5.6
	33.0	2 ± 0.6	6 ± 2.1	3 ± 0.3	7 ± 1.5	3 ± 0.3	5 ± 0.5
	100.0	4 ± 0.3	6 ± 1.5	6 ± 3.1	9 ± 0.9	2 ± 1.5	5 ± 2.0
	333.0		5 ± 0.3		6 ± 0.7		8 ± 2.7
	1000.0		5 ± 2.4		9 ± 0.7		7 ± 3.0
Trial Summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		379 ± 9.4	438 ± 16.0	86 ± 6.3	64 ± 4.8	95 ± 3.2	89 ± 3.3
TA1537	0.0	2 ± 0.3	3 ± 0.3	4 ± 0.7	6 ± 0.6	4 ± 0.7	6 ± 0.5
	1.0	2 ± 0.0		3 ± 0.6		3 ± 0.6	
	3.3	2 ± 0.9		5 ± 2.4		4 ± 0.3	
	10.0	1 ± 0.6	5 ± 2.4	4 ± 1.5	5 ± 0.7	2 ± 0.3	9 ± 5.5
	33.0	2 ± 0.3	5 ± 2.3	3 ± 0.0	3 ± 0.9	3 ± 0.9	5 ± 0.9
	100.0	2 ± 0.7	6 ± 3.5	5 ± 2.3	5 ± 1.7	1 ± 0.9	3 ± 1.0
	333.0		3 ± 0.3		3 ± 1.7		4 ± 0.9
	1000.0		3 ± 0.9		4 ± 1.5		3 ± 0.7
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		531 ± 70.5	678 ± 77.0	118 ± 3.5	70 ± 3.2	43 ± 3.0	106 ± 21.6
TA98	0.0	11 ± 1.8	14 ± 4.6	16 ± 0.3	19 ± 2.6	15 ± 2.4	19 ± 3.8
	1.0	14 ± 0.0		11 ± 0.3		11 ± 2.3	
	3.3	14 ± 2.0		14 ± 2.2		10 ± 1.8	
	10.0	11 ± 0.6	18 ± 2.3	16 ± 3.8	19 ± 0.6	11 ± 1.2	21 ± 3.4
	33.0	16 ± 2.0	18 ± 3.3	11 ± 0.0	15 ± 2.0	13 ± 2.9	21 ± 1.9
	100.0	14 ± 1.7	15 ± 1.8	9 ± 0.3	14 ± 2.7	13 ± 1.2	12 ± 2.9
	333.0		18 ± 0.6		20 ± 2.3		14 ± 2.0
	1000.0		12 ± 3.8		18 ± 3.5		18 ± 2.4
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative
Positive control		154 ± 4.2	206 ± 5.0	1180 ± 153.4	1237 ± 23.3	1384 ± 48.9	1103 ± 174.7

TABLE D1 Mutagenicity of Tetrachlorophthalic Anhydride in *Salmonella typhimurium* (continued)

[1] The detailed protocol and these data are presented in Zeiger *et al*. (1985). Cells and tetrachlorophthalic anhydride or solvent (distilled water) were incubated in the absence of exogenous metabolic activation (-S9) or with Aroclor 1254-induced S9 from male Syrian hamster liver or male Sprague-Dawley rat liver. 0 µg/plate is the solvent control.

[2] Revertants are presented as the mean ± standard error from 3 plates.

[3] Precipitate on plate.

[4] Slight toxicity.

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

TABLE D2 Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Tetrachlorophthalic Anhydride[1]

Compound	Dose (µg/mL)	Total Cells	No. of Chromo-somes	No. of SCEs	SCEs/ Chromo-some	SCEs/ Cell	Hrs in BrdU	Increase over Solvent (%)[2]
-S9								
Trial 1								
Summary: Negative								
Dimethylsulfoxide		50	1047	462	0.44	9.2	26.0	
Mitomycin-C	0.005	25	523	518	0.99	20.7	26.0	124.45
Tetrachlorophthalic anhydride	25	50	1049	458	0.43	9.2	26.0	–1.06
	75	50	1050	427	0.40	8.5	26.0	–7.84
	250	50	1050	455	0.43	9.1	26.0	–1.80
	750	50	1048	417	0.39	8.3	26.0	–9.83
								P=0.919
+S9								
Trial 1								
Summary: Weak positive								
Dimethylsulfoxide		50	1045	473	0.45	9.5	26.0	
Cyclophosphamide	1.00	50	1048	763	0.72	15.3	26.0	60.85
Tetrachlorophthalic anhydride	25	50	1049	539	0.51	10.8	26.0	13.52
	75	50	1045	520	0.49	10.4	26.0	9.94
	250	50	1049	509	0.48	10.2	26.0	7.20
	750	50	1047	590	0.56	11.8	26.0	24.50*
								P=0.003
Trial 2								
Summary: Negative								
Dimethylsulfoxide		50	1045	404	0.38	8.1	26.0	
Cyclophosphamide	1.5	50	1049	1355	1.29	27.1	26.0	234.12
Tetrachlorophthalic anhydride	125	50	1042	389	0.37	7.8	26.0	–3.44
	250	50	1048	448	0.42	9.0	26.0	10.57
	500	50	1043	429	0.41	8.6	26.0	6.39
								P=0.071

TABLE D2 Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Tetrachlorophthalic Anhydride (continued)

[1] Study performed at Columbia University. SCE = sister chromatid exchange; BrdU = bromodeoxyuridine. A detailed description of the protocol and these data are presented by Galloway *et al.* (1987).

[2] Percentage increase in SCEs/chromosome of culture exposed to tetrachlorophthalic anhydride relative to those of culture exposed to solvent.

* Positive (>20% increase over solvent control).

TABLE D3 Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Tetrachlorophthalic Anhydride[1]

-S9					+S9				
Dose (µg/mL)	Total Cells	No. of Abs	Abs/ Cell	Cells with Abs (%)	Dose (µg/mL)	Total Cells	No. of Abs	Abs/ Cell	Cells with Abs (%)
Trial 1 — Harvest time: 14.0 hours Summary: Negative					**Trial 1** — Harvest time: 14.0 hours Summary: Negative				
Dimethylsulfoxide					Dimethylsulfoxide				
	100	4	0.04	4.0		100	4	0.04	4.0
Mitomycin-C					Cyclophosphamide				
0.15	100	27	0.27	23.0	15	100	18	0.18	13.0
Tetrachlorophthalic anhydride					Tetrachlorophthalic anhydride				
75	100	6	0.06	6.0	25	100	5	0.05	4.0
250	100	5	0.05	5.0	75	100	6	0.06	6.0
750	100	7	0.07	7.0	250	100	8	0.08	8.0
				P=0.219[2]					P=0.085
					Trial 2 — Harvest time: 14.0 hours Summary: Negative				
					Dimethylsulfoxide				
						100	2	0.02	2.0
					Cyclophosphamide				
					15	100	25	0.25	22.0
					Tetrachlorophthalic anhydride				
					75	100	6	0.06	6.0
					125	100	9	0.09	6.0
					250	100	9	0.09	6.0
									P=0.112

[1] Study performed at Columbia University. Abs = aberrations. A detailed presentation of the protocol and these data are found in Galloway *et al.* (1987).

[2] Significance of percent cells with aberrations tested by the linear regression trend test vs. log of the dose.

TABLE D4 Induction of Sex-Linked Recessive Lethal Mutations in *Drosophila melanogaster* by Tetrachlorophthalic Anhydride[1]

Route of Exposure	Dose (ppm)	Incidence of Deaths (%)	Incidence of Sterility (%)	No. of Lethals/No. of X Chromosomes Tested			
				Mating 1	Mating 2	Mating 3	Total[2]
Injection	500	0	1	1/2321	0/2364	3/2259	4/6944(0.06%)
	0			3/1907	4/1873	3/1789	10/5569(0.18%)
Feeding	1000	9	13	2/1965	2/1936	3/1676	7/5577(0.13%)
	0			1/2189	0/2132	1/1698	2/6019(0.03%)

[1] Study performed at University of Wisconsin, Madison. A detailed description of the protocol and these data are presented in Valencia *et al.* (1985). F_1 daughters from the same treated parental male were kept together to identify clusters; clusters were found and removed in the control group for the injection experiment. Results of the injection study were negative (P=0.979) and results of the feeding study were equivocal (P=0.037) (Margolin *et al.*, 1983).

[2] Combined total number of lethal mutations/number of X chromosomes tested for 3 mating trials.

TABLE D5 Induction of Sister Chromatid Exchanges in Mouse Bone Marrow Cells by Tetrachlorophthalic Anhydride[1]

Treatment	Dose (mg/kg)	SCEs/Cell
Dimethylbenzanthracene[2]		12.73
Tetrachlorophthalic anhydride	0	4.44
	100	5.37
	200	5.68
	400	6.51
		P=0.012[3]

[1] Study performed at Oak Ridge Associated Universities. The detailed protocol is presented by McFee *et al.,* (1992). The harvest time was 23 hours. The 0 mg/kg dose is the solvent control (dimethylsulfoxide, 0.1 mL by intraperitoneal injection).

[2] Positive control (10 mg/kg in dimethylsulfoxide).

[3] Significance tested by the one-tailed trend test (Margolin *et al.*, 1986).

TABLE D6 Induction of Chromosomal Aberrations in Mouse Bone Marrow Cells by Tetrachlorophthalic Anhydride[1]

Treatment	Dose (mg/kg)	Cells with Aberrations (%)
Dimethylbenzanthracene[2]		18.75
Tetrachlorophthalic anhydride	0	2.00
	100	0.50
	200	1.25
	400	1.25
		P=0.012[3]

[1] Study performed at Oak Ridge Associated Universities. The detailed protocol is presented by McFee *et al.* (1992). The harvest time was 17 hours. The 0 mg/kg dose is the solvent control (dimethylsulfoxide, 0.1 mL by intraperitoneal injection).
[2] Positive control (200 mg/kg in dimethylsulfoxide).
[3] Significance tested by the one-tailed trend test (Margolin *et al.*, 1986).