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Final Report

PRELIMINARY TOXICOLOGICAL STUDIES OF TATB, TCB, AND TCTNB

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SUMMARY

The acute oral LD50s for TATB and TCTNB in both sexes of rats and mice were greater than 5 g/kg. TCB had the following LD50s: 1800 mg/kg in male rats, 2800 mg/kg in female rats, 3350 mg/kg in male mice, and 3402 mg/kg in female mice.

Primary skin irritation studies in the rabbit showed that TCB and TCTNB were mildly irritating and that TATB was nonirritating. Eye irritation studies, also in the rabbit, showed that TCB and TATB were mild but transitory irritants, with TCTNB responding as a severe irritant. The sensitization study in the guinea pig did not show TATB, TCB, or TCTNB to be sensitizers.

TCTNB, TATB, and TCB were not mutagenic when tested in five strains of Salmonella typhimurium and in Escherichia coli strain WP2. TCTNB was the most toxic of the three compounds tested in these strains, and it also showed toxic effects in the recombination-deficient Bacillus subtilis strain M45. None of the compounds showed toxic effects in repair-deficient E. coli strain p3478. Mitotic recombination in the yeast S. cerevisiae D3 was increased by TCB but not by TCTNB or TATB; however, a dose-related response could not be established.

The response of rats exposed for 1 hour to saturated TCB vapor indicates that TCB does not present an acute inhalation hazard. The only noteworthy observation was that the rate of weight gain for the treated group was less than that of the controls. The results indicated that TATB is not an acute inhalation hazard as a dust. TCTNB probably would have the same hazard value as TATB when inhaled as a dust. However, TCTNB was administered as an aerosol in DMSO, and the presence of DMSO may have enhanced the TCTNB bioavailability by increasing its solubility in biological fluids and increasing pulmonary membrane permeability. These studies have indicated the potential acute inhalation toxicity of TATB, TCB, and TCTNB. They did not address the problem of continuous daily exposures, such as might be encountered in the work environment.

INTRODUCTION

As part of its overall program to obtain safety information on potential explosives, Lawrence Livermore Laboratory (LLL) is particularly interested in TATB, TCB, and TCTNB.* At the request of LLL, SRI designed and conducted the following studies on TATB, TCB, and TCTNB:

(1) Acute oral LD50 in the rat and mouse

(2) Skin and eye irritation in the rabbit

(3) Skin sensitization in the guinea pig

(4) *In vitro* mutagenesis/carcinogenesis in microorganisms

(5) One-hour dust or vapor inhalation in the rat.

The following compound batches were used in the studies listed above:

Compound	Batch Number	Used in Study Number:
TATB	B-318	(1) through (5)
	B-365	In storage
TCB	B-320	(1) through (4)
	B-367	(5)
TCTNB	B-319	(1) through (4)
	B-366	(5)

(5) 1,3,5- (Sniner

* TATB: 1,3,5-triamino-2,4,6-trinitrobenzene; TCB: 5-trichlorobenzene; TCTNB: 1,5,5-trichloro-2,4,6-trinitrobenzene.

EXPERIMENTAL PROCEDURES

Single-Dose Oral Toxicity (LD50)

Rat

Ten young-adult Sprague-Dawley-derived rats of each sex, weighing 70 to 111 g, were used in each of the three test groups. Each compound was administered to fasted rats as a suspension in corn oil. The graded dosages were given intragastrically with a ball-point needle and syringe.

After treatment, all animals were observed daily for physiological and behavioral responses. Body weights were recorded initially and weekly thereafter. At the end of the 2-week observation period, all surviving animals were sacrificed and examined for evidence of gross pathologic changes.

Initially, we conducted range-finding studies at various dose levels to identify the zone of toxicity. Three to five animals were used per level. The results of this preliminary work are not presented in this report, but the data are on file at SRI.

SRI and LLL agreed to use 5 g/kg as the maximum dose for testing. Any compound showing 50% mortality or less at this level was reported as having an LD50 of greater than 5 g/kg.

Mouse

The procedures for the single-dose oral toxicity (LD50) study in the mouse were similar to those used for the rat. Ten young-adult ICR/SIM mice of each sex, weighing 20 to 31 g, were used in each test group.

As in the rat study, all animals were observed daily after treatment for physiological and behavioral responses. Body weights were recorded initially and then weekly. After 2 weeks of observation, all animals were sacrificed and examined for evidence of gross pathological changes. The maximum dose tested was 5 g/kg, the same as that established for the rat study.

Primary Skin Irritation

The procedure used to assess primary skin irritation was the Draize Test. Six New Zealand white rabbits, weighing 3 to 5 lb each, were used in each test group. Only one compound was applied to an individual rabbit. The back of each rabbit was clipped free of hair 24 hr before application of the compounds. Two sites were abraded and two were unabraded. Each compound was applied in 0.5-g quantities to each test site. The test sites were covered with a gauze pad, and the entire back was overwrapped with rubber dam. Twenty-four hours later, the wraps were removed, excess compound was removed, and each test site was scored for erythema and edema. Readings were recorded for the 24-hour examination and again at 72 hours. A final irritation score was calculated from the 24- and 72-hour readings.

Eye Irritation

Nine New Zealand white rabbits, weighing 3 to 5 lb each, were used in each test group. Only one compound was administered to an individual rabbit. The compound was instilled in 0.1-g quantities into the left eye of each rabbit, the right eye serving as the control. In each test group, three of the rabbits had the compound washed from the eye 30 seconds after instillation; three, 5 minutes after instillation; and three did not have the compound washed from the eye.

Each eye was graded for occular lesions at 1 and 4 hours on the day of application and again at 24, 48, 72, and 96 hours. Of particular interest was determination of whether the cornea, iris, or conjunctiva developed irritancy and/or showed a transitory response (lasting for more than a few hours). In cases of severe eye irritation, we continued observations for 7 days or more to identify more closely the recovery time. The procedure and grading system were taken from the Draize Test.

Skin Sensitization

Ten male Hartley-strain guinea pigs, weighing 300 to 400 g, were used in the vehicle control group and in each of the three treatment groups. The animals were housed individually and received a commercial

laboratory ration supplemented on alternate days with fresh lettuce or cabbage.

Each test compound was diluted to a concentration of 0.1% with a 1% solution of carboxymethylcellulose. The control group received the 1% solution of carboxymethylcellulose alone. Each compound solution was administered in a series of ten "sensitizing" injections into the lower back and flanks of the guinea pigs. Before each injection, the test site was clipped free of hair with electric small-animal clippers. Injections were made randomly over the test area every other day via a 1-cc tuberculin syringe fitted with a 26-gauge needle. The volume of the first injection was 0.05 ml; the other nine injections were each 0.1 ml. Twenty-four hours after each injection, the resulting reaction was scored for redness, height, and diameter. Redness and height were scored on a scale of 0 to 4, as described by Draize; diameters of reactions were measured in millimeters using micrometer calipers.

Two weeks after administration of the tenth sensitizing injection, the lower back and flanks of each experimental guinea pig were clipped free of hair, and a challenge injection of 0.05 ml was administered. Each animal's reactions were graded 24 hours later and compared with those from the sensitizing injections.

In Vitro Mutagenesis/Carcinogenesis

Background

SRI examined TATB, TCTNB, and TCB for mutagenicity by *in vitro* microbiological assays with *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, TA100; *Escherichia coli* strain WP2; repair-deficient and repair-proficient strains of *Bacillus subtilis* and *E. coli*; and with the yeast *Saccharomyces cerevisiae* D3. An Aroclor 1254-stimulated, rat liver-homogenate metabolic activation system was included in each procedure (except in the DNA repair assay) to provide metabolic steps that the bacteria either are incapable of conducting or do not conduct under the assay conditions. The purpose of this study was to determine whether the compounds elicited a mutagenic response in microorganisms.

The assay procedure with S. typhimurium has been proven to be 85 to 90% accurate in detecting carcinogens as mutagens, and it has about the same accuracy in identifying chemicals that are not carcinogenic.[1] The assay procedure with S. cerevisiae is about 60% accurate in detecting carcinogens as agents that increase mitotic recombination.[2] The assay with E. coli WP2 and the microbial sensitivity assay are two additional systems for detecting mutagens. The combination of these four assay procedures significantly enhances the probability of detecting potentially hazardous chemicals. However, because the test systems are not 100% accurate, neither a positive nor a negative result proves that a chemical is hazardous or nonhazardous to man.

Salmonella typhimurium strains TA1535, TA1537, TA1538, TA92, and TA100

The S. typhimurium strains used at SRI were obtained from Dr. Bruce Ames of the University of California at Berkeley.[3-5] All are histidine auxotrophs (his$^-$) by virtue of mutations in the histidine operon. In addition to the mutations in the histidine operon, five of the indicator strains have mutations in the lipopolysaccharide coat (rfa$^-$) and deletions that cover a gene involved in the repair of uv damage (uvrB$^-$). The rfa$^-$ mutation makes the strains more permeable to large molecules, thereby increasing their sensitivity to these molecules. The uvrB$^-$ mutation decreases repair of some types of chemically damaged DNA and thereby enhances sensitivity to some mutagenic chemicals. Strain TA1535 is reverted to histidine prototrophy (his$^+$) by many mutagens that cause base-pair substitutions. Strains TA1537 and TA1538 are reverted by many frameshift mutagens. TA1537 is more sensitive than TA1538 to mutations by some acridines and benzanthracenes, but the difference is quantitative rather than qualitative. TA100 is derived from TA1535 by the introduction of the R factor plasmid pKM101.[6] The introduction of this plasmid, which confers ampicillin resistance to the strain, greatly enhances the sensitivity of the strain to some base-pair substitution mutagens. We have shown that mutagens such as benzyl chloride and 2-(2-furyl)-3-(5-nitro-2furyl)-acrylamide (known as AF2) can be detected in plate assays by TA100 but not by TA1535. The presence of this plasmid also makes strain

TA100 sensitive to some frameshift mutagens--e.g., ICR-191, benzo(a)pyrene, aflatoxin B_1, and 7,12-dimethylbenz(a)anthracene. TA98 is derived from TA1538 by the addition of the same plasmid, and the plasmid makes this strain more sensitive to some mutagens. TA92 is the same as TA100 except that it is uvrB$^+$ and rfa$^+$.

All the indicator strains are stored in liquid nitrogen. For each experiment, an inoculum from frozen stock cultures is grown overnight at 37°C in a nutrient broth consisting of 1% tryptone and 0.5% yeast extract. After stationary overnight growth, the cultures are shaken for 3 to 4 hours to ensure optimal growth. Each culture is checked for sensitivity to crystal violet. The presence of the rfa$^-$ mutation makes the indicator strains sensitive to this dye, whereas the parent strain, rfa$^+$, is not sensitive to the dye. However, the mutation is reversible, leading to the accumulation of rfa$^+$ cells in the culture. Therefore, the cells must be tested routinely to ensure their sensitivity to crystal violet. Each culture also is tested by specific mutagens known to revert each test strain (positive controls).

To a sterile 13 x 100 mm test tube placed in a 43°C heating block, we add in the following order for assays in agar:

(1) 2 ml of 0.6% agar*

(2) 0.05 ml of indicator organisms

(3) 50 µl of a solution of the test chemical

(4) 0.50 ml of metabolic activation mixture (optional).

For negative controls, we use steps (1), (2), and (4) (optional) and 50 µl of the solvent used for the test chemical.

This mixture is stirred gently and then poured onto minimal agar plates.† After the soft agar has set, the plates are incubated at 37°C for 2 days. The number of his$^+$ revertants (colonies that grow on plates

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* 0.6% agar contains 0.05 mM histidine and 0.05 mM biotin.

† Minimal agar plates consist of 15 g of agar, 20 g of glucose, 0.2 g of $MgSO_4 \cdot 7\ H_2O$, 2 g of citric acid monohydrate, 10 g of K_2HPO_4, and 3.5 g of $NaHNH_4PO_4 \cdot H_2O$ per liter.

lacking a sufficient amount of histidine to support colony formation) are counted and recorded. Some of the revertants are routinely tested to confirm that they are his$^+$, require biotin, and are sensitive to crystal violet (rfa$^-$).

Escherichia coli strain WP2

The E. coli WP2 (uvrA$^-$)[7,8] used in this project was given to us by Dr. D. McCalla. A procedure similar to the one used with Salmonella is used to measure the reversion of WP2 to tryptophan independence. However, instead of containing a trace of tryptophan in the top agar, the minimal agar plates contain 1.25 g of oxoid broth per liter to provide the trace of tryptophan required for enhancement of any mutagenic effect of the test chemical.

In addition to reversion of the mutated tryptophan gene, WP2 may undergo a forward mutation in a tryptophan tRNA gene to achieve tryptophan independence. This is called phenotypic reversion. We do not distinguish experimentally between the true revertants and the phenotypic revertants (although the latter tend to form smaller colonies).

Escherichia coli strains W3110 and p3478 and Bacillus subtilis strains H17 and M45

We obtained the E. coli strains W3110 and p3478 from Dr. H. Rosenkranz.[9] Strain p3478 is a polA$^-$ derivative of strain W3110. It carries a single, revertible mutation in a gene for a DNA polymerase; Gross and Gross[10] showed that this mutation is involved in DNA repair synthesis. This mutation increases the sensitivity of strain p3478 to chemicals that lead to alternations of or damage to the DNA. Therefore, we can assay for chemicals that damage DNA by comparing the relative sensitivity of p3478 and W3110 to the test chemicals.

The B. subtilis strains H17 and M45 were obtained from Dr. Kada.[11] Strain M45 (rec$^-$) is derived from H17, but it is deficient in the genetic recombination mechanism necessary to repair DNA damage. Cells deficient in this repair mechanism are killed more easily by some chemical mutagens than are wild-type recombination-proficient cells. If a chemical

is toxic to rec$^-$ cells, but at the same concentration is not equally toxic to rec$^+$ cells, the chemical probably is a mutagen.

Inoculums from frozen stocks are grown overnight in nutrient broth* at 37°C with shaking. To 2 ml of nutrient broth containing 0.6% agar is added 0.1 ml of the test culture. The suspension is mixed and poured onto plates containing nutrient broth and 2% agar.

After the soft agar has solidified, a sterile filter disc impregnated with the test chemical is placed in the center of the plate. The plates are incubated at 37°C for 16 hours, and the width of the zone of toxicity or inhibition of growth is then measured. We usually must test several concentrations of chemical to detect accurately differences in the zones of growth inhibition, because higher initial concentrations lead to steep concentration gradients that may reduce the differences in growth inhibition of the two strains.

Positive controls for this assay are 1-phenyl-3,3-dimethyltriazene (1 mg) and N-methyl-N'-nitro-N-nitrosoguanidine (20 μg) placed on the filter disc. The sizes of the zones of toxicity caused by these mutagens correspond to the relative sensitivities of the repair-deficient strains (p3478 and M45) and their repair-proficient parent strains (W3110 and H17). The negative control is 50 μg of chloramphenicol placed on the disc. In this case, equal zones of inhibition are expected in genetically related strains since the toxicity of this control does not depend on a mechanism that leads to DNA damage. All assays are performed at least twice and in duplicate.

Saccharomyces cerevisiae D3

The yeast S. cerevisiae D3 is a diploid heterozygous for a mutation in adenine-metabolizing enzymes.[12] Cells homozygous for this mutation produce a red dye when grown on medium containing adenine. Adenine-requiring homozygotes can be generated from the heterozygotes by mitotic recombination. Many mutagens increase the frequency of mitotic recombination. Mitotic recombination is indicated by the development of colonies with red pigmentation, and the number of pigmented colonies obtained indicates the mutagenicity of a compound or its metabolite.[13]

The Saccharomyces test strain from the liquid nitrogen is grown overnight at 30°C with aeration in 1.0% tryptone and 0.5% yeast extract. The cells are washed twice in 0.067 M PO_4 buffer (pH 7.4) and resuspended in the same buffer at a concentration of 10^8 cells/ml.

The *in vitro* yeast mitotic recombination assay in suspension consists of 5 x 10^7 washed stationary-phase yeast cells in 1 ml of 0.067 M PO_4 buffer (pH 7.4) and 50 mg/ml of the test chemical (or a fraction of the concentration required to give approximately 50% killing). The suspension is incubated at 30°C for 4 hours. After incubation, the sample is diluted serially in sterile saline and plated on tryptone-yeast-agar plates. Plates of a 10^{-3} dilution are incubated for 2 days at 30°C, followed by 2 days at 4°C to enhance the development of the red pigment indicative of adenine-negative homozygosity. To detect red colonies or red sectors, we scan the plates with a dissecting microscope at 10 x magnification. Plates of a 10^{-5} dilution are incubated for 2 days at 30°C for determination of the total number of colony-forming units.

The *in vitro* yeast mitotic recombination assay with metabolic activation is conducted as above with the addition of the metabolic activation system to the incubation mixture.

Aroclor 1254-Stimulated Metabolic Activation System

Some carcinogenic mutagens (e.g., dimethylnitrosamine) are inactive unless they are converted to their active form by being metabolized. Kier et al.[14] have described the metabolic activation systems we use. Adult male rats are given a single 500-mg/kg intraperitoneal injection of a polychlorinated biphenyl (Aroclor 1254)[15] Four days after the injection, the animals are deprived of food. On the fifth day, the rats are sacrificed.

The livers are removed aseptically and placed in preweighed, sterile glass beakers. The organ weight is determined, and all subsequent operations are conducted in an ice bath. The organ is washed in an equal volume of cold, sterile 0.15 M KCl (1 ml/g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 M KCl, and homo-

genized with a Potter-Elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 9000 x g, and the supernatant is removed and stored in liquid nitrogen. To the postmitochondrial supernate are added $MgCl_2$, KCl, glucose-6-phosphate, TPN, and sodium phosphate (pH 7.4).

Inhalation Studies

Inhalation Chambers

Exposures to TCB and TATB were conducted in a cylindrical Lucite chamber approximately 12 inches in diameter and 24 inches high. Rats were exposed to the dust or vapor through two rows 1.5-inch holes in the middle part of the chamber, which allow for head-only exposures. The rats were placed in wire restrainers on shelves around the inhalation chamber in such a way that their heads protruded into the chamber. This entire exposure system is contained within a glove box so that hazardous materials can be controlled.

Exposures to TCTNB were conducted in a chamber suited for liquid aerosols. This chamber is made of stainless steel and Lucite. The animals were contained in wir restrainers and only their heads were exposed.

The exhaust from the first chamber was routed through a glass-wool column and two liquid traps to prevent the vapor or dust from escaping into the room. The exhaust from the second chamber was routed through an in-line incinerator.

Generating Systems

TCB was generated as a vapor by metering air at 33 liters/min over a layer of TCB in a flask maintained at 71 to 72°C by a water bath. The air temperature at the animal level was 24.5°C. This system provided a saturated TCB vapor for the animals to inhale.

TATB was generated as a dust. The dust was placed in a 500-ml Erlenmeyer flask fitted with a glass sidearm that allowed a stream of air to be directed toward the bottom of the flask. The flask was connected to

the top of the inhalation chamber by a polyethylene tube and was placed on a vibrator so that the dust was constantly stirred up while the air stream carried the smallest particles into the chamber. The air input into the generator was approximately 8.7 liters/min.

TCTNB was generated as a 10% solution in DMSO after agglomeration of particles precluded generating it as a dust. Four DeVilbiss nebulizers operated at 15 psi were used to generate the aerosol into a 2.5-liter mixing chamber. This aerosol was carried into the inhalation chamber through a primary dilution column at 13 liters/min.

Aerosol/Vapor Concentrations

Vapor concentrations of TCB were estimated based on the amount generated and not recovered from the generator and from the tubing to the inhalation chamber. The nominal concentration of TCB was calculated to be 9.3 g/m^3.

At least four air samples were collected from the inhalation chamber at the animal nose level during the exposures to TATB and TCTNB. Samples were collected on preweighed nucleopore filters by pulling a known amount of chamber air through the filter. The filters were weighed again with a Perkin-Elmer Model AD2 microbalance that has an accuracy of ± 1 µg. The analytical concentration of TATB was found to be 219 ± 59 mg/m^3, and that for TCTNB was 682 ± 391 mg/m^3.

Inhalation Exposures

All exposures were for 60 minutes. The animals were weighed just before they were placed in the wire restrainers. During the exposure and daily thereafter for 14 days, the animals were observed for toxic signs. They were weighed again 7 and 14 days after exposure. After 14 days, the animals were sacrificed and autopsied for gross pathological changes.

RESULTS AND DISCUSSION

Single-Dose Oral Toxicity (LD50) in the Rat

TATB

None of the male or female rats treated orally with a single 5-g/kg dose of TATB died. All animals appeared to be normal throughout dosing and during the post-treatment observation period.

All the rats steadily gained weight throughout the study and revealed no gross abnormalities at necropsy.

The oral LD50 for TATB in male and female rats is greater than 5 g/kg.

TCTNB

None of the male or female rats that received 5 g/kg TCTNB as a single oral dose died. However, all animals exhibited mild depression and inactivity during the first 48 hours after treatment. Additionally, the treated rats had a rough hair coat for the first 56 hours after treatment. Throughout the remainder of the study, all the rats appeared normal.

Body weights increased throughout the study; however, the overall increase was approximately 27% less than that of the TATB-treated rats. Necropsy of all rats at the end of the observation period revealed no gross abnormalities.

The oral LD50 for TCTNB in male and female rats is greater than 5 g/kg.

TCB

Table I summarizes the oral toxicity of TCB in corn oil to male and female rats. In general, all rats exhibited a rough hair coat during the first 48 hours after treatment. Additionally, for those animals

that eventually died, the following observations were noted: Initially, the animals showed signs of passive tremors, depression, and inactivity that led to prostration, persistent tremors, coma, and death. With the exception of one male administered 2500 mg/kg that died 10 days after treatment, all deaths occurred between 21 and 96 hours after treatment. The behavioral and physiological responses of survivors approximately 48 hours after treatment appeared normal.

Necropsy of all surviving rats (both male and female) revealed no gross abnormalities.

The oral LD50 for TCB was 1800 mg/kg for male rats and 2800 mg/kg for female rats.

Single-Dose Oral Toxicity (LD50) in the Mouse

TATB

None of the male or female mice treated orally with a single 5-g/kg dose of TATB died. All male mice had a rough hair coat during the first 4 days post-treatment, but the coat returned to normal thereafter. The females showed no adverse effects throughout the study.

All animals steadily gained weight throughout the study and revealed no gross abnormalities at necropsy.

The oral LD50 for TATB in male and female mice is greater than 5 g/kg.

TCTNB

None of the male or female mice that received 5 g/kg TCTNB as a single dose died. All animals appeared to be normal throughout dosing and during the post-treatment observation period.

The animals steadily gained weight throughout the study and revealed no gross abnormalities at necropsy.

The oral LD50 for TCTNB in male and female mice is greater than 5 g/kg.

TCB

Table 2 summarizes the oral toxicity of TCB in corn oil to male and female mice. Observations included rough hair coat, passive tremors, depression, inactivity, prostration, persistent tremors, coma, and eventually death. All animals that showed signs other than just rough hair coat eventually died. Deaths occurred between 22 and 288 hours hours after treatment. Survivors appeared to have normal body weights and normal behavioral and physiological responses approximately 48 hours after treatment.

Necropsy of all survivors revealed no gross abnormalities.

Primary Skin Irritation

TATB caused a minimum amount of edema in one of the six treated rabbits at the 24-hour reading only. The total primary irritation score for TATB was 0.04.

All six rabbits treated with TCTNB showed edema at 24 hours, with two rabbits also showing erythema. Only two rabbits showed erythema and edema at the 72-hour reading. The total primary irritation score for TCTNB was 0.67.

The TCB-treated rabbits all showed erythema and edema at the 24-hour readings, and only three showed erythema at the 72-hour reading. The total primary irritation score for TCB was 0.88.

The primary skin irritation studies showed TATB to be nonirritating and TCTNB and TCB to be mildly irritating to the skin.

Eye Irritation

Tables 3, 4, and 5 summarize the eye irritation responses for TATB, TCTNB, and TCB, respectively.

TATB

Irritation was observed in conjunctival and iris tissues only. All three rabbits in the no-wash and 5-minute wash groups showed circumcorneal injection around the iris at the 1-hour reading. This condition persisted for 3 days in the no-wash group. Occasional circumcorneal injection responses were noted in individual rabbits at all treatment levels, but all effects involving the iris had disappeared by day 4 after treatment.

Conjunctival responses were observed at all treatment levels and generally involved mild redness, chemosis, and discharge. The treated eye of all rabbits in the 30-second wash group had returned to normal within 24 hours, whereas the treated eye of rabbits in the 5-minute wash group did not completely clear until day 4, and the no-wash group was not judged normal in response until day 7. Overall, the degree of eye irritation caused by TATB was mild but transitory.

TCTNB

TCTNB caused irritation in all three ocular tissues, although the degree varied among individual rabbits.

No corneal damage was observed in rabbits that received a thorough eye wash 30 seconds after compound instillation. However, two of the three rabbits did show occasional circumcorneal injection. Redness, chemosis, and discharge were observed in all rabbits. The above responses were transitory, and the eyes of all rabbits in the 30-second wash group had returned to normal 7 days post-treatment.

Two rabbits in the group that received a thorough eye wash 5 minutes after compound instillation showed mild corneal opacity at the 4-hour reading. All three rabbits in this group showed some circumcorneal injection. Redness, chemosis, and discharge were observed in all rabbits, with one continuing to show these symptoms through a 28-day observation period; readings were not continued beyond this 4-week period.

The treated eyes of all three rabbits in the no-wash treatment group exhibited maximum swelling during the 1-, 4-, and 24-hour readings, thus

preventing us from accurately scoring the cornea and iris tissues. Readings of the cornea, iris, and conjunctival tissues remained at 20 to 50% of maximum scores throughout an extended 28-day observation period. Because of the persistent damage in all three occular tissues examined, the overall degree of eye irritation caused by TCTNB was severe.

TCB

TCB caused irritation of all three occular tissues examined. No corneal damage was observed in the 30-second and 5-minute wash treatment groups, but a minor effect was observed in the cornea of the no-wash group. The corneal tissues had returned to normal within 3 days. Occasional circumcorneal injection was observed in individual rabbits at all levels, but all effects on the iris had disappeared by day 4 post-treatment.

Conjunctival responses were observed at all treatment levels and generally involved varying degrees of redness, chemosis, and discharge. The eyes of the rabbits in the 30-second wash group had returned to normal within 24 hours, but the 5-minute wash group was not normal until day 3, and the no-wash group did not return to normal until 7 days post-treatment. The overall degree of eye irritation caused by TCB was mild but transitory.

Skin Sensitization

Careful review of the data collected from each guinea pig in each treatment group indicates that all challenge injections for all three compounds were within the limits of reactions recorded during the sensitizing period.

The guinea pig skin sensitization study did not show TATB, TCTNB, or TCB to be sensitizers.

In Vitro Mutagenesis/Carcinogenesis

S. typhimurium

Table 6 presents the results of the microbiological assays with the five S. typhimurium strains and E. coli strain WP2. None of the compounds was mutagenic toward any of the strains of bacteria tested. TCTNB was more toxic than TATB or TCB at the higher dose levels.

The DMSO solutions of TCTNB changed color, progressing from clear to bright yellow over a few hours. We do not know what chemical reactions may be involved in this color change nor whether mutagenic properties of the compound are affected.

S. cerevisiae

Table 7 presents the results for mitotic recombination in S. cerevisiae. TCB increased mitotic recombination in these assays. A clear positive response is indicated by a more than threefold increase in the absolute number of recombinants (mitotic recombinants per milliliter) as well as in the relative number of mitotic recombinants (recombinants per 10^5 survivors). Such a response was not consistently present in the assays with TCB, and a dose-related response was not obtained. Although a large enough increase in recombinants was seen to indicate that TCB is mutagenic, the increase in mitotic recombination was slight compared with the positive control (1,2,3,4-diepoxybutane).

Neither TATB nor TCTNB was mutagenic in S. cerevisiae D3, but the latter compound was toxic to the yeast; survival of the organism was less than 1% when the concentration of TCTNB was 0.004%. The order of toxicities of the compounds is TCTNB > TCB > TATB.

DNA Repair Assays

As shown in Table 8, TCTNB demonstrated a preferential killing of the recombination-deficient B. subtilis strain M45, although little inhibition of the parent strain H17 (rec^+) was observed. This presumably indicates an ability of TCTNB to cause damage to DNA that cannot be repaired in the rec^- B. subtilis cells. No zone of growth inhibition by

TCTNB was observed in the repair-deficient E. coli strain p3478. No other toxic effects related to DNA repair were seen in the assays with TATB or TCB in the E. coli or B. subtilis strains.

Inhalation Studies

TCB

Eight male and eight female rats were exposed to TCB. During the exposure three rats appeared to be salivating. As the exposure continued, some TCB was seen to crystallize on the whiskers and fur around the animals' noses. All animals appeared to have some mild irritation around the eyes. After the exposure, no mortality or toxic signs were noted, and the animals appeared to be normal over the next 14 days. However, during the 2 weeks after exposure, the weight gain for the treated animals was slightly less than that for the control animals. At autopsy, the prominent findings were mottled kidneys in seven males and three females, focal hemorrhage, and lung congestion. However, because these findings also were present in the control group, they probably are not attributable to treatment.

TATB

Ten male rats were exposed to dust aerosols of TATB for 1 hour at an analytical concentration of 212 ± 41 mg/m^3. Only male animals were used for these exposures because earlier oral toxicity studies had shown that TATB was equally toxic to male and females. During the exposure, no toxic signs were observed, although the animals' faces and noses were covered with yellow powder. No toxic signs were noted after the exposure or during the daily observation thereafter. The weekly weight gain of the test animals was comparable to that of the controls. Autopsy revealed some lung consolidation and congestion with focal areas of hemorrhage that were more pronounced in the treated animals than in the controls.

TCTNB

Ten male rats were exposed to TCTNB. Initially, we attempted to generate this compound as a dust. However, the agglomeration of the particles made this impossible. Therefore, a 60-minute aerosol inhalation exposure was conducted using a 10% solution of TCTNB in DMSO. The analytical chamber concentration of TCTNB was 684 ± 390 mg/m^3. During the exposure, the animals were quiet. Near the end of the 1-hour exposure, two to three animals appeared to be experiencing respiratory difficulty. When they were removed from the chamber, almost all the rats were in respiratory distress. Three died within 2 hours after the end of the exposure, and all the remaining animals died within 24 hours. These animals were not autopsied.

The DMSO nominal concentration during this exposure was 6.15 g/m^3. Recently, we exposed rats to 4.2 g/m^3 of DMSO for 2 hours daily for 8 consecutive days and observed no toxic signs. (The acute intravenous LD50 of DMSO in rats is 2.5 g/kg.) Therefore, that DMSO contributed directly to the TCTNB toxicity is unlikely.

Table 9 summarizes the body weight data collected throughout the inhalation studies.

Table 1

ACUTE ORAL TOXICITY OF TCB TO FASTED
MALE AND FEMALE RATS USING CORN OIL AS A VEHICLE

	Dose (mg/kg)	No. of Dead/ No. of Treated	Time to Death (hours)	LD50 (mg/kg)
Male	1250	3/10	21-45	1800
	2500	7/10	24-240	(1216-2664)*
	3750	10/10	45-70	
	5000	10/10	24-48	
Female	1250	0/10	--	2800
	2500	3/10	24-74	(2295-3416)*
	3750	9/10	45-96	
	5000	10/10	24-73	

* 95% confidence limits.

Table 2

ACUTE ORAL TOXICITY OF TCB TO FASTED
MALE AND FEMALE MICE USING CORN OIL AS A VEHICLE

	Dose (mg/kg)	No. of Dead/ No. of Treated	Time to Death (hours)	LD50 (mg/kg)
Male	1250	0/10	--	
	2500	4/10	24-72	3350
	3750	5/10	22-46	(2245-4590)*
	5000	8/10	45-120	
Female	2500	0/10	--	
	3150	5/10	46-288	3402
	3969	7/10	22-70	(3026-3825)*
	5000	9/10	24-192	

*95% confidence limits.

Table 3

SUMMARY OF EYE IRRITATION RESPONSES
IN RABBITS TREATED WITH TATB
Three Rabbits per Wash Condition

Tissue Graded*	Washing Condition	Average Irritancy Score						
		Hours		Days				
		1	4	1	2	3	4	7
Cornea	30 sec	0	0	0	0	0		
Iris	30 sec	0	1.7	0	0	0		
Conjunctivae	30 sec	5.3	4.0	0	0	0		
Cornea	5 min	0	0	0	0	0		
Iris	5 min	5.0	0	0	1.7	0		
Conjunctivae	5 min	1.7	2.7	2.0	2.0	0.7	0	
Cornea	No wash	0	0	0	0	0	0	0
Iris	No wash	5.0	1.7	1.7	1.7	1.7	0	0
Conjunctivae	No wash	4.0	2.7	1.3	0.7	0.7	0.7	0

* Maximum corneal response = 80
Maximum iris response = 10
Maximum conjunctivae response = 20.

Table 4

SUMMARY OF EYE IRRITATION RESPONSES IN RABBITS TREATED WITH TCNB
Three Rabbits per Wash Condition

Tissue Graded*	Washing Condition	Average Irritancy Score: Hours 1	Hours 4	Days 1	2	3	4	7	8	9	10	11	12	13	14	21	28
Cornea	30 sec	0	0	0	0	0	0	0									
Iris	30 sec	0	1.7	3.3	0	0	0	0									
Conjunctivae	30 sec	8.0	7.3	6.7	2.0	2.0	0.7	0									
Cornea	5 min	0	3.3	0	3.3	3.3	0	0	0	0	0	0	0	0	0	0	0
Iris	5 min	5.0	0	1.7	1.7	1.7	0	0	0	0	0	0	0	0	0	0	0
Conjunctivae	5 min	9.0	12.0	9.3	8.0	8.0	4.0	3.3	4.0	2.7	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Cornea	No wash	-†	-	-	18.3	10.0	10.0	33.3	53.3	30.0	31.7	41.7	35.0	35.0	35.0	13.3	13.3
Iris	No wash	-	-	-	5.0	5.0	5.0	3.3	5.0	3.3	3.3	3.3	3.3	3.3	3.3	1.7	1.7
Conjunctivae	No wash	16.0	17.3	-	18.0	17.3	13.3	11.3	12.7	10.7	11.3	11.3	10.7	8.0	8.0	4.0	2.7

* Maximum corneal response = 80
Maximum iris response = 10
Maximum conjunctivae response = 20.

† Eyes swollen shut--impossible to score.

Table 5

SUMMARY OF EYE IRRITATION RESPONSES
IN RABBITS TREATED WITH TCB
Three Rabbits per Wash Condition

Tissue Graded*	Washing Condition	Average Irritancy Score: Hours 1	Hours 4	Days 1	Days 2	Days 3	Days 4	Days 7
Cornea	30 sec	0	0	0	0	0		
Iris	30 sec	0	1.7	0	0	0		
Conjunctivae	30 sec	2.7	4.0	0	0	0		
Cornea	5 min	0	0	0	0	0		
Iris	5 min	3.3	0	0	0	0		
Conjunctivae	5 min	2.7	4.0	0.7	1.3	0		
Cornea	No wash	0	1.7	3.3	5.0	0	0	0
Iris	No wash	5.0	5.0	1.7	3.3	3.3	0	0
Conjunctivae	No wash	13.3	11.0	4.7	6.0	3.3	2.0	0

* Maximum corneal response = 80
Maximum iris response = 10
Maximum conjunctivae response = 20.

Table 6

IN VITRO ASSAYS WITH FIVE SALMONELLA TYPHIMURIUM STRAINS AND ESCHERICHIA COLI STRAIN WP2

Compound	Metabolic Activation	Micrograms of Compound Added per Plate	Average Number of Revertants per Plate: Histidine Revertants TA1535	TA1537	TA1538	TA98	TA100	Tryptophan Revertants WP2
Negative controls	-		23	10	14	29	160	76
	+		17	11	24	57	156	81
Positive controls								
β-propiolactone	-	50	680					
9-Aminoacridine	-	100		>2000				
2-Nitrofluorene	-	50			310			
2-Anthramine	-	20				40	160	72
	+					>2000	>2000	1170
TCB	-	5	44	8	12	44	164	75
	-	10	28	9	18	39	142	80
	-	50	24	12	13	30	133	57
	-	100	17	5	10	23	120	48
	-	500	10	5	9	16	82	49
	-	1000	7	4	9	22	112	51
	-	2500	19	6	6	13	122	58
	-	5000	8	5	10	16	104	47
	+	5	11	13	23	37	171	67
	+	10	18	17	20	43	153	85
	+	50	18	9	20	32	158	68
	+	100	17	9	21	32	142	61
	+	500	8	10	11	34	100	69
	+	1000	10	9	17	25	128	72
	+	2500	10	4	18	29	114	52
	+	5000	16	5	16	32	125	84

Table 6 (concluded)

Compound	Metabolic Activation	Micrograms of Compound Added per Plate	Average Number of Revertants per Plate: Histidine Revertants TA1535	TA1537	TA1538	TA98	TA100	Tryptophan Revertants WP2
TCTNB	-	5	21	8	18	20	156	74
	-	10	26	6	15	21	126	56
	-	50	27	5	4	11	69	47
	-	100	21	2	7	9	52	41
	-	500	4	2	2	6	32	20
	-	1000	3	2	2	4	20	18
	-	2500	6	5	2	1	15	10
	-	5000	0	0	0	1	7	12
	+	5	16	8	21	31	155	78
	+	10	13	13	25	39	164	87
	+	50	16	9	20	20	83	79
	+	100	14	7	16	20	65	44
	+	500	4	2	3	8	25	27
	+	1000	5	2	4	9	22	20
	+	2500	0	2	2	4	18	21
	+	5000	0	0	0	3	12	14
TATB	-	5	41	13	15	26	151	-
	-	10	30	7	12	32	126	81
	-	50	32	7	13	25	148	93
	-	100	36	8	16	21	148	78
	-	500	40	8	9	25	146	95
	-	1000	26	10	14	25	125	69
	-	2500	31	8	9	28	152	84
	-	5000	33	8	14	30	125	87
	+	5	23	7	19	34	128	-
	+	10	20	9	28	25	151	91
	+	50	24	10	22	42	138	108
	+	100	22	7	20	39	132	87
	+	500	20	13	21	34	145	88
	+	1000	18	6	17	35	139	88
	+	2500	20	8	16	27	143	88
	+	5000	20	6	15	33	173	94

Table 7

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE D3

Exper. No.	Compound	Metabolic Activation	Percent Concentration (x/v or v/v)	Surviving Cells per Milliliter (x 10^{-7})	Percent Survivors	Recombinants per Milliliter (x 10^{-3})	Recombinants per 10^5 Survivors
1	Negative control	-		7.9	100	5.5	7.0
		+		7.7	100	4.0	5.2
	1,2,3,4-Diepoxybutane	-	0.08	8.4	106	827	1860
		+	0.08	4.0	51	476	3000
	TATB	-	0.05	8.7	110	7	8.0
		-	0.1	8.6	109	4	4.7
		-	0.5	8.4	106	6	7.2
		-	1.0	9.1	115	5	5.5
		-	5.0	9.3	118	12	12.9
		+	0.05	7.2	94	9	12.6
		+	0.1	7.8	101	6	7.7
		+	0.5	6.8	88	11	16.2
		+	1.0	5.9	77	5	8.5
		+	5.0	7.4	96	4	5.4
2	Negative control	-		5.5	100	5	9.1
		+		5.7	100	4.5	7.9
	1,2,3,4-Diepoxybutane	-	0.1	0.018	0.3	Toxic	Toxic
		+	0.1	6.5	118	843	1300
	TATB	-	0.4	5.9	107	6	10.2
		-	0.8	5.3	96	8	15.0
		-	1.6	6.3	115	7	11.1
		-	2.4	5.9	107	5	8.4
		-	4.0	4.6	84	9	19.8
		+	0.4	5.0	88	6	12.0
		+	0.8	4.8	84	6	12.4
		+	1.6	4.9	86	5	10.1
		+	2.4	4.7	82	2	4.2
		+	4.0	5.9	104	5	8.4

Table 7 (continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (x/v or v/v)	Surviving Cells per Milliliter ($\times 10^{-7}$)	Percent Survivors	Recombinants per Milliliter ($\times 10^{-3}$)	Recombinants per 10^5 Survivors
1	Negative control	-		5.5	100	5	9.1
		+		5.7	100	4.5	7.9
	1,2,3,4-Diepoxybutane	-	0.1	0.018	0.3	Toxic	Toxic
		+	0.1	6.5	118	843	1300
	TCTNB	-	0.0004	5.2	95	2	3.9
		-	0.0008	4.5	82	1	2.2
		-	0.004	0.006	0.1	Toxic	Toxic
		-	0.008	0.011	0.2	Toxic	Toxic
		-	0.02	0	0	Toxic	Toxic
		+	0.0004	5.2	91	4	7.7
		+	0.0008	5.2	91	4	7.7
		+	0.004	0.022	0.4	Toxic	Toxic
		+	0.008	0	0	Toxic	Toxic
		+	0.02	0	0	Toxic	Toxic
2	Negative control	-		4.7	100	3.0	6.4
		+		4.5	100	2.5	5.6
	1,2,3,4-Diepoxybutane	-	0.1	0.8	17	485	5800
		+	0.1	4.4	98	625	1400
	TCTNB	-	0.00006	4.9	104	3	6.2
		-	0.00008	4.5	96	2	4.4
		-	0.0002	4.8	102	3	6.3
		-	0.0004	4.6	98	6	13
		+	0.00006	5.2	116	5	9.7
		+	0.00008	4.8	107	<1	<2.1
		+	0.0002	4.6	102	6	13
		+	0.0004	5.7	127	3	5.2

Table 7 (continued)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Surviving Cells per Milliliter ($\times 10^{-7}$)	Percent Survivors	Recombinants per Milliliter ($\times 10^{-3}$)	Recombinants per 10^5 Survivors
1	Negative control	-		7.9	100	5.5	7.0
		+		7.7	100	4.0	5.2
	1,2,3,4-Diepoxybutane	-	0.08	8.4	106	627	1860
		+	0.08	4.0	51	476	3000
	TCB	-	0.05	2.7	34	7	26.1
		-	0.1	2.4	30	4	16.9
		-	0.5	4.2	53	13	31.1
		-	1.0	6.1	77	15	24.5
		-	5.0	5.4	68	7	12.9
		+	0.05	1.4	18	10	70.6
		+	0.1	1.8	23	6	33.6
		+	0.5	4.6	60	30	64.7
		+	1.0	5.3	69	4	7.5
		+	5.0	4.1	53	4	9.7
2	Negative control	-		5.5	100	5	9.1
		+		6.7	100	4.5	7.9
	1,2,3,4-Diepoxybutane	-	0.1	0.018	0.3	Toxic	Toxic
		+	0.1	6.5	118	843	1300
	TCB	-	0.4	3.3	60	7	21
		-	0.8	3.5	64	16	46
		-	1.6	5.6	102	3	5.4
		-	2.4	4.8	87	1	2.1
		-	4.0	2.8	51	4	14
		+	0.4	3.4	60	9	26
		+	0.8	3.3	58	15	46
		+	1.6	5.3	93	6	11
		+	2.4	4.4	77	6	13
		+	4.0	4.8	84	5	10

Table 7 (concluded)

Exper. No.	Compound	Metabolic Activation	Percent Concentration (x/v or v/v)	Surviving Cells per Milliliter ($x\ 10^{-7}$)	Percent Survivors	Recombinants per Milliliter ($x\ 10^{-3}$)	Recombinants per 10^{5} Survivors
3	Negative control	-		4.7	100	3.0	6.4
		+		4.5	100	2.5	5.6
	1,2,3,4-Diepoxybutane	-	0.1	0.8	17	485	5800
		+	0.1	4.4	98	625	1400
	TCB	-	0.06	1.0	21	1	9.8
		-	0.08	1.0	21	1	10
		-	0.4	2.0	43	4	20
		-	0.6	2.7	57	1	3.7
		-	0.8	1.7	36	3	17
		+	0.06	1.0	22	4	39
		+	0.08	0.9	20	3	35
		+	0.4	1.4	31	9	64
		+	0.6	2.6	58	6	23
		+	0.8	2.8	62	8	28

Table 8

DNA REPAIR ASSAY
(Average Size of Zone of Inhibition in Millimeters*)

Compound	Dose (ug)	E. coli W3110	E. coli p3478	B. subtilis H17	B. subtilis M45
Nitroso-guanidine	20	16	24→27	13	27
	40	18	26→29	20	38
1-Phenyl-3,3-dimethyl-triazene	1000			32	76
Chloramphenicol	20	21	21	20	28
TCTNB	10	--	—	7	21
	50	--	—	7	22
	100	7	7	9	28
	500	7	7	9	32
	1000	7	7	9	33
TATB	100	7	7	7	7
	500	7	7	7	7
	1000	7	7	7	7
TCB	100	7	7	7	7
	500	7	7	7	7
	1000	7	7	7	7

* Size of filter disc is 7 mm.

Table 9

BODY WEIGHTS OF RATS BEFORE AND AFTER
A ONE-HOUR INHALATION EXPOSURE TO ATMOSPHERES OF
TCB VAPORS, TATB DUST, OR TCTNB AEROSOLS*

Compound	Number and Sex	Initial Weight	Weight Post-Exposure 1 Week	Weight Post-Exposure 2 Weeks
TCB 9.3 g/m^3	8 males	253 ± 12	273 ± 14	300 ± 16
	8 females	173 ± 14	198 ± 22	210 ± 26
TATB 212 41 mg/m^3	10 males	232 ± 11	276 ± 15	317 ± 17
TCTNB 684 390 mg/m^3	10 males	203 ± 27	---	---
Controls	5 males	226 ± 17	259 ± 19	311 ± 21
	5 females	174 ± 9	221 ± 11	268 ± 15

* Each value represents the mean and standard error in grams.