FOREWORD

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control and Prevention. In July 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. The NTP coordinates the relevant programs, staff, and resources from these Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

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

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

These studies are designed and conducted to characterize and evaluate the toxicologic potential of selected chemicals in laboratory animals (usually two species, rats and mice). Chemicals selected for NTP toxicology studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. The interpretive conclusions presented in this Toxicity Study Report are based only on the results of these NTP studies. Extrapolation of these results to other species and quantitative risk analyses for humans require wider analyses beyond the purview of these studies. Selection *per se* is not an indicator of a chemical's toxic potential.

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

National Toxicology Program
Toxicity Report Series
Number 57

**NTP Technical Report
on the Toxicity Studies of**

Benzyltrimethylammonium Chloride

(CAS No. 56-93-9)

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

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PEER REVIEW

The draft report on the toxicity studies of benzyltrimethylammonium chloride was evaluated by the reviewers listed below. These reviewers serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determine if the design and conditions of these NTP studies are appropriate and ensure that the Toxicity Study Report presents the experimental results and conclusions fully and clearly.

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ABSTRACT

BENZYLTRIMETHYLAMMONIUM CHLORIDE

CAS No. 56-93-9

Molecular Weight: 185.70

Synonyms: Ammonium, benzyltrimethyl-chloride (8CI); BTM; BTMAC; N,N,N,-trimethyl-benzenemethanaminium chloride; trimethylbenzylammonium chloride (9CI); TMBAC

Benzyltrimethylammonium chloride is widely used as a solvent for cellulose, a gelling inhibitor in polyester resins, a chemical intermediate, a paint dispersant, and an acrylic dyeing agent. It is also used in plant growth regulator compositions and synthetic processes. The National Institute of Environmental Health Sciences nominated benzyltrimethylammonium chloride for study due to its high production volume and the potential for occupational exposure, as well as the limited information on toxicity of this chemical. Male and female F344/N rats and B6C3F$_1$ mice received benzyltrimethylammonium chloride by gavage for 16 days or 13 weeks. Animals were evaluated for hematology, clinical chemistry, histopathology, neurotoxicity, and reproductive toxicity. Genetic toxicology studies were conducted in *Salmonella typhimurium* and in mouse peripheral blood erythrocytes.

In the 16-day studies, groups of five male and five female rats received 0, 16, 32, 63, 125, or 250 mg benzyltrimethylammonium chloride/kg body weight in deionized water by gavage, 5 days per week for 16 days. Groups of five male and five female mice received 0, 63, 125, 250, 500, or 1,000 mg/kg benzyltrimethylammonium chloride in deionized water by gavage, 5 days per week for 16 days. All rats in the 125 and 250 mg/kg groups, all mice in the 250, 500, and 1,000 mg/kg groups, and one 125 mg/kg female mouse died on day 1 of the studies. Clinical findings observed in 125 mg/kg male and female rats included abnormal breathing, ataxia, lethargy (males only), nasal and eye discharge, and tremors. Salivation was slightly increased in male and female rats in the 63 mg/kg groups. Female mice in the 125 mg/kg group had

a significantly greater absolute liver weight than that of the vehicle controls. No gross or microscopic changes observed in rats or mice were considered related to chemical administration.

In the 13-week studies, groups of 10 male and 10 female rats and mice received benzyltrimethylammonium chloride in deionized water by gavage at doses of 0, 12.5, 25, 50, or 100 mg/kg, 5 days per week for 13 weeks. Benzyltrimethylammonium chloride generally had little effect on the body weights of rats or mice. Final mean body weights of dosed animals were within 8% (rats) or 3% (mice) of the control group body weights. The deaths of two female rats and one male and one female mouse administered 100 mg/kg were the result of pharmacologic effects on the cardiovascular system. Some cholinergic effects including chromodacryorrhea, lacrimation, salivation, pupillary constriction, altered gait, and mild tremors were observed at nonlethal doses in rats; these effects were accompanied by alterations in body position. No significant target organ toxicity was observed in dosed rats or mice.

Benzyltrimethylammonium chloride was not mutagenic in *S. typhimurium* strain TA97, TA98, TA100, or TA1535, with or without S9 metabolic activation enzymes. However, significant increases in the frequency of micronucleated normochromatic erythrocytes were found in the peripheral blood of male and female mice administered benzyltrimethylammonium chloride by gavage for 13 weeks.

Based on the mortality observed in the 16-day and 13-week studies, rats and mice appeared to be equally sensitive to benzyltrimethylammonium chloride. The minimally toxic dose for rats and mice was estimated to be 50 mg/kg.

INTRODUCTION

CHEMICAL AND PHYSICAL PROPERTIES

Benzyltrimethylammonium chloride is a quaternary ammonium compound with a structural resemblance to acetylcholine and other chemicals having cholinergic activity. It is an off-white to yellow powder (*Sigma-Aldrich*, 1988) with a melting point of 236° to 239° C (Karsai *et al.*, 1986). It decomposes at 239° C, and the decomposition products include carbon monoxide, carbon dioxide, nitrogen oxides, hydrochloride gas, and ammonia (*Sigma-Aldrich*, 1988; Sax and Lewis, 1989). Benzyltrimethylammonium chloride is hygroscopic (*Sigma-Aldrich*, 1988) and soluble in water, ethanol, and butanol and slightly soluble in butyl phthalate and tributyl phosphate (Sax and Lewis, 1987; Weast, 1989).

PRODUCTION, USE, AND HUMAN EXPOSURE

Benzyltrimethylammonium chloride is prepared by boiling benzyl chloride and trimethylamine in absolute ethanol. In addition, a reaction involving benzyl dimethylamine and methyl chloride, with or without solvent, may be used to manufacture this compound (Karsai *et al.*, 1986). Benzyltrimethylammonium chloride is also prepared by dissolving phenylmethyl chloride in ether and adding 25% trimethylamine in methanol. The product is collected and recrystallized from alcohol and ether (Hume and Holland, 1965).

In 1983, 16 companies were listed as manufacturers of benzyltrimethylammonium chloride. Ten of the manufacturers reported a total production volume ranging from 1.5 to 15.5 million pounds (USEPA, 1990). In 1986 and 1988, respectively, 4,132,000 and 3,985,000 pounds of benzyltrimethylammonium chloride were produced (USITC, 1986, 1987, 1989).

Benzyltrimethylammonium chloride is used as a solvent for cellulose, a gelling inhibitor in polyester resins, a chemical intermediate (Sax and Lewis, 1987), and a paint dispersant for the rubber industry (*Chemical Marketing Reporter*, 1983). It is also used extensively as an acrylic dyeing agent in the textile industry (Moore *et al.*, 1987). Benzyltrimethylammonium chloride is patented for use in plant growth regulator compositions and synthetic processes (Karsai *et al.*, 1986).

Occupational exposure may result from the use of this compound in the chemical (Sax and Lewis, 1987), rubber (*Chemical Marketing Reporter*, 1983), and textile industries (Moore *et al.*, 1987). Data from the National Occupational Exposure Survey, conducted by the National Institute for Occupational Safety and Health during the years 1981 to 1983, estimated that 5,000 workers were potentially exposed to benzyltrimethylammonium chloride (NIOSH, 1990). No exposure or threshold limit values for benzyltrimethylammonium chloride have been established.

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

Male Wistar rats intravenously administered benzyltrimethylammonium iodide eliminated the compound from plasma in two phases. One phase had a half-life of 3 minutes, and the other had a half-life of 70 minutes. Benzyltrimethylammonium chloride had an overall half-life of 30 minutes following intravenous administration. Greater than 90% of the dose was eliminated in the urine; 0.9% was eliminated in the bile. The highest tissue concentration following intravenous dosing was found in the liver and kidney 10 minutes after injection. No metabolites were found in the urine, bile, or plasma (Neef *et al.*, 1984).

Absorption from the gastrointestinal tract did not exceed 40% for F344/N rats or 15% for $B6C3F_1$ mice administered 0.63 mg benzyltrimethylammonium chloride (ring-U-^{14}C)/kg body weight by gavage in water and did not exceed 10% for rats or mice administered 63 mg/kg dermally for 24 hours. Greater than 90% of the gavage dose was excreted in the urine and feces within 24 hours of administration. There was no evidence of metabolism of the parent compound in rats or mice because greater than 90% of the excreted radiolabel was composed of the parent compound (Sanders *et al.*, 1995).

Humans

No absorption, distribution, metabolism, or excretion studies of benzyltrimethylammonium chloride in humans were found in a review of the literature.

TOXICITY

Experimental Animals

An acute median lethal oral dose of 250 mg/kg has been reported for rats of an unspecified strain (Dewitt *et al.*, 1953). The acute oral lethality of 1,600 mg benzyltrimethylammonium chloride/kg body weight in an unspecified number of male TAC:SWfBr mice was reported to be 100% (Ellis *et al.*, 1980). In that study, benzyltrimethylammonium chloride was administered orally as a 2% suspension in 0.5% methylcellulose. The results of those studies indicate that rats are more sensitive than mice to the lethality of benzyltrimethylammonium chloride; these results were used to select the doses for the 16-day NTP studies. A 48-hour median lethal concentration for benzyltrimethylammonium chloride in water fleas *(Daphnia pulex)* was determined to be 11.94 ppm in an aquatic static bioassay (Moore *et al.*, 1987). Sanders *et al.* (1995) studied the acute oral toxicity of 125, 175, 210, and 250 mg benzyltrimethylammonium chloride/kg body weight in groups of five male F344/N rats (300 to 350 g). Mortality was observed in the 175, 210, and 250 mg/kg groups. In addition, benzyltrimethylammonium chloride induced muscarinic cholinergic symptoms of salivation and chromodacryorrhea. These symptoms were relieved by atropine injection, but atropine administration did not reduce mortality.

Humans

No data were found on the acute toxicity of benzyltrimethylammonium chloride in humans. However, 10 human fatalities resulting from overexposure to aryl/alkyl quaternary ammonium compounds have been reported. Three of these fatalities involved benzalkonium chloride, and the subjects suffered complete cardiovascular collapse. A common symptom observed in these fatalities was curariform paralysis. Intramuscular injection reportedly resulted in liver and kidney necrosis (Gosselin *et al.*, 1984).

NEUROTOXICITY

The cholinergic activities of benzyltrimethylammonium chloride were studied in isolated muscle preparations *in vitro* and through quantitation of saliva collected from the submaxillary duct of cats following intravenous administration (Hamilton and Rubinstein, 1968). Researchers concluded that benzyltrimethylammonium chloride is capable of stimulating both nicotinic and muscarinic receptors and speculated that it acts at the same ganglionic site as acetylcholine. This was confirmed by studies in which 25 mg benzyltrimethylammonium chloride/kg body weight, dissolved in tetrahydrofurfuryl alcohol and administered intravenously, induced a gastrocnemius muscle twitch in an unspecified strain of rats (Ellis *et al.*, 1980). Benzyltrimethylammonium chloride (40 μg/kg), administered without atropine to mongrel dogs via a femoral vein canula, mimicked

muscarinic acetylcholinergic activity and caused a decrease in blood pressure. A 400 μg/kg dose administered to atropinized dogs induced a nicotinic increase in blood pressure (Hume and Holland, 1965).

Anticholinergic actions of benzyltrimethylammonium bromide have been demonstrated *in vitro* using superfused frog rectus abdominis muscle and rabbit sciatic nerve gastrocnemius muscle preparations (Dretchen *et al.*, 1971). At low concentrations, the salt inhibited cholinergic receptors. At high concentrations, the salt appeared to have acted by a nonspecific mechanism that may have involved increased potassium efflux.

CARCINOGENICITY

No carcinogenicity studies of benzyltrimethylammonium chloride in experimental animals or humans were found in a review of the literature.

GENETIC TOXICITY

Only one set of published mutagenicity data for benzyltrimethylammonium chloride was identified; therefore, there is insufficient information to fully characterize the genetic activity of this compound. Results of mutagenicity testing in *Salmonella typhimurium* strains TA97, TA98, TA100, and TA1535, with and without induced rat or hamster liver S9 activation enzymes, were uniformly negative (Zeiger *et al.*, 1988). Concentrations of benzyltrimethylammonium chloride ranged from 100 to 10,000 μg/plate. Slight toxicity was noted at the higher doses in some trials, but no increases in the number of mutant colonies were seen.

STUDY RATIONALE AND DESIGN

Benzyltrimethylammonium chloride was nominated for toxicity testing by the National Institute of Environmental Health Sciences because of its high production volume, the potential for occupational exposure, and the limited information on toxicity. Sixteen-day and 13-week toxicity studies were conducted in F344/N rats and B6C3F$_1$ mice administered benzyltrimethylammonium chloride by gavage. This route was selected because benzyltrimethylammonium chloride was found to be poorly absorbed via the dermal route (Sanders *et al.*, 1995). Micronucleus tests were conducted on mouse peripheral blood erythrocytes following treatment with benzyltrimethylammonium chloride by gavage for 13 weeks.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF BENZYLTRIMETHYLAMMONIUM CHLORIDE

Benzyltrimethylammonium chloride was obtained from Fluka Chemical Corporation (Ronkonkoma, NY) in one lot (306793/1). Information on the identity, purity, and stability of the bulk chemical was provided by the manufacturer; identity was confirmed by the study laboratory. Reports on analyses performed in support of the benzyltrimethylammonium chloride studies are on file at the National Institute of Environmental Health Sciences.

The manufacturer identified the chemical, an off-white to yellow crystalline powder, as benzyltrimethylammonium chloride by nuclear magnetic resonance spectroscopy. The purity of lot 306793/1, determined by argentometric titration, was 100.4% or greater. The study laboratory confirmed the identity of the chemical with infrared spectroscopy. The spectrum was consistent with a literature reference for benzyltrimethylammonium bromide (*Aldrich*, 1990).

Based on the manufacturer's stability information, the bulk chemical was stored at room temperature in sealed containers flushed with nitrogen to expel moisture.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations for the 16-day studies were prepared once within 7 days of the beginning of dosing. Dose formulations for the 13-week studies were prepared within 8 days of the first day of dosing, then every 2 to 3 weeks until the end of the study. To prepare the dose formulations, a weighed amount of benzyltrimethylammonium chloride was dissolved in deionized water and diluted to achieve the highest concentration required. Serial dilutions of the highest concentration were made to obtain each of the lower concentrations. Dose formulations were stored in sealed serum vials (16-day studies) or amber glass bottles (13-week studies) at room temperature.

Stability studies of 3.2 and 1.25 mg/mL dose formulations were performed by the study laboratory using ultraviolet spectroscopy. Stability was confirmed for at least 21 days (3.2 mg/mL) or 28 days (1.25 mg/mL) for formulations stored in sealed containers at room temperature.

The study laboratory analyzed the dose formulations used in the 16-day studies and the initial, mid-point, and final dose formulations used in the 13-week studies. The study laboratory also analyzed animal room samples of the same dose formulations after they had been in use for 2 to 3 weeks. All dose formulations administered to rats and mice and all animal room samples were within 10% of the target concentrations.

16-Day Studies

Male and female F344/N rats and $B6C3F_1$ mice were obtained from Taconic Farms (Germantown, NY). On receipt, the rats and mice were 4 weeks old. Animals were quarantined for 13 (rats) or 14 (mice) days and were 6 weeks old on the first day of the studies. Groups of five male and five female rats received 0, 16, 32, 63, 125, or 250 mg benzyltrimethylammonium chloride per kg body weight in deionized water by gavage, 5 days per week for 16 days. Groups of five male and five female mice received 0, 63, 125, 250, 500, or 1,000 mg/kg in deionized water by gavage, 5 days per week for 16 days. Feed and water were available *ad libitum*. Rats and female mice were housed five per cage; male mice were housed individually. Animals were observed daily, and clinical findings and body weights were recorded initially, on day 8, and at the end of the studies. Prior to terminal sacrifice, a functional observation battery was performed on all surviving rats. Details of the study design and animal maintenance are summarized in Table 1.

At the end of the 16-day studies, the animals were anesthetized with a mixture of carbon dioxide and oxygen, and blood was collected from the retroorbital sinus of all rats and mice for hematology and clinical chemistry analyses. Blood samples for hematology analysis were collected in tubes containing EDTA as an anticoagulant. Blood samples for clinical chemistry analysis were collected in untreated clot tubes, and the sera were separated by centrifugation. Hematology parameters were analyzed on a Serono-Baker 9000 Automated Cell Counter (Serono-Baker Diagnostics, Allentown, PA). Manual hematocrit values were determined using an Adams Microhematocrit Centrifuge, CT2900 (Clay Adams, Sparks, MD). Leukocyte differentials and erythrocyte, leukocyte, lymphocyte, and platelet morphology were determined by light microscopy from blood smears stained with modified Wright's stain using an Ames Hematek Slide Stainer (Miles Laboratory, Ames Division, Elkhart, IN). Reticulocyte counts were determined by light microscopy using blood samples stained with new methylene blue (Sigma Chemical Company, St. Louis, MO). Clinical chemistry parameters were measured

using a Hitachi 717® chemistry analyzer (Boehringer Mannheim, Indianapolis, IN). All reagents were obtained from the manufacturer with the exception of the reagents for sorbitol dehydrogenase and total bile acid determinations, which were obtained from Sigma Chemical Company. The parameters measured are listed in Table 1.

A necropsy was performed on all rats and mice. The heart, right kidney, liver, lung, spleen, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 µm, and stained with hematoxylin and eosin. Histopathologic examinations were performed on vehicle control rats and mice, rats receiving 63, 125, or 250 mg/kg, and mice receiving 125, 250, 500, or 1,000 mg/kg. Table 1 lists the tissues and organs examined.

13-WEEK STUDIES

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

Core study groups of 10 male and 10 female rats and mice received benzyltrimethylammonium chloride in deionized water by gavage at doses of 0, 12.5, 25, 50, or 100 mg/kg, 5 days per week for 13 weeks. Feed and water were available *ad libitum*. Rats and female mice were housed five per cage; male mice were housed individually. Clinical findings were recorded and animals were weighed initially, on day 8, and weekly until the end of the studies. A functional observation battery was performed on core study rats on days 10 and 85. Details of the study design and animal maintenance are summarized in Table 1.

On days 3 and 21, blood was collected from the retroorbital sinus of groups of 10 special study rats administered the same doses as core study rats for hematology and clinical chemistry analyses. At the end of the 13-week studies, blood was collected from the retroorbital sinus of all core study rats for hematology and clinical chemistry analyses and from all core study mice for clinical chemistry analyses. Methods used for

hematology and clinical chemistry analyses were the same as those used in the 16-day studies. The parameters measured are listed in Table 1.

At the end of the 13-week studies, samples were collected for sperm motility and vaginal cytology evaluations on core study rats and mice receiving 0, 25, 50, or 100 mg/kg. The parameters evaluated are listed in Table 1. Methods used were those described in the NTP's sperm morphology and vaginal cytology evaluations protocol (NTP, 1991). For 12 consecutive days prior to the scheduled terminal sacrifice, the vaginal vaults of the females were moistened with saline, if necessary, and samples of vaginal fluid and cells were stained. Relative numbers of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were determined and used to ascertain estrous cycle stage (i.e., diestrus, proestrus, estrus, and metestrus). Male animals were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk (rats) or modified Tyrode's buffer (mice) was applied to slides and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were minced, and the tissue was incubated in the saline solution and then heat fixed at 65° C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

A necropsy was performed on all core study rats and mice. The heart, right kidney, liver, lung, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 5 μm, and stained with hematoxylin and eosin. A complete histopathologic examination was performed on vehicle control groups and all 100 mg/kg rats and mice. Table 1 lists the tissues and organs routinely examined.

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

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Benzyltrimethylammonium Chloride

16-Day Studies	13-Week Studies
Study Laboratory	
Microbiological Associates, Inc. (Bethesda, MD)	Microbiological Associates, Inc. (Bethesda, MD)
Strain and Species	
Rats: F344/N Mice: $B6C3F_1$	Rats: F344/N Mice: $B6C3F_1$
Animal Source	
Taconic Farms (Germantown, NY)	Taconic Farms (Germantown, NY)
Time Held Before Studies	
Rats: 13 days Mice: 14 days	Rats: 12 days (males) or 13 days (females) Mice: 14 days (males) or 15 days (females)
Average Age When Studies Began	
6 weeks	6 weeks
Date of First Dose	
Rats: 10 August 1992 Mice: 11 August 1992	Rats: 12 (males) or 13 (females) October 1993 Mice: 14 (males) or 15 (females) October 1993
Duration of Dosing	
16 days (5 days/week)	13 weeks (5 days/week)
Date of Last Dose	
Rats: 25 August 1992 Mice: 26 August 1992	Rats: 10 (males) or 11 (females) January 1994 Mice: 12 (males) or 13 (females) January 1994
Necropsy Dates	
Rats: 26 August 1992 Mice: 27 August 1992	Rats: 11 (males) or 12 (females) January 1994 Mice: 13 (males) or 14 (females) January 1994
Average Age at Necropsy	
8 weeks	19 weeks
Size of Study Groups	
5 males and 5 females	10 males and 10 females
Method of Distribution	
Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 16-day studies
Animals per cage	
Rats: 5 Mice: 1 (males) or 5 (females)	Rats: 5 Mice: 1 (males) or 5 (females)
Method of Animal Identification	
Tail tattoo	Tail tattoo
Diet	
NIH-07 open formula pelleted diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed weekly	NIH-07 open formula meal diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*, changed weekly

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Benzyltrimethylammonium Chloride

16-Day Studies	13-Week Studies
Water Tap water (Washington Suburban Sanitary Commission Potomac Plant) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available *ad libitum*	Same as 16-day studies
Cages Polycarbonate (Lab Products, Inc., Rochelle Park, NJ), changed twice weekly for group-housed animals and once weekly for individually housed animals	Same as 16-day studies
Bedding Sani-Chips® (P.J. Murphy Forest Products, Montville, NJ) changed twice weekly for group-housed animals and once weekly for individually housed animals	Same as 16-day studies
Racks Stainless steel (Lab Products, Inc., Rochelle Park, NJ), changed and rotated every 2 weeks	Same as 16-day studies
Animal Room Environment Temperature: 20.6°-23.9° C Relative humidity: 35%-65% Room fluorescent light: 12 hours/day Room air changes: 10/hour	Temperature: 20.6°-23.9° C Relative humidity: 35%-65% Room fluorescent light: 12 hours/day Room air changes: 10/hour
Doses Rats: 0, 16, 32, 63, 125, or 250 mg/kg in deionized water by gavage (dosing volume=5 mL/kg body weight) Mice: 0, 63, 125, 250, 500, or 1,000 mg/kg in deionized water by gavage (dosing volume=10 mL/kg body weight)	0, 12.5, 25, 50, or 100 mg/kg in deionized water by gavage (dosing volume=5 mL/kg body weight for rats and 10 mL/kg body weight for mice)
Type and Frequency of Observation Observed twice daily; clinical findings and body weights were recorded initially, on day 8, and at the end of the studies.	Observed twice daily; animals were weighed and clinical findings were recorded initially, on day 8, and weekly thereafter until the end of the studies.
Method of Sacrifice CO_2 asphyxiation	Same as 16-day studies
Necropsy Necropsy performed on all animals. Organs weighed were the heart, right kidney, liver, lung, spleen, right testis, and thymus.	Necropsy performed on all core study animals. Organs weighed were the heart, right kidney, liver, lung, right testis, and thymus.

TABLE 1
Experimental Design and Materials and Methods in the Gavage Studies of Benzyltrimethylammonium Chloride

16-Day Studies	13-Week Studies
Clinical Pathology	
Blood was collected from the retroorbital sinus of all rats and mice surviving to the end of the studies for hematology and clinical chemistry. ***Hematology***: automated and manual hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and nucleated erythrocyte counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; platelet count; and total leukocyte count and differentials ***Clinical chemistry***: urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, serum cholinesterase, erythrocyte cholinesterase, and bile acids	Blood was collected from the retroorbital sinus of all special study rats on days 3 and 21 and from all core study rats surviving until the end of the study for hematology and clinical chemistry. Blood was collected from the retroorbital sinus of all mice surviving to the end of the study for clinical chemistry. ***Hematology***: automated and manual hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and nucleated erythrocyte counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; platelet count; and total leukocyte count and differentials ***Clinical chemistry***: urea nitrogen, creatinine, total protein, albumin, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, serum cholinesterase, and bile acids
Histopathology	
Complete histopathology was performed on all vehicle control animals, 63, 125, and 250 mg/kg rats, and 125, 250, 500, and 1,000 mg/kg mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone and marrow, brain, clitoral gland, esophagus, gallbladder (mice), heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland (except male mice), nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, uterus, and Zymbal's gland (rats).	Complete histopathology was performed on all core study vehicle control and 100 mg/kg rats and mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, gallbladder (mice), heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland, muscle, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spinal cord, spleen, stomach (forestomach and glandular), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, urinary bladder, uterus, and Zymbal's gland (rats). The lung of all 50 mg/kg rats was also examined.
Sperm Motility and Vaginal Cytology	
None	At the end of the studies, sperm samples were collected from all core study male rats and mice in the 0, 25, 50, and 100 mg/kg groups for sperm motility evaluations. The following parameters were evaluated: spermatid heads per testis and per gram testis, spermatid counts, and epididymal spermatozoal motility and concentration. The left cauda epididymis, left epididymis, and left testis were weighed. Vaginal samples were collected for up to 12 consecutive days prior to the end of the studies from all core study females administered 0, 25, 50, or 100 mg/kg for vaginal cytology evaluations. The parameters evaluated were estrous cycle length and the percentage of cycle spent in the estrous cycle stages.
Functional Observation Battery	
At the end of the study, all surviving rats were subjected to a functional observation battery. The parameters evaluated were body position, activity level, coordination, gait, general behavior, head-flick, head-searching, compulsive licking or biting, backward walking, self-mutilation, circling, convulsions, tremors, lacrimation or chromodacryorrhea, salivation, piloerection, pupillary dilation or constriction, unusual respiration, diarrhea, excessive or diminished urination, and vocalization.	Core study rats were subjected to a functional observation battery on days 10 and 85. The parameters evaluated were the same as those evaluated in the 16-day studies.