National Toxicology Program
Toxicity Report Series
Number 13

NTP Technical Report on Toxicity Studies of

Trinitrofluorenone

(CAS No. 129-79-3)

Administered by Dermal Application and Dosed Feed to F344/N Rats and B6C3F$_1$ Mice

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NIH Publication 92-3132
July 1992

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

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The NTP report on the toxicity studies of trinitrofluorenone is based primarily on the 14-day dermal study that began in August, 1987, and ended in September, 1987; the 14-day dosed feed study that began in September, 1987, and ended in October, 1987; and the 13-week dosed feed study that began in April, 1988, and ended in July, 1988, at the Southern Research Institute, Birmingham, AL.

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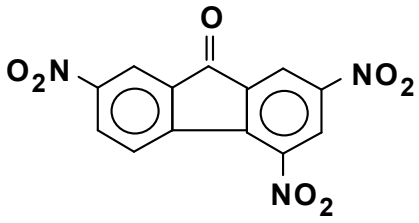
Trinitrofluorenone



Molecular Formula: $C_{13}H_5N_3O_7$

CAS Number: 129-79-3

Molecular Weight: 315.19

Synonyms: TNF; 2,4,7-trinitro-9H-fluoren-9-one

ABSTRACT

Trinitrofluorenone (TNF) is a major component of a toning formulation that at one time was used widely in certain photocopy processes. Because the principal route of exposure of humans to TNF probably would be dermal, studies were conducted to compare chemical absorption, distribution, excretion, and tissue retention, as well as toxicity in 14-day studies, by oral and dermal routes of exposure. Further, 13-week toxicity studies were carried out with TNF incorporated into the feed of rats and mice of both sexes. In genetic toxicity evaluations, TNF was found to be mutagenic in *Salmonella typhimurium*, with and without metabolic activation.

In disposition and metabolism studies, excretion patterns following oral administration of radiolabeled TNF (in doses ranging between 1 and 100 mg/kg body weight) were similar; 20% and 70% of the administered dose appeared in urine and feces, respectively, during the first 72 hours. Residual radiolabel in tissues comprised less than 1% of the dose. The appearance in the feces of 60-70% of a 1 mg/kg i.v. dose provided evidence for substantial biliary excretion of TNF; studies of the radiolabeled materials extracted from urine and feces suggested that glucuronidation is a major biotransfomation of TNF and its metabolites.

In the dermal exposure studies, groups of 5 F344/N rats and 5 B6C3F$_1$ mice of each sex were administered TNF in acetone by topical application once a day, 5 days per week, for 14 days. Doses were 0, 7.5, 15, 30, 60, or 120 mg/kg body weight for rats and 0, 12.5, 25, 50, 100, or 200 mg/kg for mice. There were no deaths, no adverse clinical signs, and no gross or microscopic changes related to treatment in either species, except for discoloration of skin at the site of application. Disposition studies with female rats showed that less than 10% of a dermal dose of 47 mg and less than 3% of a dermal dose of 400 mg were available systemically.

In contrast, toxicity was observed in the 14-day feeding studies with TNF. Groups of 5 rats and 5 mice of each sex were fed diets containing TNF at concentrations of 0, 500, 1600, 5000, 16000, or 50000 ppm. There were no deaths of rats or mice, but body weight gains of rats receiving 50000 ppm were reduced by as much as 45%. Animals receiving diets with 5000 ppm or higher TNF had a black discoloration of the skin and hair and enlarged and/or dark thyroid glands. Mild follicular cell hypertrophy and pigmentation of the epithelium and colloid were noted in the thyroid gland. Among mice, the brain and gallbladder were dark; the spleen of females was dark and also enlarged by hematopoiesis. Thymic lymphoid depletion and atrophy of the seminal vesicles were present in top-dose male rats (50000 ppm).

In 13-week studies, groups of 10 animals of each sex received diets containing TNF at concentrations of 0, 1000, 2000, 4000, 8000, or 16000 ppm for rats, and 0, 3125, 6250, 12500, 25000, or 50000 ppm for mice. No rats died, but the deaths of several mice in the 50000 ppm groups indicated a possible relation to TNF ingestion. Body weight gains of dosed rats were lower than controls and were dose-related. Top-dose male mice gained markedly less weight than controls. A mild macrocytic anemia and increase in methemoglobin was present in dosed rats at the end of the study. In both species, there was a widespread occurrence of a dark brown pigment in dosed animals, with little evidence of toxicity related to the pigment accumulation. Other treatment-related effects in male rats included mesenteric vascular inflammation, renal inflammation, testicular degeneration with reduced sperm count and motility, splenic hematopoiesis, and oval cell hyperplasia, cytoplasmic alteration, and mixed cell foci in the liver. Top-dose female rats had centrilobular hepatocyte cytoplasmic alteration and splenic hematopoiesis. Dosed mice of both sexes showed cystic degeneration of the thyroid gland, liver hypertrophy, and splenic hematopoiesis.

In summary, TNF caused a variety of lesions in oral feeding studies. The no-observed-adverse-effect-level (NOAEL) for microscopic changes other than pigment accumulation was 1000 ppm for rats. A NOAEL could not be determined for mice from this study. Limited dermal absorption likely would prevent significant systemic toxicity resulting from contact of TNF with the skin.

PEER REVIEW

Peer Review Panel

The members of the Technical Reports Review Subcommittee of the National Toxicology Program's Board of Scientific Counselors who evaluated the draft report on the toxicity studies of trinitrofluorenone on March 11-12, 1991, are listed below. Panel members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, panel members act to determine if the design and conditions of the NTP studies were appropriate and to ensure that the toxicity study report presents the experimental results and conclusions fully and clearly.

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Summary of Peer Review Comments

Dr. Frank Kari, NIEHS, NTP staff scientist, introduced the short-term toxicity studies of trinitrofluorenone (TNF) by reviewing the rationale for study, the experimental design, and the results.

Dr. Carlson, a principal reviewer, said this was a well-written report. He noted that the preferred route of exposure, based on human exposure, would have been dermal, yet dosed feed was chosen because chemical disposition studies showed that less than 10% of a dermal dose was absorbed. Therefore, he said that further information from the disposition studies should be summarized in the abstract, in addition to that for the topical application. Dr. Kari said additional information about the disposition studies would be added to the abstract.

Dr. Garman, a second principal reviewer, said the report presented a large body of information in a well-organized and succinct manner. He found the pigment accumulation in the large neurons in the brain stem to be particularly unusual. Since ultrastructural studies were performed, he asked that consideration be given to including an electron micrograph in the text showing the membrane-bound pigment particles. Dr. Davis seconded this request, and Dr. Kari agreed to add a plate.

Following a short discussion of editorial points and other concerns, Dr. Longnecker announced that the panel would accept the report with the indicated corrections and additions.

INTRODUCTION

Physical Properties, Production, Uses, and Exposure

Trinitrofluorenone (TNF) is a trinitro-derivative of fluorene that was used extensively as a toning agent in certain photocopy processes. TNF exists as pale yellow needles or crystals, has a melting point of 175-176°C, and a vapor pressure of 1.1×10^{-10} torr at room temperature. It is slightly soluble in water and very soluble in acetone, benzene, and chloroform. TNF has been used widely in the transfer process in photocopiers and printers; it is the photoelectrically active ingredient of the coating mixture deposited on the photoconductor film on the exterior of the printing drum. Use was limited to certain copier models produced by International Business Machines (IBM), of which approximately 60,000 were in use at the end of 1979 (NIOSH, 1981).

Possibilities for significant human exposure to TNF include contact with new or spent toners containing the chemical, dermal exposure to photocopy paper containing TNF, and proximity to the atmosphere around photocopy equipment that uses TNF. IBM estimates that the average mass of TNF thermally bonded to a sheet of photocopy paper (8 1/2 x 11 inches) is 2.6 □g; the worst-case scenario places this value at 4.5 □g. There is no information available on TNF extractability in this form. The maximum measured concentration of TNF in a "spent toner" collection bag is 360 ppm; the maximum measured concentration of TNF in ambient air samples collected at the operator position is 0.09 □g/m^3 (NIOSH, 1981). Lesser applications for TNF include use in the analysis of indoles by mass spectrophotometry, and as a fungicide against rice leaf blight (Hutzinger and Jameson, 1970).

TNF was reported to the U.S. International Trade Commission in 1976 and 1977, implying that the annual production was greater than 2,300 kg**.** Production information was not reported in either 1978 or 1979. In 1977, the TSCA Inventory reported a single manufacturer of this compound, MacKenzie Chemical Works, Inc.; however, personal communications with MacKenzie's regulatory affairs official revealed that the company no longer manufactures TNF. Two other companies that advertised distribution of this compound (Polysciences Inc., Warrington, PA; and Southland Corp., Great Meadows, NJ) revealed that their combined sales of TNF from October, 1984, to September, 1985, totaled less than 50 grams. In 1983, an IBM corporate toxicologist reported to the NTP that IBM had discontinued using TNF and had incinerated its remaining inventory.

Human Toxicity

Twenty-three IBM employees have been characterized as having had extensive exposure to TNF and have received additional medical evaluation during and after exposure. A NIOSH physician examined medical records of these employees; no remarkable pattern of diagnoses was perceived. Routine blood test reviews (blood counts, electrolytes, kidney function, liver functions, liver enzymes) and urine values revealed no abnormal patterns among the 23 workers. Ongoing medical surveillance of workers with a history of possible exposure to TNF did not record data relating to fertility or reproductive outcomes (NIOSH, 1981).

Animal Studies

No reports of animal studies on the toxicity of TNF, or studies of absorption, distribution, metabolism, or excretion of TNF, were located in the published literature. Copies of proprietary reports of toxicity studies were made available to the NTP; this information aided in designing the current study.

Chronic Toxicity/Carcinogenicity

Huggins and Yang (1962) reported administering by gavage a single oral bolus of TNF (100 mg), dissolved in sesame oil, to 50-day-old, female Sprague-Dawley rats. The animals were observed for 310 days. Seven of 20 treated rats (35%) developed mammary tumors, compared to 8/164 (5%) in an untreated control group. It was not specified, however, if these were concurrent controls. Under similar protocols, 8/9 (89%) rats given an oral dose of 100 mg of benzo(a)pyrene developed mammary tumors; 0/18 rats developed mammary tumors after a dose of 200 mg of benz(a)anthracene; 700/700 historical positive control rats developed mammary tumors after oral intubation with 20 mg of 7,12-dimethylbenzanthracene (Huggins and Yang, 1962)

Although TNF's carcinogenic potential has not been evaluated rigorously, it is well established that various nitro and amine derivatives of fluorene are carcinogenic. The carcinogenicity of aromatic amines and their amide derivatives appears dependent upon these compounds' metabolic activation by N-hydroxylation. For example, in the N-hydroxy derivatives of 2-acetylaminofluorene (2-AAF), there is a further activation step resulting in the formation of the O-acyl derivative, regarded as the ultimate carcinogenic metabolite. Species resistant to the carcinogenic action of 2-AAF fail to metabolize the amine to the hydroxamic acid form and/or fail to conjugate the latter to the O-acyl derivative (Kadlubar *et al.*, 1977; Irving, 1977)

Genetic Toxicology

Prokaryotic Cells

TNF has been assayed several times for mutagenic activity in *Salmonella typhimurium*, with uniformly positive results. In amounts of up to 2.5 mg per plate, TNF caused a dose-dependent increase in reversion frequencies, without exogenous metabolic activation (S9), in strains TA98, TA100, TA1537, and TA1538. Responses ranged from a 15- to 100-fold increase (2.5 mg concentration) in strains TA98 and TA100, to more than a 1000-fold increase above background in strains TA1537 and TA1538, indicating that TNF is a frameshift mutagen (Levin *et al.*, 1979). Probst *et al.* (1980, 1982) confirmed the mutagenicity of TNF, without activation, in 7 strains of *Salmonella typhimurium*. McCoy *et al.* (1981) evaluated the mutagenicity of a number of nitrated derivatives of fluorene, noting a significant reduction in TNF's mutagenic activity in strain TA98NR, a nitroreductase-deficient derivative of TA98. This finding supports the notion that the nitro group must be reduced for subsequent expression of mutagenic activity. Results from an NTP *Salmonella* gene mutation assay, using strains TA98 and TA100, were strongly positive both in the

presence and the absence of induced S9 (Appendix D, Table D1). Test concentrations in the NTP assay ranged from 0.001 to 66 mg/ml.

Eukaryotic Cells (in vitro)

Mutagenesis assays, measuring forward mutation at the thymidine kinase locus in L5178Y mouse lymphoma cells, were conducted with TNF. Concentrations ranging from 0.7 to 3.0 mg/ml significantly and reproducibly increased the number of mutant colonies above the spontaneous control level in a dose-related manner, both in the presence and the absence of metabolic activation (Burrell *et al.*, 1980).

Probst *et al.* (1980, 1982) reported that TNF caused unscheduled DNA synthesis (UDS) in primary rat hepatocyte cultures, at concentrations ranging from 1 to 500 mM. An additional report confirmed UDS induction in TNF-treated rat hepatocytes, in both *in vitro* and *in vivo/in vitro* assays (Joachim and Decad, 1983).

Incubation of TNF with human peripheral lymphocytes *in vitro* (Tucker and Ong, 1984), at concentrations ranging from 0.1 to 3.0 mg/ml, caused dose-related increases in sister chromatid exchange (SCEs); the highest dose caused an approximately 3-fold increase over the solvent control. Chromosomal aberrations also were induced in these cells at concentrations ranging from 3-10 mg/ml TNF; higher concentrations of TNF-produced toxicity were characterized by cell-cycle delay, fewer observable metaphases, and poor chromosome morphology. The authors also noted that TNF-treated blood cultures turned considerably darker than control cultures, suggesting the conversion of hemoglobin to methemoglobin in the treated cells. A strong, dose-related increase in SCE also was observed in mouse bone marrow treated *in vitro* with 1.0 to 2.0 mg/ml TNF (Krishna *et al.,* 1986); the response at the highest dose was comparable (a 3-fold increase) to that observed in experiments with human lymphocytes.

Study Rationale and Design

TNF was nominated for study by the American Federation of State, County, and Municipal Employees, in part because of the large number of people potentially exposed to TNF through photocopiers containing the chemical, or printed materials that might contain TNF; and because of the lack of published toxicity or carcinogenicity information on the chemical. TNF also was nominated by the National Cancer Institute, based on photocopier use of TNF, limited animal data suggestive of a carcinogenic effect, and positive results in genotoxicity test systems.

Since the principal route of exposure of humans to TNF likely would be dermal, the current evalutions were designed to compare chemical absorption, distribution, excretion, and tissue retention, as well as toxicity in 14-day studies using both oral and topical application. Results of the dermal studies suggested absorption was insufficient to allow full evaluation of the systemic toxicity of TNF; therefore, 13-week toxicity studies were carried out by incorporating TNF into the feed of F344/N rats and B6C3F_1 mice of each sex.

MATERIALS AND METHODS

Procurement and Characterization of Trinitrofluorenone

2,4,7-Trinitrofluoren-9-one (TNF, CAS 129-79-3) for use in toxicity studies was purchased from MacKenzie Chemical Works (Central Islip, NY). Cumulative analytical data indicated a purity of greater than 99%. Infrared, ultraviolet/visible, and nuclear magnetic resonance spectra were consistent with the structure of TNF and with available literature references. Elemental analyses for carbon, hydrogen, and nitrogen agreed with theoretical values, and Karl Fischer analyses indicated less than 0.1% water. No impurities greater than 0.1%, relative to the TNF peak, were observed by high-performance liquid chromatography (HPLC). Bulk chemical was stored at room temperature (~22°C) and protected from light. Quantitative HPLC reanalysis was undertaken at approximately 4-month intervals, including analyses within 30 days of the start and end of the subchronic studies. No degradation of the bulk chemical was detected during the course of the studies.

[^{14}C]-2,4,7-Trinitrofluorenone labelled at the carbonyl carbon was prepared by Amersham International (Arlington Heights, IL), with a radiochemical purity of >98% and a specific activity of 25 mCi/mmol. [^{14}C]-TNF was analyzed with both normal and reverse phase HPLC systems, using a Waters Associates HPLC system (Waters Chromatography, Milford MA) equipped with two model 6000A pumps, a model 720 solvent programmer, a model U6K injector, and a Kratos 733 Spectroflow UV detector (Kratos Analytical Inc., Ramsey, NJ). UV$_{\lambda max}$ was monitored at 254 nm. Unlabeled TNF for radiolabel dilution was purchased from Aldrich Chemical Company (Milwaukee, WI, Lot No. BP02128CE) and was characterized by ^{1}H- and ^{13}C-NMR.

Dose Formulations

Doses were administered to animals by a variety of routes. Toxicity studies were performed by topical application and dosed feed; absorption, distribution, and excretion studies were done by topical application, oral gavage, and intravenous and intraintestinal injection.

Analytical grade acetone was the vehicle used to administer TNF for topical application toxicity studies. Stability studies indicated that TNF/acetone solutions at a concentration of 0.5 mg/ml were stable in sealed glass vials for 3 weeks in the dark at room temperature. Solutions stored for 3 hours, open to air and light, also were stable. Chemical preparations administered to the animals were stored for a maximum of 20 days at room temperature and were protected from light prior to and during use. Analyses of TNF concentrations were performed on each dose level from the initial mixing of TNF/acetone preparations applied to the animals. Results indicated that all dose levels were within 10% of the targeted concentrations.

Topical doses for absorption and distribution studies were prepared by combining [^{14}C]-TNF and unlabeled TNF (if required) with acetone to obtain the appropriate amount of TNF in 200 µl of dosing solution. Topical dose formulations were analyzed by HPLC, with radioactivity monitored by a Ramona LS detector (IN/US, Fairfield, NJ).

NIH-07 Open Formula mash rodent feed (Zeigler Brothers, Inc., Germantown, PA) was used to prepare dosed-feed mixtures for toxicity studies. An initial stability study was conducted on a feed blend concentration of 0.5 mg TNF/g feed; the study showed no significant loss of TNF after 3 weeks' storage, in darkness, in a sealed glass container at room temperature. Dosed feed stored open to air and light for 7 days in a rat cage also was stable. Dosed feed preparations were stored for a maximum of 20 days at room temperature and protected from light prior to and during use. HPLC analysis for each dose concentration was performed on the first, middle, and final mixing of TNF/feed preparations given the animals. Results indicated that all dose levels were within acceptable limits (± 10% of theoretical values).

Oral doses for metabolism and disposition studies were administered by gavage. Dose formulations were prepared to contain approximately 10 µCi [^{14}C]-TNF, an appropriate amount of unlabeled TNF, and sufficient dose vehicle for a single oral dose of 5 ml/kg. TNF was dissolved in a minimum amount of acetone, then added to rapidly stirred corn oil to produce a final concentration of 2.0 mg TNF/ml. The radiochemical purity of the oral dose formulations (>98%) was determined by HPLC analysis on the day of dose administration.

Intravenous and intraintestinal doses were prepared using **[**^{14}C]-TNF dissolved in DMSO to obtain a solution containing 2.0 mg TNF/ml DMSO. An appropriate amount of serum then was added with rapid mixing to produce a final dose formulation of 0.5 mg TNF/ml DMSO.

Disposition Study Designs

Female F344/N rats were purchased form Charles River Laboratories, Inc. (Raleigh, NC). The animals were quarantined for at least a week prior to being used in an experiment. They were fed Certified Purina Rodent Chow #5002, furnished tap water *ad libitum*, and housed in individual glass metabolism cages, which allowed for the separate collection of urine, feces, and exhaled $^{14}CO_2$.

Twenty-four hours prior to topical dose administration, the animals were sedated with a Ketamine®-Xylazine® mixture (7:1) at a dose of 60 mg/kg of body weight, and the hair was clipped from a 3 x 4 cm area on the back of each animal. The clipped area was wiped with acetone, dried, and examined for nicks; all animals found to have nicks were excluded from the study. A 2 cm^2 circular area was inscribed with an indelible marker as the designated dose area. Two hundred microliters of the dosing solution was applied to the designated area and spread evenly. A non-occlusive, metal grid protective device (a Lipshaw tissue capsule) was adhered over the dose site with cyanoacrylate adhesive, and the animal was returned to the metabolism cage.

Oral dose levels for the metabolism and disposition studies were 1, 10, and 100 mg/kg, administered by gavage. The animals were returned to individual metabolism cages after dosage.

For distribution studies, intravenous and intraintestinal doses of TNF were administered at 1 mg/kg body weight. The dose formulation for the intravenous and initial bile cannulation study was injected in a lateral tail vein. The intraintestinal dose for a second bile cannulation study was instilled into the proximal end of the small intestine of animals prepared for bile duct cannulation studies, as described below.

Urine and feces were collected separately in round-bottom flasks over dry ice. Urine was collected at 6, 12, and 24 hours. Urine and feces were stored at -20°C in the dark until analyzed. Bile was collected continuously from anesthetized, cannulated rats over a 6-hour period at regular time intervals. Rats initially were anesthetized by i.p. injection using 60 mg/kg Ketamine® and 8.6 mg/kg Xylazine®. Anesthesia was maintained by i.p. injections of 20 mg/kg of sodium pentobarbital administered approximately 1 and 4 hours after dosing.

At the end of each experiment, the rats were anesthetized with an i.p. injection of 60 mg/kg Ketamine® and 8.6 mg/kg Xylazine®, and blood was withdrawn by cardiac puncture. Urine was removed from the bladder and added to the last urine collection. Rats were killed by intracardiac injection of sodium pentobarbital (300 mg/kg). Following the sacrifice of orally dosed animals, the contents of the gastrointestinal tract were segmented into stomach, small intestine, cecum, and large intestine, collected in individual vials, and stored at -20°C until analyzed.

The skin application site from dermally dosed animals was excised and secured to a glass funnel. A stream of acetone was used to wash off the remaining dose on the skin and to remove the adhesive where the tissue capsule had adhered to the skin. The application site was scrubbed with 5 separate gauzes soaked in soapy water, using 50 scrubbing strokes per gauze, and further scrubbed with gauzes soaked in tap water using 50 scrubbing strokes per gauze. The combined rinses were collected into preweighed containers. The skin was finally immersed in 2N ethanolic NaOH for dissolution. Gauzes were placed in individual scintillation vials.

Adipose tissue (3 sample locations), adrenals, brain, gastrointestinal tract (segmented into stomach, small intestine, and large intestine, each washed free of contents), heart, kidney, liver, lung, skeletal muscle (3 sample locations), ovaries, and skin (3 sample locations) were removed, weighed, and placed into vials containing solubilizing media (Soluene®-350). Aliquots of urine, bile, skin wash solutions, and dermal dose site homogenates were added directly to vials containing scintillation cocktail (Scintiverse E., Fisher Scientific, Pittsburgh, PA). Samples of tissues, feces, gastrointestinal tract contents, and blood (0.2-0.3 g) were digested in 2 ml Soluene®-350. After digestion, samples requiring bleaching were decolorized with perchloric acid/hydrogen peroxide prior to addition of scintillation cocktail. Samples were quenched with base and kept in darkness overnight before assay by liquid scintillation spectrometry.

Urine samples from 2 rats were collected and analyzed by HPLC to identify TNF and its metabolites. Urine was collected and analyzed for time intervals of 0-6 hours, 6-12 hours, and 12-24 hours from rats dosed orally at 1 and 10 mg/kg. Urine also was collected over the time interval of 24-48 hours from the 100 mg/kg dose group and analyzed by HPLC. The urine was filtered, and 25 µl aliquots of neat filtrate were analyzed by HPLC. Radioactivity was monitored using a Ramona LS detector equipped with a 230 µl solid scintillate cell; the effluent was collected into a tared receiving vessel. Aliquots then were assayed for total ^{14}C content. Recoveries were determined by comparing the total ^{14}C collected versus a 25 µl aliquot of the neat filtered urine.

Bile from two cannulated rats was collected over time intervals of 15-30 minutes and 5-6 hours; the bile (10 µl) was analyzed using the above-described methods for analysis of the urine samples. Scintiverse-LC was pumped through the cell at a rate of 4 times that of column effluent. Fractions

were collected and assayed for ^{14}C by scintillation spectrometry; percent radioactivity was determined for each peak using Ramona® version 7.7 software.

Fecal samples were collected over 12-48 hours from all animals orally administered 100 mg/kg TNF; the samples were extracted with equal volumes of 0.1M ammonium bicarbonate buffer (pH 7.8) and methanol. The aqueous extract was lyophilized, reconstituted in methanol, and filtered through a 0.22 µm Durapore® filter (Millipore Corp., Bedford MA). A 10 µl aliquot of the resultant filtrate then was analyzed by the same methods described for bile analysis. The methanolic extract was concentrated by rotary evaporation; analysis of the concentrate was accomplished in the same manner as for the aqueous extract.

Urine, bile, and aqueous fecal extracts were treated with the enzyme preparation ß-glucuronidase/sulfatase to determine the presence of glucuronide conjugates. Acetate buffer, pH 5, was added to urine, bile, and aqueous fecal extract to create a final concentration of 0.1M. Twenty microliters of the enzyme preparation were added per ml of sample, and the mixture was maintained at 37 ± 1°C for 4 hours. Fecal extract incubation with the enzyme was repeated for a 23-hour period; resulting mixtures were analyzed by HPLC using the system described for bile profiles.

Toxicity Study Designs

Male and female Fischer-344/N rats and B6C3F$_1$ mice used in these studies were produced under strict barrier conditions at Taconic Farms, Germantown, NY (14-day studies), or at Simonsen Laboratories, Inc., Gilroy, CA (13-week studies). Animals were the progeny of defined microflora-associated parents transferred from isolators to barrier-maintained rooms. Rats and mice were shipped to the study laboratory at 4 weeks of age, quarantined at the study laboratory for 11 days, and placed on study at 6 weeks of age. Blood samples were collected and the sera analyzed for viral titers from 5 animals/sex/species at study start and at termination in the 13-week studies. Data from 5 viral screens performed in rats and 12 viral screens performed in mice (Boorman *et al.,* 1986; Rao *et al.,* 1989, 1989a) showed there were no positive antibody titers.

Groups of 5 rats and 5 mice of each sex were administered TNF by topical application. Each group received doses of 0, 3, 6, 12, 24, or 48 mg TNF/ml in acetone, administered once daily for 14 days, excluding weekends. The dosing volume was adjusted weekly based on changes in group mean body weight, with target doses of 0, 12.5, 25.0, 50.0, 100.0, or 200.0 mg TNF/kg body weight for mice, and 0, 7.5, 15.0, 30.0, 60.0, or 120.0 mg TNF/kg body weight for rats. When the dosing volume exceeded 100 microliters, the volume was applied in 2 equal doses. Complete necropsies were performed on all animals; microscopic evaluations were conducted on skin from the application site, control skin (inguinal area), and all gross lesions observed at necropsy from the vehicle and treated groups.

In the 14-day dosed feed studies, groups of 5 rats and 5 mice of each sex received diets containing TNF at concentrations of 0, 500, 1600, 5000, 16000, or 50000 ppm (0.05, 0.16, 0.5, 1.6, or 5%) daily. Complete necropsies were performed on all animals; microscopic evaluations were conducted on all gross lesions observed at necropsy from the vehicle and treated groups. Organs examined

included thyroid gland, skin, testes, epididymides, seminal vesicles, thymus, and cecum in rats, and thyroid gland, brain, and spleen in mice.

In the 13-week dosed feed studies, groups of 10 rats and 10 mice of each sex received TNF mixed in their diets at concentrations of 0, 1000, 2000, 4000, 8000, or 16000 ppm for rats, and 0, 3125, 6250, 12500, 25000, or 50000 ppm for mice. Ten additional rats/sex were included at each dietary level for evaluation of hematologic and clinical chemistry parameters. Sperm morphology and vaginal cytology examinations were performed on rats receiving diets containing 0, 4000, 8000, and 16000 ppm TNF, and on mice receiving 0, 6250, 12500, and 25000 ppm. Details of clinical examinations and pathology procedures are outlined in Table 1. Animals surviving to the end of the studies were killed using CO_2 anesthesia; weights were recorded for the liver, right kidney, heart, lungs, thymus, and right testicle.

For clinical pathology studies, male and female rats were anesthetized with a CO_2:O_2 mixture (70%:30%), and blood samples were collected from the retroorbital sinus using heparinized microcapillary tubes. Samples for determination of hematologic and biochemical variables were collected from additional study animals on study days 5 and 21, and from the regular study animals at 13 weeks. Blood samples for hematologic analyses (approximately 0.50 ml) were collected in plastic tubes coated with potassium EDTA (Microvette CB 1000, Sarstedt, Numbrecht, Germany) and held at room temperature. Samples for biochemical analyses (approximately 0.75 ml) were collected in plastic tubes containing serum separator gel (Microtainer® serum separator tube, Becton Dickinson, Rutherford, NJ). These samples were allowed to clot for 30 minutes at room temperature. At the end of this period, samples were centrifuged at 5000g for 10 minutes and serum was removed for biochemical analyses.

Automated hematologic analyses were performed with an Ortho ELT-8 hematology system (Ortho Diagnostics Systems, Inc., Westwood, NJ). The following variables were measured: erythrocyte, leukocyte, and platelet counts, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), hematocrit (HCT), and hemoglobin concentration (HGB). Leukocyte differentials were determined by microscopic evaluation of Wright-stained blood smears.

Analyses of biochemical variables in serum were performed using a Roche Cobas Fara chemistry system (Roche Diagnostics Systems, Nutley, NJ). For the following variables, reagent kits and applications developed by the manufacturer were used: alanine aminotransferase (ALT), total protein, albumin, urea nitrogen (UN), creatinine, creatine kinase (CK), and alkaline phosphatase (AP). For determinations of sorbitol dehydrogenase (SDH) and total bile acids, reagent kits were obtained from Sigma Chemical Company (St. Louis, MO); applications were developed in-house for the chemistry analyzer.

Complete necropsies were performed on all animals, and organs and tissues were examined for gross lesions. Tissues were fixed in 10% neutral buffered formalin; those intended for microscopic evaluation were trimmed, dehydrated, and embedded; sectioned at approximately five microns; stained with hematoxylin and eosin; and examined. A complete histopathologic examination, including all protocol-required tissues (Table 1), was performed on all high-dose and control rats of both sexes, high-dose and control female mice, and all early-death mice. All protocol-required

tissues also were examined from all male mice in the 25000 ppm group. In addition to all gross lesions, the brain, heart, spleen, liver, kidney, thyroid gland, skeletal muscle, and lymph node from all lower-dose-level animals of both species were examined, as were the ovary and uterus from all females at lower dose levels. The adrenal gland, bone, epididymis, intestine, mesentery, pituitary gland, prostate, skin, testes and thymus also were examined in rats. Other tissues examined in mice included mammary gland and urinary bladder (males only).

Upon completion of the histologic evaluation by the laboratory pathologist, the slides, paraffin blocks, and residual wet tissues were sent to the NTP Archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were sent to an independent pathology laboratory for quality assessment; the results were reviewed and evaluated by the NTP Pathology Working Group (PWG). Tissues evaluated by the PWG included the brain, thyroid gland, liver, kidney, testes, epididymis, skin, heart, bone, and mammary gland. 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).

Reproductive Toxicity

To screen for potential reproductive toxicity, epididymal sperm motility was evaluated at necropsy, and vaginal cytology was evaluated on animals during the 2 weeks just preceding necropsy, using procedures outlined by Morrissey *et al.* (1988). For the 12 days prior to sacrifice, females were subject to vaginal lavage with saline. Aspirated cells were air-dried onto slides, stained with Toluidine Blue O, and cover slipped. The relative preponderance of leukocytes, nucleated epithelial cells, and large squamous epithelial cells were used to identify the stages of the estrual cycle.

Sperm motility was evaluated at necropsy as follows: The left epididymis was removed and quickly weighed; the cauda epididymis was removed at the junction of the vas deferens and the corpus epididymis, and weighed. Warm (37°C) Tyrodes buffer (mice, 80 µl) or test yolk buffer (rats, 80 µl) was applied to 2 pre-warmed slides, and a small cut made in the distal cauda epididymis. The sperm that extruded from the epididymis were dispersed throughout the solution, cover slipped, and counted immediately on a warmed microscope stage. Two independent observers counted the number of moving and non-moving sperm in 5 fields of 30 sperm or less per field. After sperm sampling for motility evaluation, the cauda was placed in phosphate buffered saline (PBS), gently chopped with a razor blade, and allowed to sit for 15 minutes. The remaining clumps of tissue were removed, the solution mixed gently, and heat-fixed at 65°C. Sperm density was subsequently determined using a hemocytometer.

TABLE 1 Experimental Design and Materials and Methods in the Dermal and Dosed Feed Studies of Trinitrofluorenone

Study Laboratory	Southern Research Institute, Birmingham, AL
Study Dates	14-Day Dermal Studies: August -- September, 1987 14-Day Dosed Feed Studies: September -- October, 1987 13-Week Dosed Feed Studies: April -- July, 1988
Strain and Species	F344/N rats; B6C3F$_1$ mice
Animal Source	14-Day Studies: Taconic Farms, Inc., Germantown, NY 13-Week Studies: Simonsen Laboratories, Inc., Gilroy, CA
Chemical Source	MacKenzie Chemical Works, Central Islip, NY
Size of Study Groups	14-Day Studies: 5 males and 5 females of each species. Rats were housed 5 per cage and mice were individually caged. 13-Week Studies: 10 males and 10 females of each species. Rats were housed 5 per cage and mice were individually caged.
Doses	14-Day Dermal Studies: Rats: 0, 7.5, 15, 30, 60, or 120 mg TNF/kg body weight in acetone. Mice: 0, 12.5, 25, 50, 100, or 200 mg TNF/kg body weight in acetone . 14-Day Dosed Feed Studies: Rats and mice: 0, 500, 1600, 5000, 16000, or 50000 ppm TNF in feed. 13-Week Dosed Feed Studies: Rats: 0, 1000, 2000, 4000, 8000, or 16000 ppm in feed. Mice: 0, 3125, 6250, 12500, 25000, or 50000 ppm TNF in feed.
Method of Animal Distribution	Animals were stratified by weight and assigned to cages and dose groups according to a table of random numbers.
Diet	NIH 07; available *ad libitum*
Animal Room Environment	14-Day Studies: Temp: 69-75°F; relative humidity: 35-65%; fluorescent light 12 h/d; 10 room air changes/h 13-Week Studies: Temp: 68-76°F; relative humidity: 23-87%; fluorescent light 12 h/d; 10 room air changes/h
Time Held Before Study	14-Day Studies: Rats: 11 d; Mice: 13 d 13-Week Studies: Rats: 11 d, Mice: 11 d
Age When Placed on Study	14-Day and 13-Week Studies: 6 wks
Age When Killed	14-Day Studies: 8 wks 13-Week Studies: 19 wks
Type and Frequency of Observation	14-Day Dermal Studies: Observed 2 x d; weighed at days 1 and 8 (mice) or 9 (rats), and at study termination. 14-Day Dosed Feed Studies: Observed 2 x d; weighed at days 1 and 8, and at study termination. 13-Week Dosed Feed Studies: Observed 2 x d; weighed weekly and at study termination.

TABLE 1 Experimental Design and Materials and Methods in the Dermal and Dosed Feed Studies of Trinitrofluorenone (continued)

Necropsy and Histologic Examinations (13-week studies)	Necropsies performed; the following tissues were examined histologically for all controls, all 16000 ppm rats and all 50000 ppm mice and 25000 ppm male mice: adrenals, bone (femur including marrow and epiphysis), brain, esophagus, gallbladder (mice), gross lesions and tissue masses, heart, cecum, colon, rectum, duodenum, jejunum, ileum, kidneys, liver, lungs and mainstem bronchi, mandibular and mesenteric lymph nodes, mammary gland, nasal cavity and turbinates, ovaries, pancreas, parathyroids, pituitary, preputial or clitoral glands, prostate, salivary gland, skin, spleen, stomach, testes/epididymis/ seminal vesicle, thigh muscle, thymus, thyroid, trachea, urinary bladder, uterus, and vagina. Target organs [Rats]: adrenal gland, bone, brain, epididymis, heart, intestine, kidney, liver, lung, lymph node, mesenteric vessels, ovary, pituitary gland, prostate, skeletal muscle, skin, spleen, testis, thymus, thyroid gland, uterus; [Mice]: brain, liver, kidney, heart, lymph node, ovary, spleen, skeletal muscle, thyroid gland, mammary gland, uterus, urinary bladder (males only). Gross lesions examined at all lower doses. Hematologic and serum chemical analyses performed; sperm morphology, vaginal cytology evaluated in rats from untreated controls and three highest dose groups; in mice from untreated controls, 6250, 12500, and 25000 ppm groups.

To quantify spermatogenesis, the left testis was weighed, frozen and stored. After thawing, testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the testis in PBS containing 10% DMSO. Homogenization-resistant spermatid nuclei were enumerated using a hemocytometer; the data were expressed as spermatid heads per total testis, and per gram of testis.

Statistical Methods

For the 13-week studies, the significance of differences between dosed and control groups was assessed using nonparametric multiple comparisons procedures designed to protect against false positive inferences. Either Dunn's test or Williams' modification of Shirley's multiple comparisons procedure was applied, based on the occurrence of a dose-related response in the data (Dunn, 1964; Shirley, 1977; Williams, 1986). If the P value from Jonckheere's test (Hollander and Wolfe, 1973) for a dose-related trend is greater than or equal to 0.10, Dunn's test is used rather than Shirley's test. Tables for each individual parameter show the results of Shirley's or Dunn's test (reported at the P = 0.05 and 0.01 levels). The outlier test of Dixon and Massey (1951) was employed to detect extreme values. Treatment effects for vaginal cytology 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.

Quality Assurance

The studies of TNF were performed in compliance with FDA Good Laboratory Practices regulations (21 CFR 58). The Quality Assurance Unit of Southern Research Institute performed audits and inspections of protocols, procedures, data, and reports throughout the conduct of the studies. The operations of the Quality Assurance Unit were monitored by the NTP.

RESULTS

Disposition and Metabolism

Approximately 10% of a dermally administered dose of 47 µg TNF was absorbed in 24 hours of exposure. Less than 1% of the applied dose remained in the tissues at that time. The amount of the administered dose that appeared in the urine and feces, 24 hours after dosing, was 3.5% and 4%, respectively. Approximately 0.8% of the dose was recovered from the dissected tissues. Elevated tissue/blood ratios (values ranging 4-5) were found for small and large intestine, kidney, and liver; 1% of the administered dose was present in the skin at the application site. Total absorption of radiolabel was 9.4% (4.4 µg) of the applied dose. The amount of label unabsorbed on skin at the application site was 76.8% (35.9 µg).

An 8-fold increase in the administered dose (400 µg) resulted in a decrease in the relative proportion of TNF penetrating the skin. Approximately 1% of the dose was excreted in urine and 1% in the feces. After 72 hours, 0.3% of the applied dose remained in the collected tissues. Thus, of 400 µg of an applied dermal dose, approximately 2.6% of the dose (10 µg) was systemically available.

TNF was administered by gavage at 1, 10, and 100 mg/kg body weight. For each dose level, excretion during the first 72 hours was similar, with 17-22% and 67-68% of the label excreted in urine and feces, respectively. The time course of whole body elimination of radiolabel following a 10 mg/kg dose is shown in Table 2. Tissue residues of the radiolabel comprised less than 1% of the administered dose.

TABLE 2 Elimination and Total Tissue Retention of Radioactivity After Gavage Administration of [^{14}C]-TNF (10 mg/kg) to Female F344/N Rats

	ORAL DOSE 10 MG/KG TNF Cumulative % Dose[a]				
Time (h)	Urine	Feces	Contents[b]	Tissues[c]	% Recovery
0 - 6	5.7 ± 1.8				
6 - 12	11.0 ± 2.4	0.07 ± 0.01			
12 - 24	19.1 ± 3.7[d]	2.8 ± 2.6			
24 - 48	21.7 ± 4.5[d]	57.6 ± 9.3			
48 - 72	21.9 ± 4.6[d]	67.0 ± 5.2	0.18 ± 0.13	0.50 ± 0.37	89.9 ± 1.0

a Values are means ± standard deviations for 4 animals.
b Sum of % dose recovered from contents of stomach, small and large intestine, and cecum.
c Sum of % dose recovered from adipose, blood, cecum, large and small intestine, kidney, liver, muscle, plasma, skin, stomach.
d Values include cage rinses.

Tissue/blood ratios (values ranging from 0.1-0.2) in adipose tissue indicated the remaining residues in the tissues were considerably more polar than the lipophilic parent compound, TNF. The distribution of radiolabel in tissues following oral dosing with 10 mg/kg is shown in Table 3 and is representative of the results seen following administration of 1 or 100 mg TNF/kg body weight.

TABLE 3 Tissue Distribution of Radioactivity 72 Hours After Gavage Administration of [^{14}C]-TNF (10 mg/kg) to Female F344/N Rats[a]

Tissue	ng-eq TNF/g Tissue	% Administered Dose in Tissue
Adipose	32 ± 27	0.03 ± 0.01
Blood	140 ± 33	0.08 ± 0.02
Cecum	154 ± 56	0.01 ± 0.003
Large Intestine	100 ± 30	0.01 ± 0.001
Small Intestine	117 ± 43	0.01 ± 0.004
Kidney	847 ± 184	0.06 ± 0.01
Liver	578 ± 160	0.22 ± 0.06
Muscle	32 ± 3.8	0.14 ± 0.01
Plasma	160 ± 107	0.05 ± 0.03
Skin	157 ± 47	0.21 ± 0.05
Stomach	1132 ± 720	0.05 ± 0.03
Total (excluding Plasma)		0.81 ± 0.18

[a] Values are means ± S.D. for 4 animals.

Oral doses of TNF (10 mg/kg) were given to groups of animals pretreated with lincomycin/neomycin in experiments designed to estimate the effect of gut microflora on the disposition of TNF. The pretreated animals did not differ from controls in the amount of the radiolabel appearing in excreta, tissues, or gastrointestinal tract contents. In both cases, nearly 60% of the dose remained in the gastrointestinal tract 6 hours following dose administration, with 15-20% of the dose remaining in the stomach. This suggested that reduction of intestinal microflora does not alter absorption or elimination of TNF.

A comparison of excretion profiles following an intravenous or an oral dose of TNF (Table 4) shows that in both cases, 60-70% of the administered radiolabel was excreted in the feces, primarily during the first 24 hours. Fecal elimination of an i.v. dose provides evidence for biliary excretion and the potential for enterohepatic circulation of TNF or its metabolites. The biliary excretion of radiolabel following intravenous or intraintestinal administration of TNF is shown in Table 5. These data confirm the suspected involvement of biliary excretion in the elimination of TNF and provide evidence for an enterohepatic circulation as well.

The profile of metabolites appearing in urine, bile, and feces was determined by reverse phase HPLC. Radioactivity eluted in 2 distinct regions, one corresponding to very polar metabolites and the other corresponding to fairly lipophilic metabolites. The profile for urine collected in the first 6 hours after oral administration of 10 mg/kg TNF was typical of that found for most other timepoints and routes of administration. The resultant chromatogram showed a cluster of peaks which eluted during the polar mobile phase (H_2O:CH_3CN-95:5) followed by a major region of radioactive peaks eluting during the nonpolar mobile phase of 100% CH_3CN.

After treatment of the urine with β-glucuronidase, composition of the urinary metabolites changed markedly. The amount of polar metabolites greatly diminished concomitant with an increase in the proportion of later eluting peaks, particularly a major peak eluting between 19.0 and 19.5 min-

TABLE 4 Elimination and Total Tissue Retention of Radioactivity after Oral (1 mg/kg) and Intravenous (1 mg/kg) Administration of [^{14}C]-TNF to Female F344/N Rats[a]

	ORAL Cumulative % Dose			
Time (h)	Urine	Feces	Tissues [d]	% Recovery
0 - 6	8.5 ± 2.8 [b]			
6 - 12	13.6 ± 0.4	0.02 ± 0.00		
12 - 24	17.2 ± 0.5 [c]	55.3 ± 4.3		
24 - 48	17.8 ± 0.5 [c]	67.8 ± 3.4		
48 - 72	17.9 ± 0.5 [c]	68.6 ± 3.4	0.53 ± 0.11	87.1 ± 3.6

	INTRAVENOUS Cumulative % Dose			
Time (h)	Urine	Feces	Tissues [e]	% Recovery
0 - 6	15.3 ± 2.3			
6 - 12	19.7 ± 2.3	3.3 ± 2.2		
12 - 24	23.3 ± 1.8	46.1 ± 8.2		
24 - 48	24.3 ± 1.8	53.1 ± 9.4		
48 - 72	24.7 ± 1.8	61.7 ± 2.7	4.4 ± 0.3	90.8 ± 3.5

[a] Values are means ± standard deviation for 4 animals.
[b] n = 3.
[c] Values include cage rinses.
[d] Sum of % dose recovered from adipose, cecum.
[e] Sum of % doses recovered from adipose, adrenals, brain, heart, lung, ovaries.

TABLE 5 Biliary Excretion of ^{14}C after Intravenous or Intraintestinal Administration of [^{14}C]-TNF (1 mg/kg)

Bile Collection Interval (hr)	% Intravenous Dose Excreted in Bile [a]	% Intraintestinal Dose Excreted in Bile [a]
0.0 - 0.25	8.2 ± 2.5	0.5 ± 0.1
0.25 - 0.50	9.7 ± 3.5	2.5 ± 1.1
0.5 - 0.75	6.0 ± 3.4	2.4 ± 1.8
0.75 - 1.0	3.7 ± 1.4	2.5 ± 1.7
1.0 - 1.5	6.9 ± 1.5	4.8 ± 1.9
1.5 - 2.0	3.5 ± 2.0	2.8 ± 1.5
2.0 - 3.0	6.9 ± 1.4	2.9 ± 1.5
3.0 - 4.0	4.0 ± 0.8	2.1 ± 0.2
4.0 - 5.0	2.8 ± 0.4	1.2 ± 0.9
5.0 - 6.0	1.8 ± 0.3	1.1 ± 0.4
Total	53.5 ± 13.4	21.8 ± 9.5

[a] Mean ± standard Deviation (N = 2 - 5 female rats per time point)

utes. The transformation was inhibited by 1,4-saccharolactone, an inhibitor of β-glucuronidase. It is concluded that the polar metabolites are glucuronides of the later eluting peaks. Biliary profiles were very similar to urinary profiles; most of the early eluting components in the biliary profiles also were converted to later eluting peaks on treatment with β-glucuronidase.

As previously noted, approximately two-thirds of the orally administered doses of TNF were excreted in the feces. At the 100 mg/kg dose, 62% of the radioactivity was contained in the feces collected during the 12-to-48-hour time period. The homogenate was first extracted with buffer, then with methanol; approximately 60% of the radioactivity extracted into the buffer. The chromatogram of this extract showed the same components found in urine and bile. However, the nonpolar compound eluting at 19.5 minutes was present in feces to a greater extent than in urine

or bile. The early eluting peaks were converted to this component by the action of b-glucuronidase. The methanolic extract, containing about 30% of the fecal radioactivity, contained this component as the major peak (70%), as well as a new peak which coeluted with TNF. Peak enhancement experiments with authentic sample confirmed this assignment. Apparently, about 15% of orally administered TNF was excreted unchanged.

14-Day Dermal Study in F344/N Rats

There was no mortality nor any clinical signs of toxicity, although body weight gains were slightly less than controls in most dose groups (Table 6). With the exception of a tan to yellow discoloration of the skin at the site of chemical application, there were no gross or microscopic changes related to treatment in rats.

TABLE 6 Survival and Weight Gain of F344/N Rats in the 14-Day Dermal Studies of Trinitrofluorenone

Dose Concentration (mg/ml)	Survival[a]	Mean Body Weight (grams)			Final Weight Relative to Controls (%)[c]
		Initial	Final	Change[b]	
MALE					
0	5/5	136.0	210.2	74.2	100.0
3	5/5	134.2	195.2	61.0	92.9
6	5/5	138.5	212.7	74.2	101.2
12	5/5	137.8	209.7	71.9	99.8
24	5/5	138.5	199.6	61.1	95.0
48	5/5	137.6	200.4	62.8	95.3
FEMALE					
0	5/5	108.0	144.9	36.9	100.0
3	5/5	106.8	138.8	32.0	95.8
6	5/5	111.2	144.1	32.9	99.4
12	5/5	108.1	142.6	34.5	98.4
24	5/5	112.4	144.7	32.3	99.9
48	5/5	107.6	138.8	31.2	95.8

[a] Number surviving at 14 days/number of animals/dose group.
[b] Mean weight change of the animals in each dose group.
[c] (Dosed group mean/Control group mean) x 100.

14-Day Dosed Feed Study in F344/N Rats

No deaths occurred in any group (Table 7). Mean body weight gains were lower than controls in a dose-dependent fashion and were significantly decreased at the 3 highest doses. Weight loss and a decrease of approximately 45% in feed consumption were noted in the high-dose groups (50000 ppm).

Clinical signs and other changes attributed to treatment were present in rats administered TNF at doses of 5000 ppm or greater in the feed. Urine had a brown discoloration and brown urine stains were present on the hair coat; the entire hair coat and skin had a black discoloration characterized microscopically by a brown pigmentation of the hair shafts in these albino rats. The thyroid gland was dark and enlarged. Microscopically, there was a brown granular pigment in the epithelium and colloid of the thyroid follicles; there was also a mild to moderate hypertrophy of the thyroid follicular epithelium. At the 50000 ppm dose level, there was dilatation of the cecum, and the thymus and seminal vesicles were decreased in size. Lymphoid depletion (atrophy) was present in

TABLE 7 Survival and Weight Gain of F344/N Rats in the 14-Day Dosed-Feed Studies of Trinitrofluorenone

Dose (ppm)	Survival[a]	Mean Body Weight (grams) Initial	Final	Change[b]	Final Weight Relative to Controls (%)[c]	Average Feed Consumption[d]	Estimated TNF Consumed[e]
MALE							
0	5/5	135.5	209.4	73.9	100.0	81.6	
500	5/5	130.3	206.6	76.3	98.7	77.7	40
1600	5/5	130.1	198.8	68.7	94.9	69.1	114
5000	5/5	126.4	183.6	57.2	87.7	69.8	361
16000	5/5	132.6	187.6	55.0	89.6	72.6	1200
50000	5/5	130.9	114.8	-16.1	54.8	48.2	2489
FEMALE							
0	5/5	112.4	149.0	36.6	100.0	73.8	
500	5/5	112.3	147.8	35.5	99.2	70.8	37
1600	5/5	114.6	147.1	32.5	98.7	71.4	118
5000	5/5	114.0	137.4	23.4	92.2	69.4	359
16000	5/5	114.6	139.1	24.5	93.4	67.2	1112
50000	5/5	114.6	99.1	-15.5	66.5	39.3	2032

a Number surviving at 14 days/number of animals per dose group.
b Mean weight change of the animals in each dose group.
c (Dosed group mean/Control group mean) x 100.
d Time-weighted food consumption in gm/day/kg body weight.
e Time-weighted average chemical consumed during study in g/day/kg body weight.

the thymus, and atrophy of the seminal vesicles was present in males from the 50000 ppm group. Several male rats from the 16000 ppm dose group exhibited enlarged testes and epididymides. Edema of the testes also was present in 3 rats examined from the 16000 ppm group. The epididymis examined microscopically from 1 rat in the 16000 ppm group had edema and inflammation of the stroma between the tubules.

13-Week Dosed Feed Study in F344/N Rats

All animals survived to the end of the study. Body weight gains and terminal body weights were decreased in all dose groups of both sexes (Table 8; Figure 1). Food consumption for all dose groups was decreased relative to that of controls.

A variety of organ weight changes were observed in these studies (Appendix A). Interpretation of these changes was complicated by the large differences in body weights between the groups, particularly in males. The organs most often affected included liver and kidney, which were increased in weight on an absolute and relative basis in dosed groups of both sexes. Thymus weights were reduced in dosed groups of males and females, and the testis was significantly lighter in the high-dose group of rats than in controls. In contrast, absolute heart and lung weights were decreased, while relative weights were increased in dosed males, suggesting that the weight changes in these organs were secondary to changes in body weight.

Treatment-related clinical changes and other gross changes in rats included the dark colors (brown/gray /black) of urine, hair coat, thyroid gland, spleen, brain, skin, and carcass. In high-dose males, the testes were decreased in size and nodules were present on the epididymis. One or multiple brown foci which formed depressions in the renal capsular surface were seen in male rats from the 4000 and 8000 ppm groups. Microscopically, a brown granular pigment was present in

TABLE 8 Survival, Weight Gain, and Feed Consumption of F344/N Rats in the 13-Week Dosed-Feed Studies of Trinitrofluorenone

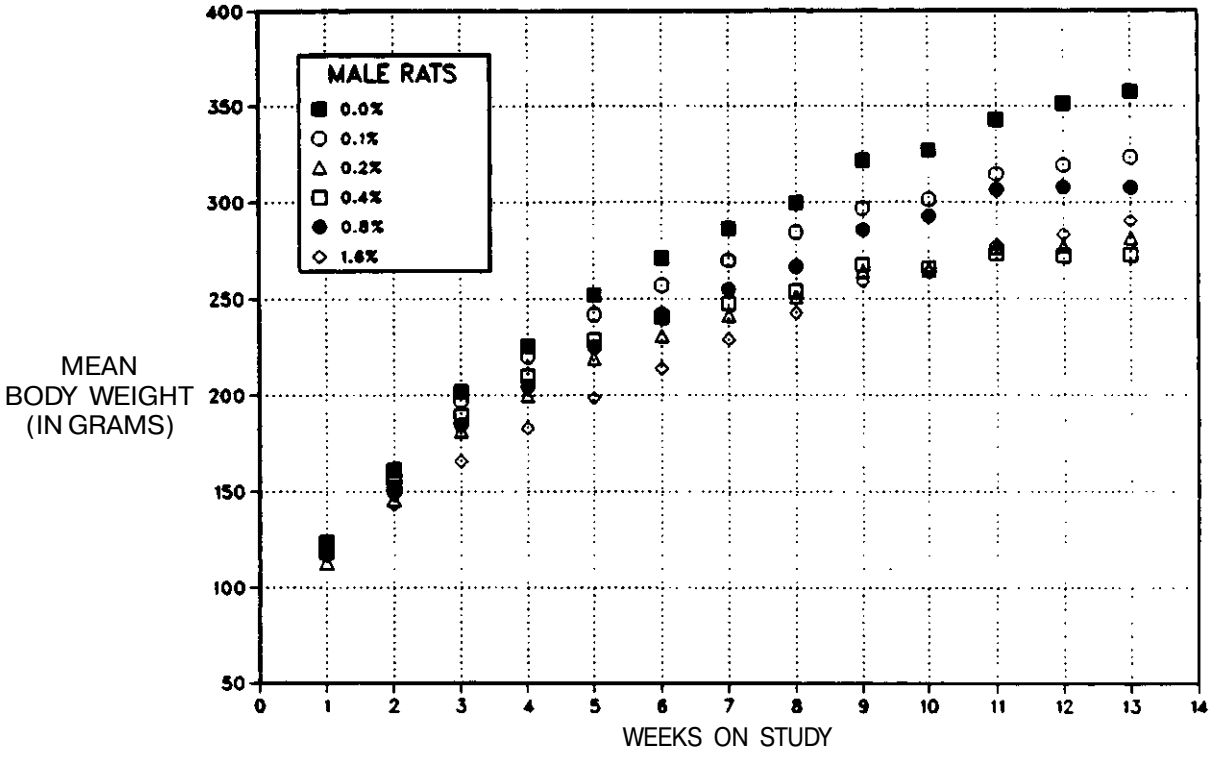
Dose (ppm)	Survival[a]	Mean Body Weight (grams) Initial	Final	Change[b]	Final Weight Relative to Controls(%)[c]	Average Feed Consumption[d]	Estimated TNF Consumed[e]
MALE							
0	10/10	124	358	234		16.8	
1000	10/10	120	323	203	90	15.8	63
2000	10/10	113	281	168	78	14.1	127
4000	10/10	118	273	154	76	13.9	245
8000	10/10	118	308	190	86	14.9	504
16000	10/10	118	290	171	81	13.9	1034
FEMALE							
0	10/10	100	195	95		10.7	
1000	10/10	99	187	89	96	10.3	66
2000	10/10	94	174	80	89	9.5	128
4000	10/10	97	168	71	86	9.5	259
8000	10/10	99	184	85	94	10.2	525
16000	10/10	97	180	83	92	9.9	1073

a Number of animals surviving at 13 weeks/number/dose group.
b Mean weight change of the animals in each dose group.
c (Dosed group mean/Control group mean) x 100.
d Average food consumption in gm/animal/day.
e Time-weighted chemical consumption in mg/kg/day.

numerous organs. The incidence and severity of pigment in organs more commonly affected is shown in Table 9.

Pigment accumulation was most extensive in the thyroid gland, where dark brown granules were observed in the cytoplasm of the follicular epithelium, with lesser amounts of pigment in the colloid secretion and interfollicular macrophages. Pigment granules in the brain were most prominent in the cytoplasm of large neurons in the basal ganglia; a smaller amount was present in the neurons of the cerebral cortex (Plate 1, Figure 2). A few pigment granules were present in the cerebellar folia and hippocampus. Pigment was in myofibers in the heart and skeletal muscle; minimal amounts were in the periosteum of the femur. Cytoplasm of the tubular epithelium of the renal cortex contained pigment granules; brown pigment-stained casts were present in the lumen of some tubules. In the kidneys of male rats, protein droplets were prominent in the cytoplasm and lumen of tubules. Interstitial cells of the ovary, epithelium of the prostate, adrenal cortical cells, and hair shafts in the skin also contained pigment. In the pituitary gland, a brown, homogeneous secretion was present in the hypophyseal cavity (Plate 1, Figure 3). Pigment also was present (primarily within macrophages) in the lymph nodes, thymus, uterus, lung, epididymis, small and large intestines, and liver (Kupffer cells and periportal macrophages).

Further attempts were made to characterize pigment in brain, thyroid, kidney, spleen, liver, and lymph nodes. In the brain, thyroid, and kidney, pigment did not stain positive with periodic acid-Schiff, acid-fast, Perl's stain for iron, or Hall's stain for bilirubin. There was some PAS-positive staining associated with brown pigment granules in macrophages in the lymph node, spleen, and liver. Brown pigment was isotropic by examination with polarized light; it also appeared brown in cleared, unstained histologic sections. By ultrastructural examination of brain and kidney, pigment appeared as electron-dense globules within membrane-bound structures in cytoplasm of neurons (Plate 2, Figures 7, 8) and in the renal tubular epithelium. In addition to pigment, other



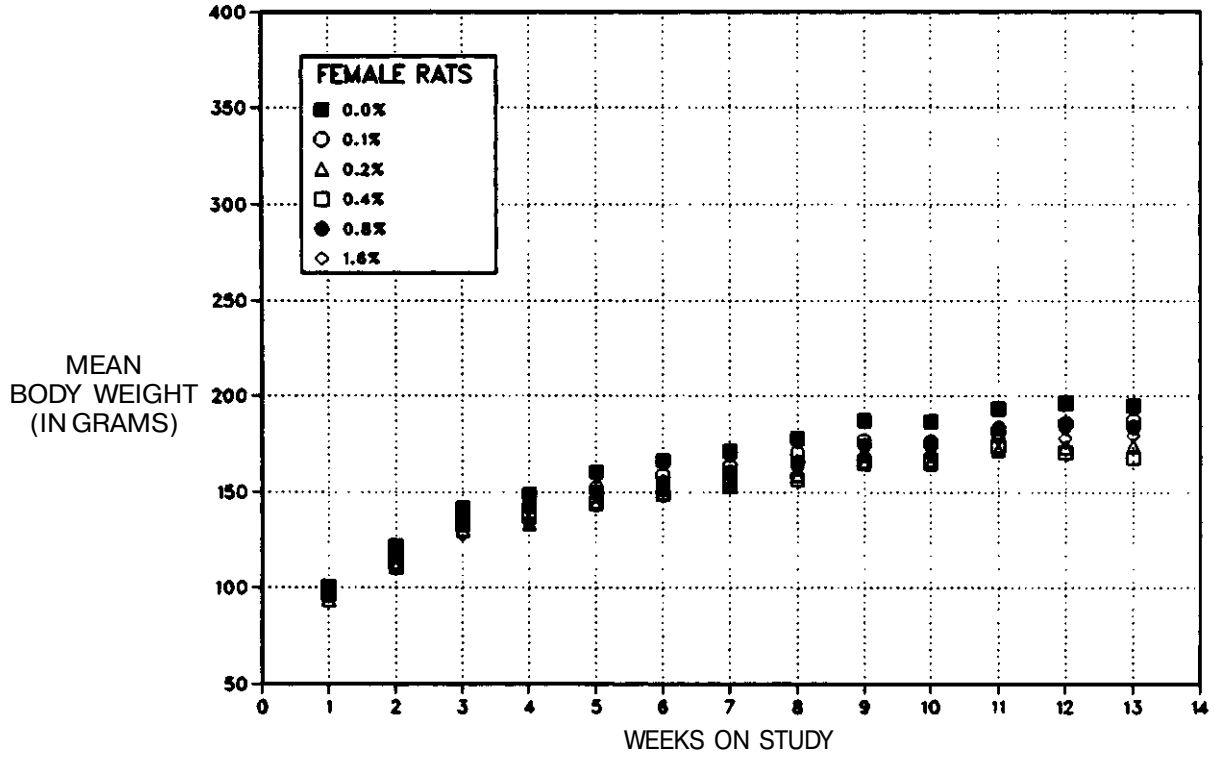


Figure 1 Growth Curves for F344/N Rats Administered Trinitrofluorenone by Dosed Feed for 13 Weeks

TABLE 9 Incidence and Severity of Pigment Accumulation in F344/N Rats Administered Trinitrofluorenone in the Feed for 13 Weeks

Dose (ppm)	0	1000	2000	4000	8000	16000
MALE						
Thyroid	0	0	0	10 (1.7)[a]	10 (4.0)	10 (4.0)
Brain	0	0	0	0	3 (1.0)	10 (3.0)
Spleen	0	0	0	10 (1.0)	10 (2.0)	10 (2.7)
Liver	0	0	0	0	10 (1.0)	10 (1.8)
Heart	0	0	0	0	7 (1.0)	10 (1.6)
Kidney	0	0	0	10 (1.0)	10 (1.8)	10 (1.7)
Mesenteric lymph node	0	0	0	0	10 (1.0)	10 (1.0)
Pituitary	0	0	0	0	3 (2.0)	6 (1.5)
Adrenal cortex	0	0	0	0	9 (1.0)	10 (1.0)
Skin (hair)	0	0	0	0	10 (2.0)	10 (3.0)
Bone	0	0	0	0	10 (1.0)	10 (1.0)
Skeletal Muscle	0	0	0	0	0	10 (1.5)
Thymus	0	0	0	0	5 (1.0)	10 (1.0)
Epididymis	0	0	0	0	2 (1.5)	9 (1.9)
Prostate	0	0	0	0	0	2 (1.5)
FEMALE						
Thyroid	0	0	0	10 (1.7)	10 (4.0)	10 (4.0)
Brain	0	0	0	0	0	10 (3.0)
Spleen	0	0	0	10 (1.0)	10 (2.0)	10 (3.0)
Liver	0	0	0	0	1 (1.0)	10 (1.3)
Heart	0	0	0	0	5 (1.0)	10 (1.0)
Kidney	0	10 (1.0)	10 (1.0)	10 (1.0)	10 (2.0)	10 (2.0)
Mesenteric lymph node	0	0	0	0	0	10 (1.0)
Pituitary	0	0	0	0	7 (1.7)	8 (1.9)
Adrenal cortex	0	0	0	10 (1.0)	10 (1.0)	10 (1.0)
Skin (hair)	0	0	0	0	10 (1.0)	10 (3.0)
Bone	0	0	0	0	1 (1.0)	10 (1.0)
Thymus	0	0	0	0	0	10 (1.0)
Skeletal Muscle	0	0	0	0	0	10 (2.0)
Ovary	0	0	0	0	0	10 (1.0)
Uterus	0	0	0	0	0	8 (1.0)

[a] Average severity score based on a scale of 1 to 4; 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. Scores are averages based on number of animals with lesions from groups of 10.

lesions attributed to TNF were present in the liver and spleen of male and female rats and in the kidney, testes, and epididymis, and mesenteric blood vessels of male rats (Table 10).

In dosed male and female rats, cytoplasmic alteration of hepatocytes in the centrilobular areas of the liver was characterized by increased eosinophilic staining of the cytoplasm. In the liver of male rats, mixed-cell foci and oval cell hyperplasia also were present in the high dose group. Multiple mixed-cell foci consisted of a mixture of clear cells with a centrally located nucleus and irregular-shaped vacuolar spaces in the cytoplasm, and eosinophilic cells with finely granular cytoplasm (Plate 1, Figure 4). Oval cell hyperplasia was in the periportal areas where pigment accumulation also was more prominent. A minimal increase in the amount of hematopoiesis, normally present in the spleen, was seen in rats at the 2 highest doses.

In male rats, chronic inflammation was present in the kidney of most animals in the 4000 and 8000 ppm dose groups. Single or multiple foci of chronic inflammation, observed grossly, consisted of fibrosis and inflammatory cell infiltrate of lymphocytes and macrophages that replaced most renal tubules in these areas (Plate 1, Figure 5). This lesion was variable in its distribution and severity. In some rats, most of 1 kidney was affected, while little or no inflammation was present in the other kidney section. In less severely affected kidneys, 1 or 2 small foci of inflammation

TABLE 10 Incidence and Severity of Non-Pigmented Lesions in F344/N Rats Administered Trinitrofluorenone in the Feed for 13 Weeks

Dose (ppm)	0	1000	2000	4000	8000	16000
MALE						
Liver						
Cytoplasmic alteration	0	0	0	0	3 (2.0)[a]	10 (3.0)
Mixed cell focus	0	0	0	0	0	9
Oval cell hyperplasia	0	0	0	0	1 (1.0)	10 (1.9)
Testes						
Degeneration	0	0	0	1 (1.0)	2 (4.0)	10 (4.0)
Epididymis						
Inflammation, chronic	0	0	0	0	3 (2.0)	10 (3.0)
Granuloma	0	0	0	0	4 (2.2)	6 (2.7)
Epithelial hyperplasia	0	0	0	0	0	4 (2.0)
Kidney						
Inflammation, chronic	0	0	0	6 (2.0)	8 (3.0)	0
Mesenteric vessels						
Inflammation, chronic	0/5	0/5	4/7 (1.5)	1/5 (3.0)	2/6 (2.0)	4/6 (2.5)
Spleen						
Hematopoiesis	0	0	0	0	2 (1.0)	3 (1.0)
FEMALE						
Liver						
Cytoplasmic alteration	0	0	0	0	0	6 (1.3)
Mesenteric vessels						
Inflammation, chronic	0/4	0/6	0/4	1/6	0/5	0/4
Spleen						
Hematopoiesis	0	0	0	0	2 (1.0)	4 (1.0)

a Average severity score based on a scale of 1 to 4; 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. Scores are averages based on number of animals with lesions from groups of 10; denominator for mesenteric vessels indicates number of rats with sufficient tissue present for examination.

were sharply demarcated from surrounding normal tissue. Often these were linear or wedge-shaped, with the base near the capsule and the apex extending into the inner medulla. The capsular surface of the kidney was sometimes depressed over a focus of inflammation. Intertubular fibrosis and tubular cell regeneration were prominent in these foci of chronic inflammation, and the tubular lumens frequently contained protein casts, cell debris, and neutrophils.

In the mesenteric vessels (adjacent to the mesenteric lymph node), chronic inflammation of arteries was characterized by fibrosis, a mixed inflammatory cell infiltrate in the adventitia and tunica media, and degeneration of the smooth muscle in the tunica media. This vascular lesion was seen primarily in male rats. There was no clear dose relationship in incidence or severity for vasculitis, although it was not present in the low dose group.

At the highest dose level, there was a marked degeneration and atrophy of the testes. The testes were reduced in size, and most seminiferous tubules contained only Sertoli cells. Microscopic foci of chronic inflammation with fibrosis and mineralization were present in some tubules of the degenerative testes. In the epididymides, chronic inflammation and fibrosis were present, and the nodules observed grossly represented focal granulomas, sometimes containing spermatozoa (Plate 1, Figure 6). Most epididymal ductular lumens were empty or contained a few inflammatory cells and cell debris. Pigmentation was present in macrophages of the interductular connective tissue, and at the highest dose there was hyperplasia of the ductular epithelium.

In other reproductive tissue evaluations, left epididymal weights were significantly decreased at the 4000 ppm level (Appendix C, Table C1). There was a dose-related decrease in left testicular weights

with significant differences at 8000 and 16000 ppm. Left cauda and whole epididymal weights were slightly increased at the 16000 ppm dose; however, the small difference in caudal weights was significant. Epididymal sperm motility could not be determined in rats at the 16000 ppm level because no motile sperm were detected in any of the 10 rats. Sperm density could be estimated for 1 animal only in the 16000 ppm dose group. Total spermatid heads per testis and total spermatid heads per gram testis were also severely affected in the top dose group (only 2 animals could be evaluated). There were no effects on estrual cyclicity or average estrous cycle length (Appendix C, Table C2).

Significant hematologic changes in animals assayed on day 5 were mild and confined to female rats (Appendix B, Table B1). These included an increased concentration of methemoglobin (high-dose group), decreased leukocyte counts produced by inconsistent lymphopenia and neutropenia (multiple dose groups), and thrombocytosis (high-dose group). In male rats on day 21, there was a mild but significant neutrophilia and methemoglobinemia. At the same time point, female rats had mild but significant decreases in MCH, MCV, and concentrations of HGB. There were increases of similar magnitude in platelet counts and concentrations of methemoglobin. In male and female rats at day 21, effects were confined to animals in the 2 highest dose groups. At 13 weeks, male and female rats in the 3 highest dose groups had significant decreases in erythrocyte counts; those in 2 highest groups showed decreased concentrations of HGB and HCT. These changes were accompanied by increases in MCV, MCH, MCHC (males), and concentrations of methemoglobin. Platelet counts were increased in animals in the same dose groups.

Relevant changes in clinical chemistry variables included significant decreases in activities of AP (all time points, both sexes, multiple dose groups); and in concentrations of total serum bile acids in the highest dose groups of both sexes on days 5 and 21, and in the top dose group of female rats at 13 weeks (Appendix B). At most time points, there was a trend for concentrations of bile acids to increase in animals in lower dose groups. Other changes that were significant but minimal in magnitude included increased activities of ALT at days 5 and 21 in female rats, increased concentrations of creatinine in female rats at all time points and in male rats at day 5, decreased concentrations of protein in male rats at days 5 and 21 in high-dose groups, and increased concentrations of UN in animals of both sexes at day 5.

Plate 1

Figure 1. Thyroid gland from male mouse fed a diet containing 12500 ppm trinitrofluorenone. Focal area of cystic degeneration consists of irregular shaped follicles lined by a flattened epithelium and containing pigment granules and cell debris. Note pigment-filled epithelium (arrows) in surrounding follicles.

Figure 2. Brain from male rat fed a diet containing 16000 ppm trinitrofluorenone. Note accumulation of dark pigment granules (arrows) in the cytoplasm of large neurons in brain stem.

Figure 3. Pituitary gland from male rat fed a diet containing 16000 ppm trinitrofluorenone. Hypophyseal cavity (C) between pars inter-media and pars distalis contains dark brown pigment.

Figure 4. Liver from male rat fed a diet containing 16000 ppm trinitrofluorenone. Mixed cell focus (arrows) adjacent to portal area (P) consists of enlarged hepatocytes with vacuolated or eosinophilic granular cytoplasm.

Figure 5. Kidney from male rat fed a diet containing 8000 ppm trinitrofluorenone. Inflammation within tubules consists of aggregates of neutrophils (arrows) and foci of granulomatous inflammation with giant cell formation (G). Inflammatory cell infiltrate is also prominent in the interstitium.

Figure 6. Epididymis from male rat fed a diet containing 16000 ppm trinitrofluorenone. Sperm granuloma (arrows) consists of central area of spermatozoa and cell debris surrounded by zone of macrophages, giant cells, and darkly staining lymphocytes. Note absence of spermatozoa in lumen of the adjacent epididymal ducts (D).

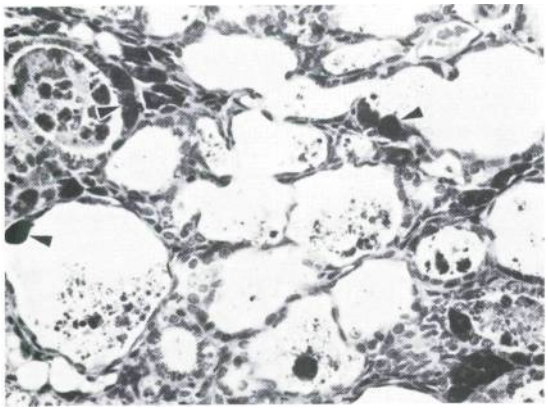


Figure 1

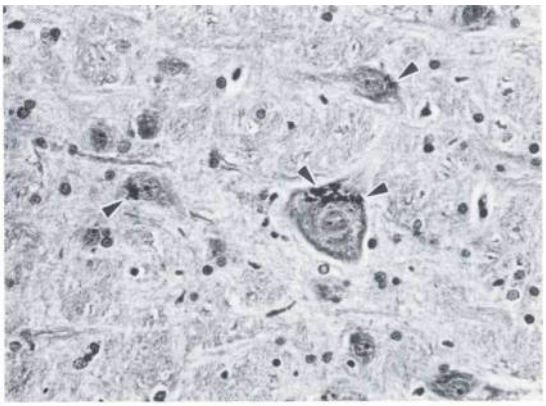


Figure 2

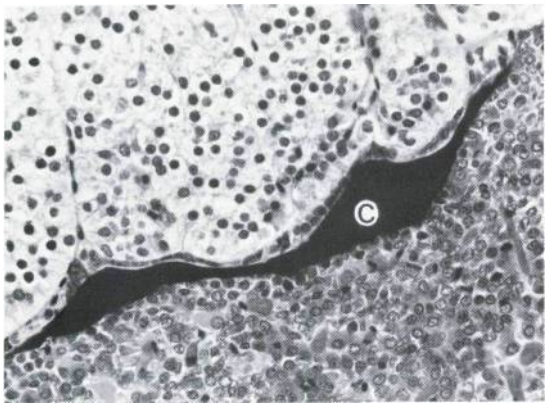


Figure 3

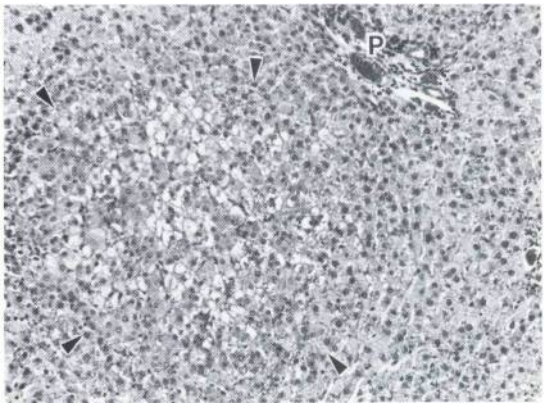


Figure 4

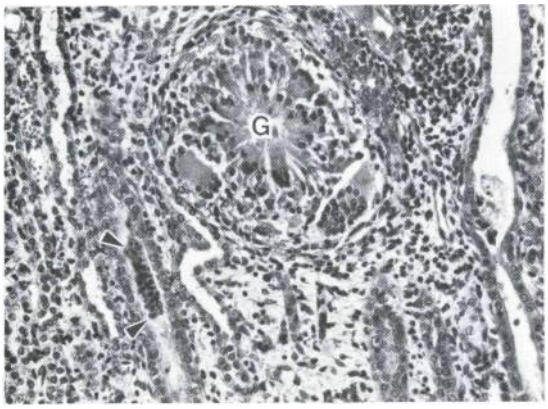


Figure 5

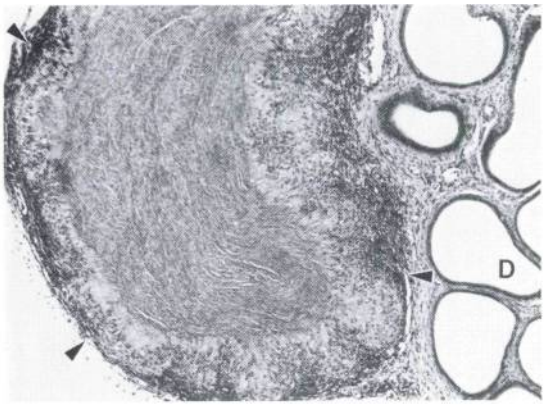


Figure 6

Reverse of Plate 1

14-Day Dermal Study in B6C3F$_1$ Mice

There were no deaths in any group and no differences in body weight gains attributed to chemical administration (Table 11). With the exception of a tan to yellow discoloration of the skin at the site of chemical application, there were no gross or microscopic changes related to treatment in mice.

TABLE 11 Survival and Weight Gain of B6C3F$_1$ Mice in the 14-day Dermal Studies of Trinitrofluorenone

Dose Concentration (mg/ml)	Survival[a]	Mean Body Weight (grams)			Final Weight Relative to Controls (%)[c]
		Initial	Final	Change[b]	
MALE					
0	5/5	25.1	27.1	2.0	100.0
3	5/5	25.2	27.6	2.4	101.8
6	5/5	25.5	27.3	1.8	100.7
12	5/5	24.9	27.6	2.7	101.8
24	5/5	25.7	28.1	2.4	103.7
48	5/5	25.0	27.5	2.5	101.5
FEMALE					
0	5/5	19.8	23.0	3.2	100.0
3	5/5	20.8	23.8	3.0	103.5
6	5/5	20.1	22.2	2.1	96.5
12	5/5	20.7	23.5	2.8	102.2
24	5/5	18.8	23.1	4.3	100.4
48	5/5	20.8	24.1	3.3	104.8

a Number surviving at 14 days/number of animals per dose group.
b Mean weight change of the animals in each dose group.
c (Dosed group mean/Control group mean) x 100.

14-Day Dosed Feed Study in B6C3F$_1$ Mice

All animals survived to the end of the study (Table 12). Body weight gains were not affected consistently by the administration of TNF. Clinical and other gross changes attributed to treatment were present in mice administered TNF at doses of 5000 ppm or greater in the feed. There was a brown discoloration of the urine, and brown urine stains were present on the hair coat. The thyroid gland was dark. At the highest dose, the brain and gall bladder were dark, and the spleen (females only) was dark and enlarged.

Microscopically, a brown granular pigment was prominent in the epithelium and colloid of the thyroid follicles; at the highest dose level there was a minimal to mild hypertrophy of the thyroid follicular epithelium. A scant amount of granular brown pigment was present in the cytoplasm of neurons in the brain. The spleen enlargement in female mice was the result of increased hematopoiesis.

TABLE 12 Survival and Weight Gain of B6C3F$_1$ Mice in the 14-day Dosed Feed Studies of Trinitrofluorenone

Dose Concentration (ppm)	Survival[a]	Mean Body Weight (grams) Initial	Final	Change[b]	Final Weight Relative to Controls (%)[c]	Average Feed Consumption[d]	Estimated TNF Consumed[e]
MALE							
0	5/5	21.7	23.4	1.7	100.0	283.4	
500	5/5	22.1	24.3	2.2	103.8	242.7	126
1600	5/5	21.4	23.8	2.4	101.7	225.7	373
5000	5/5	22.0	24.5	2.5	104.7	259.5	1341
16000	5/5	22.4	24.2	1.8	103.4	235.9	3903
50000	5/5	21.5	22.6	1.1	96.6	268.3	13869
FEMALE							
0	5/5	18.4	22.0	3.6	100.0	251.6	
500	5/5	18.2	21.3	3.1	96.8	345.6	179
1600	5/5	18.3	21.1	2.8	95.9	295.0	488
5000	5/5	19.0	21.8	2.8	99.1	324.9	1680
16000	5/5	18.8	21.3	2.5	96.8	307.8	5091
50000	5/5	19.0	21.2	2.2	96.4	333.6	17245

a Number surviving at 14 days/number of animals per dose group
b Mean weight change of the animals in each dose group.
c (Dosed group mean/Control group mean) x 100.
d Time-weighted food consumption in gm/day/kg body weight.
e Time-weighted average chemical consumption during the study in mg/day/kg body weight.

13-Week Dosed Feed Study in B6C3F$_1$ Mice

Of mice receiving diets containing TNF at concentrations of 0, 3125, 6250, 12500, 25000, or 50000 ppm, 11 died prior to study termination. Five deaths occurred in the top-dose group of males; 4 in lower dose groups of males; 2 in the top-dose group of females. Deaths of top-dose mice were considered possibly chemically-related; causes of death at lower doses could not be determined (Table 13). Body weight gains and final body weights decreased in male mice in the 25000 and 50000 ppm dose groups and in female mice in the 50000 ppm dose groups (Table 13; Figure 2).

TABLE 13 Survival, Weight Gain, and Feed Consumption of B6C3F$_1$ Mice in the 13-Week Feed Studies of Trinitrofluorenone[a]

Dose (ppm)	Survival[b]	Mean Body Weight (grams) Initial	Final	Change[c]	Final Weight Relative to Controls (%)[d]	Average Feed Consumption[e]	Estimated TNF Consumed[f]
MALE							
0	10/10	22.2	32.1	9.9		5.2	
125	9/10	22.6	32.8	10.2	102.2	5.2	587
250	8/10	21.8	31.6	9.8	98.4	5.2	1268
12500	9/10	22.2	30.4	8.2	94.7	5.5	2663
25000	10/10	21.6	29.4	7.8	91.6	6.5	6228
50000	5/10	21.2	24.7	3.5	76.9	6.1	13266
FEMALE							
0	10/10	17.7	27.6	9.9		6.0	
125	10/10	17.4	27.2	9.8	98.6	6.3	850
250	10/10	17.4	26.8	9.4	97.1	5.8	1594
12500	10/10	17.4	27.1	9.7	98.2	6.5	3509
25000	10/10	17.1*	25.8	8.7	93.5	7.0	7840
50000	8/10	17.1	25.4	8.3	92.0	7.4	17425

a Includes animals from core and special study groups.
b Number of animals surviving at 13 weeks/number per dose group.
c Mean weight change of the animals in each dose group.
d (Dosed group mean/Control group mean) x 100.
e Average food consumption in gm/animal/day.
f Time-weighted chemical consumption in mg/kg/day.

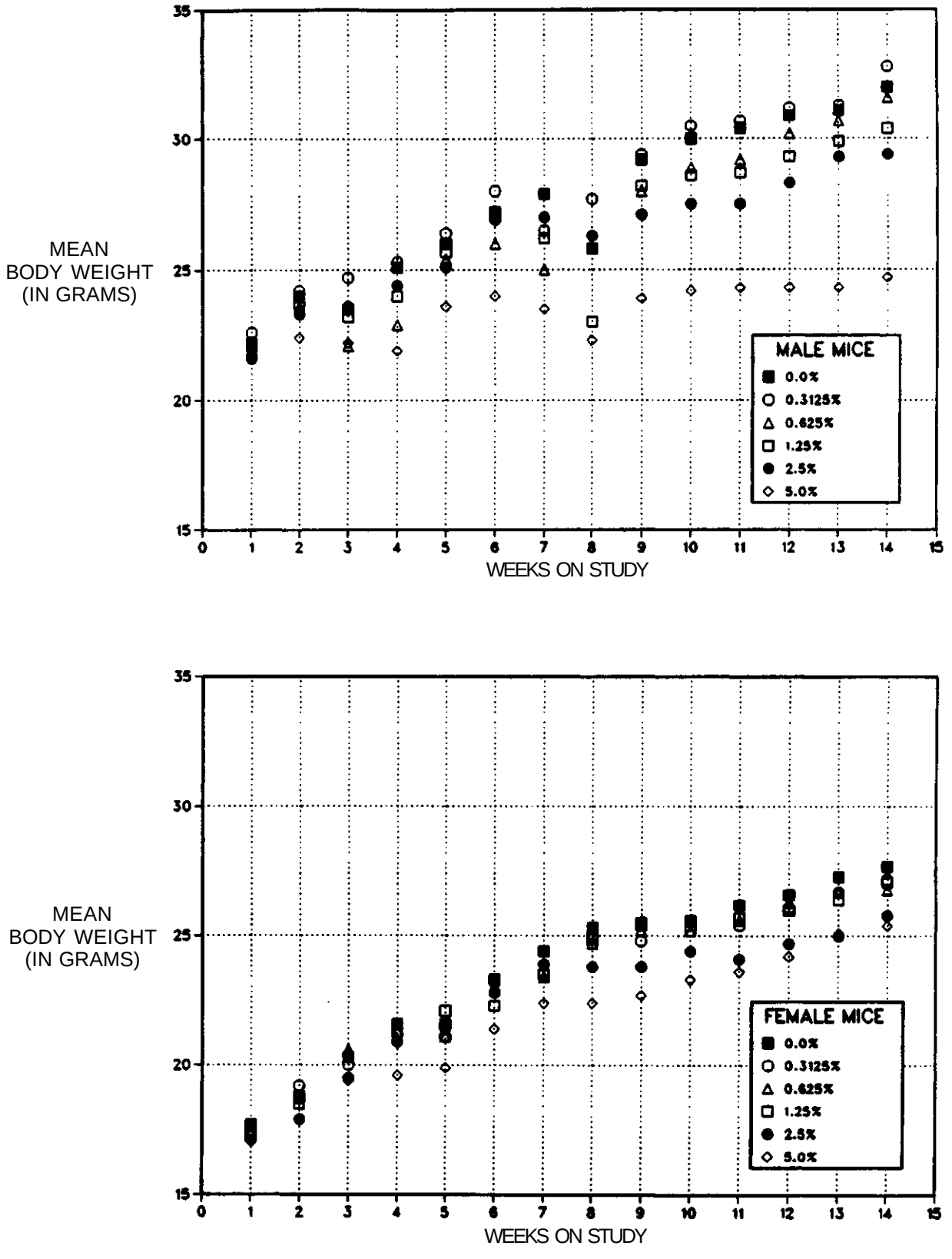


Figure 2 **Growth Curves for B6C3F$_1$ Mice Administered Trinitrofluorenone by Dosed Feed for 13 Weeks**

Plate 2

Figure 7. Electron micrograph of brainstem from male rat fed a diet containing 16000 ppm trinitrofluorenone shows a large neuron with a pale staining centrally located nucleus (N) and the typically prominent granular endoplasmic reticulum (*) in the peripheral portion of the neuronal cytoplasm. Within the cytoplasm are numerous electron-dense pigment granules associated with administration of trinitrofluorenone. TEM 9000X.

Figure 8. Higher magnification of pigment granules in Figure 1 demonstrates the membrane-bound (arrows), electron-dense granules adjacent to the granular endoplasmic reticulum (Nissl substance) in the neuronal cytoplasm. TEM 42000X.

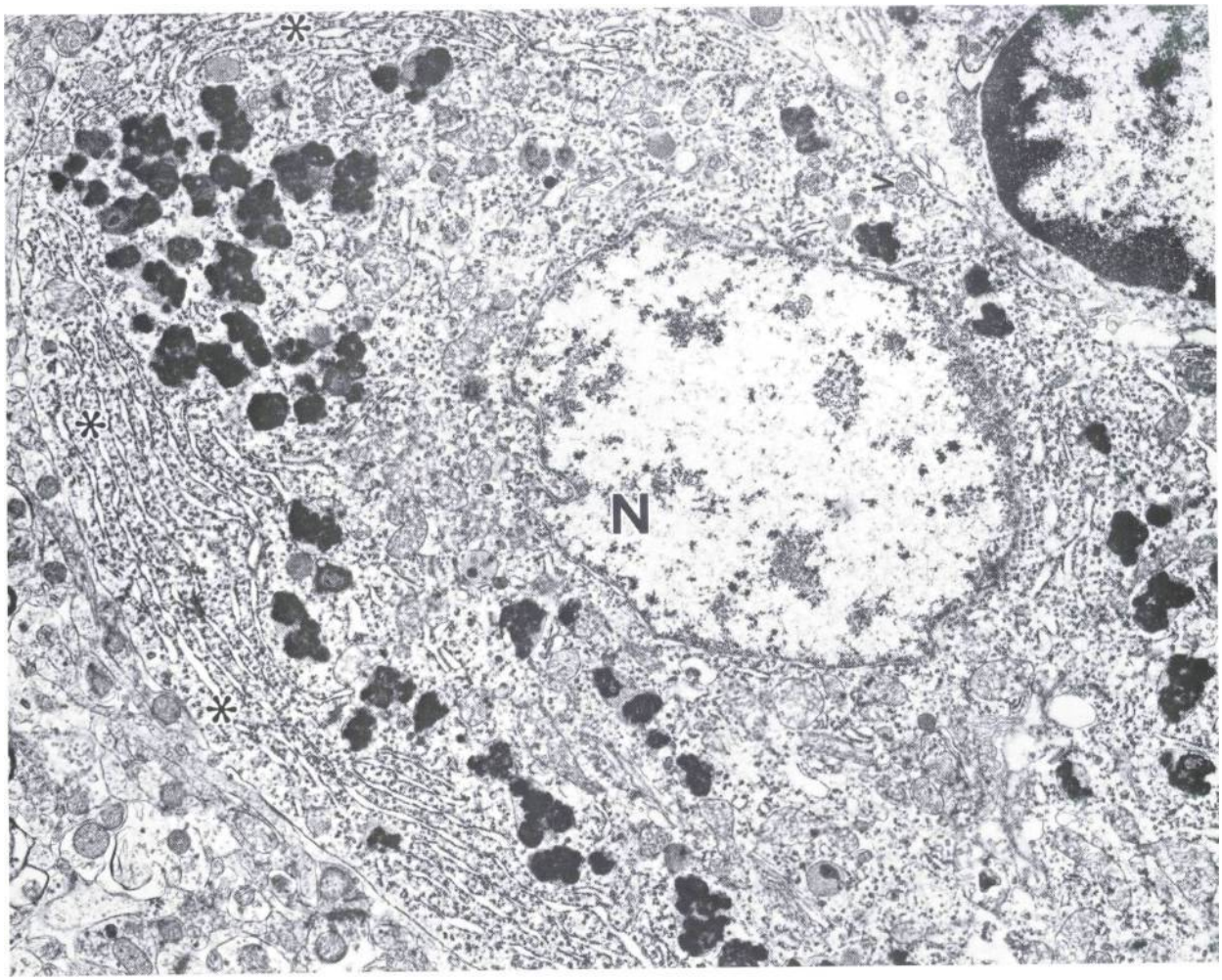


Figure 7

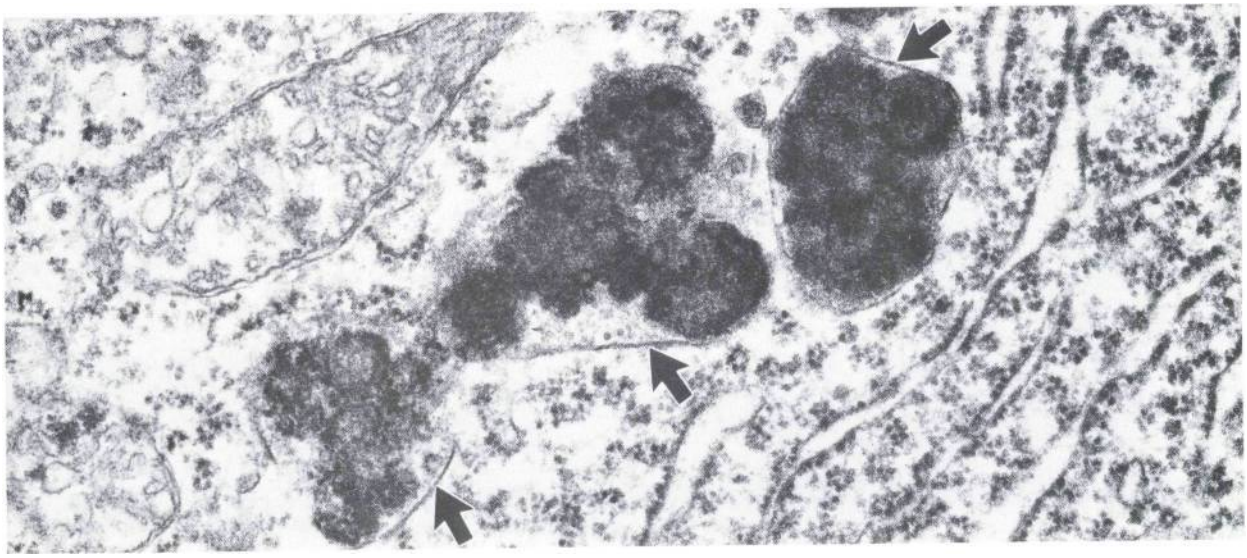


Figure 8

Reverse of Plate 2

Relative liver weights were increased and thymus weights decreased in dosed male and female mice, when compared to controls (Appendix A, Table A2). Other changes in organ weights appeared to be secondary to differences in body weights between the dosed and control groups of mice.

Treatment-related clinical and gross findings were present in mice in all dose groups. Observations included brown discoloration of the urine and bile and gray discoloration of the hair coat. The color of a number of tissues was darker than normal; this was most apparent in the thyroid gland but was also seen in the liver, spleen, brain, lymph node, and muscle. Microscopically, brown pigment was present in the thyroid gland, brain, spleen, liver, cardiac and skeletal muscle, kidney, mesenteric and mandibular lymph nodes, urinary bladder, uterus, ovary, and mammary gland. Dose levels where pigment was present and the average severity are shown in Table 14. Brown granular pigment was most prominent in the cytoplasm of the thyroid gland follicular cell epithelium and interfollicular macrophages; a small number of pigment granules were present in the colloid secretion of follicles (Plate 1). Pigment-laden follicular cells were larger and the cytoplasm stained more basophilic than in follicular cells in the thyroid gland of controls. In the brain, the cytoplasm of the larger neurons in the basal ganglia and cerebral cortex contained the most pigment; smaller aggregates of pigment granules scattered throughout the neuropil of the cerebral cortex appeared to be mainly within nerve cell processes. Most of the cerebellum, hippocampus, and white matter contained no pigment, or only scant amounts. Pigment was prominent in the spleen (red pulp and lymphoid follicles), lymph node (sinusoidal macrophages), interstitial cells of the ovary, uterine endometrial stroma, and smooth muscle layers. Minimal amounts of pigment were present in cardiac and skeletal muscle fibers, Kupffer cells of the liver, and urinary bladder epithelium. Secretions in ducts of the mammary glands and collecting ducts in the renal medulla were stained dark brown.

TABLE 14 Incidence and Severity of Pigment Accumulation in B6C3F$_1$ Mice Administered Trinitrofluorenone in the Feed for 13 Weeks

Dose (ppm)	0	3125	6250	12500	25000	50000
MALE						
Thyroid	0	9 (1.2)[a]	10 (2.6)	10 (3.7)	10 (4.0)	10 (4.0)
Brain	0	0	0	0	10 (1.1)	10 (2.8)
Spleen	0	0	0	7 (1.0)	10 (1.2)	9 (2.2)
Liver	0	0	0	4 (1.0)	10 (1.0)	7 (1.3)
Heart	0	0	0	3 (1.0)	10 (1.0)	10 (1.4)
Kidney	0	0	0	1 (1.0)	1 (1.0)	8 (1.0)
Mesenteric lymph node	0	0	0	0	5 (1.4)	4 (2.0)
Skeletal Muscle	0	0	0	0	0	6 (1.2)
Urinary bladder	0	0	0	0	0	3 (1.7)
FEMALE						
Thyroid	0	10 (1.0)	10 (2.0)	10 (3.8)	10 (4.0)	10 (3.9)
Brain	0	0	0	0	10 (1.0)	10 (2.0)
Spleen	0	0	6 (1.2)	10 (1.0	10 (2.0)	10 (2.9)
Liver	0	0	0	1 (1.0)	7 (1.0)	9 (1.0)
Heart	0	0	0	0	9 (1.0)	9 (1.6)
Kidney	0	0	0	0	0	2 (1.0)
Mesenteric lymph node	0	0	0	0	3 (1.0)	5 (1.6)
Skeletal Muscle	0	0	0	0	0	1 (1.0)
Ovary	0	1 (1.0)	3 (1.7)	3 (1.7)	6 (2.2)	9 (1.8)
Mammary gland	0	0	0	10 (2.8)	10 (2.4)	10 (2.6)
Uterus	0	0	0	0	2 (1.0)	6 (1.3)

a Average severity score based on a scale of 1 to 4; 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. Scores are averages based on the number of animals with lesions from groups of 10..

TABLE 15 Incidence and Severity of Non-Pigmented Lesions in B6C3F$_1$ Mice Administered Trinitrofluorenone in the Feed for 13 Weeks

Dose (ppm)	0	3125	6250	12500	25000	50000
MALE						
Thyroid gland						
cystic degeneration	0	1 (1.0)[a]	2 (1.0)	2 (2.0)	7(1.3)	4 (1.8)
Liver						
glycogen depletion	1(1.0)	3 (2.0)	5 (2.8)	10 (2.0)	9 (2.1)	10 (3.2)
hypertrophy	0	0	0	0	10 (1.9)	10 (2.0)
Spleen						
hematopoiesis	0	0	0	1 (1.0)	9 (1.4)	4 (1.5)
FEMALE						
Thyroid gland						
cystic degeneration	0	1 (2.0)	0	5(1.0)	5 (1.6)	5 (1.6)
Liver						
glycogen depletion	0	0	0	1 (1.0)	0	6 (2.8)
hypertrophy	0	0	0	0	4 (1.5)	5 (1.6)
Spleen						
hematopoiesis	0	1 (1.0)	2 (1.0)	7 (1.3)	9 (1.7)	7 (1.7)

[a] Average severity score based on a scale of 1 to 4; 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. Scores are averages based on the number of animals with lesions from groups of 10.

Secretions in ducts of the mammary glands and collecting ducts in the renal medulla were stained dark brown.

In addition to pigment, other lesions attributed to TNF were seen in the thyroid, liver, and spleen of both male and female mice (Table 15). Cystic degeneration of thyroid follicles was present in mice mainly at the higher doses and consisted of distended follicles lined by an attenuated follicular epithelium; in some instances, there was disruption of the follicle structure with coalescence of adjacent follicles (Plate 1, Figure 1). In the liver of male mice, and to a lesser extent in female mice, hypertrophy consisted of a zone of slightly enlarged hepatocytes which had more eosinophilic staining cytoplasm than hepatocytes in controls. An additional liver change, glycogen depletion, consisted of a decrease in the granular/vacuolar appearance of the cytoplasm typically present in mice due to the normal presence of glycogen in hepatocytes. A minimal to mild increase in hematopoiesis was present in the red pulp of the spleen of male and female mice.

The left caudal, epididymal and testicular weights, epididymal sperm motility, sperm count per gram cauda epididymis, total spermatid heads per testis, and total spermatid heads per gram testis were unaffected in mice by chemical treatment in reproductive tissue evaluations (Appendix C, Table C3). There were no effects on estrual cyclicity or average estrous cycle length (Appendix C, Table C4).

Genetic Toxicology

TNF tested with a preincubation protocol over a concentration range of 0.001-66 mg/plate, induced a dose-related increase in gene mutations in *Salmonella typhimurium* strains TA100 and TA98, with and without exogenous metabolic activation (Appendix D).

DISCUSSION

These studies were conducted to identify an appropriate exposure route and to characterize the toxicity of TNF. Although topical application best mimics the known human exposure route, a comparison of systemic toxicity following administration of TNF by topical application vs. dosed feed revealed no evidence of toxicity following dermal application, whereas evidence of systemic toxicity was obtained in the feeding studies. For these studies, the highest concentration of TNF applied dermally (48 mg/ml acetone) represents the greatest amount of TNF which could be dissolved in acetone. Results from disposition studies in female rats indicated that less than 10% of a dermal dose is available systemically, as evidenced by the sum of urine and fecal excretion, and tissue accumulation. Using this value, it is calculated that top-dose mice received a systemic body burden of TNF not exceeding 20 mg/kg body weight, and rats received a maximum of approximately 12 mg TNF/kg. These doses were not adequate to produce systemic toxicity.

In contrast, findings from the 14-day dosed-feed studies ranged from no effect to clear chemical-related toxicity. Rats were fed diets containing TNF at concentrations of 0, 500, 1600, 5000, 16000, and 50000 ppm, providing estimated doses as high as 2.5 g/kg/day. Although there was no mortality in any group, weight gains of high-dose rats were markedly depressed (~ 33 to 45% compared to controls), with males being somewhat more adversely affected than females. This was accompanied by dose-dependent increases in thyroid follicular cell pigmentation, testicular edema, and seminal vesicle atrophy. In the 14-day dosed feed studies with mice, diets containing 0, 500, 1600, 5000, 16000, and 50000 ppm TNF were fed *ad libitum*. Based on consumption data, these diets yielded TNF doses as high as 17 g/kg/day. No deaths resulted in any group, and the in-life and terminal body weights were relatively unaffected. Dose-related toxicity was limited to increased splenic hematopoiesis, pigmentation in neurons of the brain, and thyroid follicular cell pigmentation and hypertrophy.

Dosed feed administration was the route selected for further characterization of the toxicity of TNF. The disposition studies following a single oral dose of radiolabeled TNF in female rats provided a quantitative estimation of accumulation in several organs. Unfortunately, the thyroid, brain, and testis were not evaluated in these studies. Except for gastrointestinal contents, the liver and kidney accumulated the greatest amount of radiolabel on a tissue-mass basis, with a tissue-to-blood ratio about 5 times greater than in other organs. The lack of specific accumulation in adipose tissue was especially notable, suggesting that the radiolabeled chemicals were water soluble conjugates and not the lipophilic parent compound. This is consistent with metabolism studies that showed TNF is metabolized to several glucuronide conjugates.

Mice generally were more resistant than rats to the toxic effects of TNF administered in the diet for 13 weeks. Some male and female mice died after receiving diets containing 50000 ppm TNF. However, this dose level was approximately 3 times the highest concentration which could be administered to rats, based on the marked body weight loss in rats seen with 50000 ppm TNF in the 14-day study. In addition to slightly decreased body weight gains and the clinical observation of discoloration of the skin and hair, there were a number of gross, microscopic, and hematologic, treatment-related changes in both sexes of rats and mice.

The hematologic changes observed at 13 weeks in male and female rats were consistent with a mild, macrocytic anemia resulting from a treatment-related decrease in erythrocyte life span (hemolysis). These findings were associated with mild increases in methemoglobin concentrations. Although an increased methemoglobin concentration is not directly responsible for the production of anemia, it does indicate the presence of oxidative stress to hemoglobin. In advanced stages, this can result in denaturation of hemoglobin with the production of Heinz bodies, a decrease in the fluidity of erythrocyte membranes, and an increase in the lysis or removal of cells from the peripheral circulation by the spleen.

Other hematologic changes were minimal to mild and not considered biologically relevant. Increased platelet counts can result from a generalized regenerative stimulus to the bone marrow, a decrease in peripheral pools (for example, spleen and lungs), and, spuriously, from the counting of other small particles in the blood (such as separated Heinz bodies). Similarly, changes in leukocyte counts at 5 and 21 days were mild and inconsistent.

Consistent clinical chemistry findings included decreases in activities of AP and concentrations of total bile acids. The former is a frequent finding in rats and generally correlates with decreased feed consumption. The major isoenzyme of AP in the rat is of intestinal origin; its serum activities increase after eating and decrease with fasting or with decreased feed consumption. Among treated animals in the current study, average feed consumption was decreased more in male than in female rats. There was a good correlation between the decreased AP activities at 13 weeks and the decreased feed consumption.

Decreases in concentrations of total bile acids in serum are generally produced by factors that decrease intestinal absorption of bile acids. These include increased intestinal motility, increased binding to intestinal contents, and functional or mechanical impairment of absorption. There was no clinical evidence of increased motility or histopathologic evidence of significant intestinal effects. Other possibilities for the decreases in bile acids could be decreased synthesis, or an artifact due to chemical-induced interference with the assay. To explore the latter (in the clinical pathology laboratory at NIEHS), TNF in DMSO, was added to serum samples from rats to produce final concentrations of 0.1, 1.0, and 10.0 mM. Control samples with DMSO were also prepared. Measured concentrations of total bile acids were not decreased by 0.1 and 1.0 mM TNF. The formation of a precipitate in the samples at 10.0 mM TNF precluded measurement of bile acids in these samples. Because serum concentrations of TNF in the 13-week study are not known, and because the *in vitro* experiment did not explore the possibility that metabolites of TNF could be interfering with the assay, these findings are not conclusive. Further, the possibility of a treatment-related effect on the synthesis of bile acids cannot be excluded.

Other clinical pathology changes were mild and inconsistent. These included increases in UN and creatinine (possibly related to mild dehydration), and decreases in total protein at early time points in male rats. In female rats at days 5 and 21, there was evidence of mild hepatocellular damage as shown by an increase in the activity of ALT. This change was not evident at 13 weeks.

The accumulation of brown pigment in many organs was seen in both sexes of rats and mice administered TNF in the diet. Attempts to identify this pigment were unsuccessful; results of special histologic stains demonstrated that the pigment did not have staining characteristics of the

more common endogenous brown pigments, including hemosiderin, lipofuscin, or bile. This pigment was not birefringent, and the brown color of the granules also was apparent in unstained paraffin sections. Although TNF was administered in the feed, there was very little pigment seen the intestine or mesenteric lymph nodes compared to that in the thyroid gland, brain, spleen, or kidney. The pigment was present in the cytoplasm or glandular secretions of cells in many organs, but it was clearly most prominent in the thyroid gland. Dosed feed studies with unrelated compounds including minocycline (Tajima *et al.,* 1985) and hair dyes (Ward *et al.,* 1979) have resulted in the accumulation of brown pigment in the thyroid gland follicular cells and colloid; staining characteristics of pigments in those studies were similar to the pigment observed in TNF-treated rats and mice. The brown pigment associated with minocycline administration to rats was considered to be a metabolic derivative of the compound. The brown pigment seen in this study is also considered most likely a metabolite of TNF. The differences observed in skin and haircoat discoloration seen in the dermal (yellow to tan) and feed studies (brown to grey) suggest systemic metabolism of TNF.

Pigmented thyroid glands from rats that received minocycline released less thyroxine than the thyroid glands in controls (Tajima *et al.,* 1985). Thyroid function was not assessed in rats or mice administered TNF, but there was morphologic evidence of thyroid follicle degeneration in mice. However, in most tissues of rats and mice, there was little or no evidence of toxicity related to pigment deposition; pigment granules in the cytoplasm of neurons in the brain were not associated with clinical signs or other morphologic changes. In some organs (kidney, spleen, lymph node) the brown pigment granules were closely associated with minimal PAS positive stained granules, suggesting an increase in lipofuscin/ceroid pigment in these cells. For example, the slight increase in PAS-positive staining seen with the brown pigment granules in the spleen may be a reflection of the mild anemia, increased degradation of erythrocytes, and increased hematopoiesis in the spleen of TNF-treated rats and mice.

Chronic inflammation and degeneration of mesenteric vessels was seen primarily in male rats. This resembled the spontaneously occurring vascular lesion seen occasionally in the mesenteric arteries of 2-year-old F344/N rats. A similar vascular lesion has been reported following treatment of rats with theophylline (Collins *et al.,* 1988), caffeine (Johansson, 1981), or phenoldopam mesylate (Yuhas *et al.*, 1985) which acts as a vasodilator. While this vascular lesion appears to be clearly related to treatment in male rats, the incidence was not dose-related. This lesion was seen only in the portions of mesenteric arteries adjacent to the mesenteric lymph node. Variations in the amount and presence of mesenteric artery in the histologic section available for evaluation from each rat may have contributed to the apparent lack of a dose response.

Testicular degeneration was observed in all rats given the highest dose of TNF in the diet. At 8000 ppm, only 2 of 10 rats were affected, but the lesion was of marked severity. Although mean testicular weights were slightly reduced, there were no testicular lesions in the other 8 rats from this dose group. While a direct chemical effect on the germinal epithelium is possible, the pattern for the incidence and severity of this degenerative lesion was not typical for a testicular toxicant. Inflammation and/or granulomas were present in the epididymis of all high-dose males, but they also were present in 2 rats from the 8000 ppm group which had no testicular lesions. Other chemicals have caused similar testicular lesions. DL-Ethionine produced both testicular degeneration and granuloma formation in the epididymis of rats (Benson and Clare, 1966), and the

antihypertensive drug, guanethidine, produced epididymal granulomas in the absence of testicular effects (Bhathal *et al.*, 1974). The pathogenesis of the testicular and epididymis lesions in rats administered TNF is not known. The presence of granulomas in rats, in the absence of testicular changes, suggests the epididymal changes are not secondary to marked degeneration of the testis. TNF may have a direct effect on germinal epithelium of the seminiferous tubules and similarly disrupt the integrity of the epididymal ducts, resulting in a foreign body/inflammatory response to the spermatozoa. Further studies using shorter dosing periods would be necessary to address these questions.

Chronic inflammation in the kidney consisted of focal lesions in one or both kidneys that were limited to male rats in the 4000 and 8000 ppm dose groups. In addition to the chronic inflammation, there was brown pigment in tubular epithelial cells and pigment-stained casts in renal tubules. There was an increased number of protein droplets in renal tubules, but the spectrum of renal lesions typically seen in the "hyaline droplet nephropathy" of male rats (Alden, 1986; Short *et al.,* 1986) was not present in this study. The focal, well-demarcated inflammatory lesions are not typical of a direct cytotoxic effect in the kidney. This lesion may be the result of obstruction and disruption of renal tubules, with a subsequent inflammatory response to tubular contents or basement membrane antigen. The pathogenesis of this kidney lesion could not be determined. The absence of this inflammation in the kidney of the high-dose group is further evidence that multiple factors may be involved in the development of this lesion.

Microscopic evidence of liver toxicity was limited to hypertrophy and an increased intensity of cytoplasmic staining of hepatocytes in mice and female rats. However, in male rats, mixed cell foci and oval cell hyperplasia were observed in the liver. Based upon the hepatic changes, the evidence for accumulation of TNF in the liver, and its potent mutagenicity, it is considered likely that TNF would prove to be a liver carcinogen in male rats in longer-term studies.

In summary, TNF was toxic in oral feeding studies and resulted in a marked pigment accumulation in various organs. The no-observed-adverse-effect-level (NOAEL) for microscopic lesions other than pigment accumulation was 1000 ppm in the diet for rats; doses were not sufficiently low enough to determine a NOAEL for mice. The likelihood of systemic toxicity resulting from dermal contact appears remote due to limited absorption of the compound through the skin.

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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 Feed Study of Trinitrofluorenone A-2

Table A2 Organ Weights and Organ-Weight-to-Body-Weight Ratios for B6C3F$_1$ Mice in the 13-Week Feed Study of Trinitrofluorenone A-3

Table A1 Organ Weight and Organ-Weight-to-Body-Weight Ratios for F344/N Rats in the 13-Week Feed Study of Trinitrofluorenone[1]

	0 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm	16,000 ppm
Male						
n	10	10	10	10	10	10
Necropsy body wt (g)	365 ± 3	320 ± 4**	284 ± 6**	274 ± 6**	312 ± 6**	293 ± 5**
Heart						
Absolute	1 00 ± 0 02	0 95 ± 0 01	0 91 ± 0 03**	0 91 ± 0 02*	0 91 ± 0 02**	0 90 ± 0 02**
Relative	2 73 ± 0 06	2 97 ± 0 04**	3 21 ± 0 08**	3 33 ± 0 09**	2 92 ± 0 04**	3 06 ± 0 09**
Right Kidney						
Absolute	1 22 ± 0 02	1 24 ± 0 03	1 17 ± 0 03	1 15 ± 0 02	1 22 ± 0 01	1 17 ± 0 02
Relative	3 35 ± 0 05	3 85 ± 0 08**	4 11 ± 0 08**	4 19 ± 0 07**	3 92 ± 0 10**	3 98 ± 0 04**
Liver						
Absolute	13 56 ± 0 29	15 36 ± 0 68[2]	14 01 ± 0 36[2]	13 48 ± 0 54	13 60 ± 0 40	13 91 ± 0 38[2]
Relative	37 2 ± 0 87	48 1 ± 1 89**	49 2 ± 0 57**	49 1 ± 1 41**	43 6 ± 0 83**	47 7 ± 0 84**
Lungs						
Absolute	1 36 ± 0 04	1 32 ± 0 03	1 23 ± 0 04**	1 25 ± 0 03*	1 28 ± 0 03*	1 24 ± 0 02**
Relative	3 73 ± 0 11	4 13 ± 0 11*	4 32 ± 0 09**	4 58 ± 0 12**	4 12 ± 0 09**	4 23 ± 0 09**
Right Testis						
Absolute	1 50 ± 0 02	1 50 ± 0 03	1 44 ± 0 03	1 41 ± 0 03[2]	1 46 ± 0 03[2]	0 74 ± 0 04**
Relative	4 12 ± 0 05	4 68 ± 0 08	5 08 ± 0 08**	5 19 ± 0 07**	4 69 ± 0 07	2 51 ± 0 14
Thymus						
Absolute	0 31 ± 0 01	0 27 ± 0 01*	0 23 ± 0 01**	0 17 ± 0 01**[2]	0 23 ± 0 02**	0 21 ± 0 01**
Relative	0 85 ± 0 04	0 83 ± 0 03	0 80 ± 0 04	0 61 ± 0 03**	0 74 ± 0 04**	0 72 ± 0 04*
Female						
n	10	10	10	10	10	10
Necropsy body wt (g)	193 ± 4	186 ± 3	173 ± 3**	167 ± 3**	184 ± 3**	179 ± 2**
Heart						
Absolute	0 65 ± 0 02	0 63 ± 0 02	0 61 ± 0 02	0 59 ± 0 02	0 63 ± 0 02	0 62 ± 0 01
Relative	3 35 ± 0 09	3 41 ± 0 07	3 52 ± 0 09	3 51 ± 0 09	3 42 ± 0 06	3 46 ± 0 07
Right Kidney						
Absolute	0 71 ± 0 02	0 74 ± 0 02	0 69 ± 0 02	0 70 ± 0 01	0 73 ± 0 01	0 74 ± 0 02
Relative	3 67 ± 0 07	3 98 ± 0 07*	3 97 ± 0 08*	4 18 ± 0 06**	3 98 ± 0 05**	4 11 ± 0 06**
Liver						
Absolute	6 21 ± 0 15[2]	6 46 ± 0 09	6 37 ± 0 17	6 52 ± 0 15	6 99 ± 0 11**	6 83 ± 0 14**
Relative	32 3 ± 0 39	34 8 ± 0 57**	36 8 ± 0 64**	39 0 ± 0 41**	38 1 ± 0 70**	38 2 ± 0 53**
Lung						
Absolute	0 97 ± 0 03	0 93 ± 0 04	0 89 ± 0 02	0 88 ± 0 02	0 93 ± 0 02[2]	0 88 ± 0 02
Relative	5 02 ± 0 09	5 01 ± 0 19	5 12 ± 0 07	5 27 ± 0 05	5 12 ± 0 11	4 93 ± 0 09
Thymus						
Absolute	0 23 ± 0 01	0 20 ± 0 08	0 21 ± 0 07	0 13 ± 0 08**	0 20 ± 0 08**	0 20 ± 0 05**
Relative	1 18 ± 0 05	1 07 ± 0 04	1 18 ± 0 03	0 80 ± 0 04**	1 06 ± 0 04	1 12 ± 0 03

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

[2] n=9

* Statistically significantly different (P≤0 05) from the control group by Dunn's test or Shirley's test

** Statistically significantly different (P≤0 01) from the control group by Dunn's test or Shirley's test

Table A2 Organ Weight and Organ-Weight-to-Body-Weight Ratios for B6C3F$_1$ Mice in the 13-Week Feed Study of Trinitrofluorenone[1]

	0 ppm	3125 ppm	6250 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Male						
n	10	9	8	9	10	5
Necropsy body wt (g)	32 3 ± 0 9	32 5 ± 0 5	31 4 ± 0 9	30 1 ± 0 4*	30 0 ± 0 6*	24 9 ± 1 4**
Heart						
Absolute	0 159 ± 0 008	0 152 ± 0 003	0 140 ± 0 005*	0 148 ± 0 008	0 139 ± 0 004*	118 0 ± 0 006**
Relative	4 92 ± 0 20	4 70 ± 0 14	4 48 ± 0 22	4 91 ± 0 25	4 65 ± 0 13	4 76 ± 0 12
Right Kidney						
Absolute	0 300 ± 0 013	0 312 ± 0 006	0 301 ± 0 008	0 294 ± 0 007	0 271 ± 0 007*	0 210 ± 0 011**
Relative	9 27 ± 0 25	9 62 ± 0 21	9 61 ± 0 23	9 79 ± 0 22	9 05 ± 0 20	8 45 ± 0 12*
Liver						
Absolute	1 46 ± 0 05	1 54 ± 0 02	1 52 ± 0 06	1 54 ± 0 02	1 61 ± 0 05	1 31 ± 0 08
Relative	45 2 ± 0 92	47 6 ± 0 48	48 2 ± 0 73*	51 3 ± 0 76**	53 6 ± 1 22**	52 7 ± 0 77**
Lung						
Absolute	0 164 ± 0 005[2]	0 168 ± 0 005	0 166 ± 0 007	0 173 ± 0 008	0 155 ± 0 005	0 154 ± 0 006
Relative	5 14 ± 0 14	5 18 ± 0 21	5 32 ± 0 26	5 76 ± 0 23	5 18 ± 0 16	6 28 ± 0 49*
Right Testis						
Absolute	0 120 ± 0 004	0 125 ± 0 002	0 122 ± 0 004[3]	0 123 ± 0 002	0 116 ± 0 004	0 105 ± 0 006*
Relative	3 73 ± 0 09	3 84 ± 0 04	3 97 ± 0 14	4 10 ± 0 09*	3 88 ± 0 11	4 23 ± 0 06**
Thymus						
Absolute	0 041 ± 0 002	0 040 ± 0 001	0 042 ± 0 002	0 041 ± 0 002	0 037 ± 0 002	0 031 ± 0 006
Relative	1 27 ± 0 03	1 22 ± 0 03	1 36 ± 0 08	1 35 ± 0 05	1 23 ± 0 05	1 21 ± 0 21
Female						
n	10	10	10	10	10	8
Necropsy body wt (g)	28 0 ± 0 6	27 1 ± 0 4	27 3 ± 0 6	27 1 ± 0 4	25 7 ± 0 5**	25 0 ± 0 4**
Heart						
Absolute	0 150 ± 0 007	0 130 ± 0 008	0 135 ± 0 007	0 132 ± 0 003*	0 126 ± 0 003**	0 119 ± 0 004**
Relative	5 39 ± 0 28	4 79 ± 0 25	4 93 ± 0 19	4 87 ± 0 12	4 91 ± 0 12	4 75 ± 0 13
Right Kidney						
Absolute	0 212 ± 0 011	0 222 ± 0 008	0 217 ± 0 007	0 218 ± 0 006	0 206 ± 0 003	0 201 ± 0 004
Relative	7 57 ± 0 34	8 20 ± 0 29	7 95 ± 0 18	8 05 ± 0 23	8 02 ± 0 09	8 05 ± 0 16
Liver						
Absolute	1 33 ± 0 04	1 37 ± 0 03	1 40 ± 0 04	1 47 ± 0 03*	1 43 ± 0 03*	1 47 ± 0 04*
Relative	47 3 ± 0 87	50 5 ± 0 57**	51 2 ± 0 61**	54 3 ± 1 18**	55 8 ± 0 82**	58 9 ± 1 61**
Lung						
Absolute	0 189 ± 0 010	0 167 ± 0 008	0 174 ± 0 007	0 175 ± 0 007	0 162 ± 0 006*	0 160 ± 0 003*
Relative	6 79 ± 0 40	6 16 ± 0 27	6 38 ± 0 21	6 45 ± 0 22	6 33 ± 0 30	6 41 ± 0 17
Thymus						
Absolute	0 052 ± 0 003	0 051 ± 0 002	0 046 ± 0 002	0 047 ± 0 002	0 043 ± 0 002**	0 043 ± 0 002**
Relative	1 87 ± 0 11	1 88 ± 0 05	1 69 ± 0 08	1 75 ± 0 06	1 68 ± 0 08	1 71 ± 0 08

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

[2] n=9

[3] n=7

* Statistically significantly different (P≤0 05) from the control group by Dunn's test or Shirley's test

** Statistically significantly different (P≤0 01) from the control group by Dunn's test or Shirley's test

APPENDIX B

Hematology and Clinical Chemistry

Table B1 Hematology Data for F344/N Rats in the 13-Week Feed Studies of Trinitrofluorenone B-2

Table B2 Chemistry Data for F344/N Rats in the 13-Week Feed Studies of Trinitrofluorenone B-5

Table B1 Hematology Data for F344/N Rats in the 13-Week Feed Study of Trinitrofluorenone[1]

Analysis	0 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm	16,000 ppm
MALE						
n	10	10	10	10	10	10
Hematocrit (%)						
Day 5	38 6 ± 0 4	39 5 ± 0 3	39 2 ± 0 5	39 1 ± 0 5[3]	38 6 ± 0 5	39 8 ± 0 3
Day 21	44 9 ± 0 6	45 7 ± 0 3	45 8 ± 0 3	46 1 ± 0 5	44 9 ± 0 3	46 0 ± 0 6
Day 90	45 6 ± 0 3	45 0 ± 0 6	45 5 ± 0 2	45 4 ± 0 4	42 9 ± 0 7**	42 1 ± 0 5**
Hemoglobin (g/dL)						
Day 5	13 4 ± 0 1	13 6 ± 0 1	13 5 ± 0 2	13 5 ± 0 2[3]	13 5 ± 0 2	14 0 ± 0 1*
Day 21	15 6 ± 0 2	15 8 ± 0 1	16 0 ± 0 1	16 0 ± 0 2	15 5 ± 0 1	15 9 ± 0 2
Day 90	15 5 ± 0 1	15 3 ± 0 1	15 7 ± 0 1	15 6 ± 0 2	14 7 ± 0 2**	14 6 ± 0 2**
Erythrocytes (10^6/μL)						
Day 5	6 49 ± 0 11	6 85 ± 0 10	6 81 ± 0 12	6 75 ± 0 10[3]	6 67 ± 0 08	6 85 ± 0 12
Day 21	8 06 ± 0 10	8 18 ± 0 06	8 23 ± 0 07	8 26 ± 0 09	8 07 ± 0 08	8 29 ± 0 08
Day 90	9 12 ± 0 05	8 99 ± 0 10	8 94 ± 0 06	8 85 ± 0 09**	8 61 ± 0 13**	8 15 ± 0 13**
Mean cell volume (fL)						
Day 5	59 5 ± 0 8	57 6 ± 0 6	57 7 ± 0 6	57 8 ± 0 6[3]	57 8 ± 0 3	58 2 ± 0 7
Day 21	55 6 ± 0 2	55 9 ± 0 3	55 7 ± 0 3	55 8 ± 0 4	55 6 ± 0 4	55 4 ± 0 3
Day 90	50 0 ± 0 2	50 0 ± 0 3	51 0 ± 0 2**	51 3 ± 0 3**	49 8 ± 0 2	51 8 ± 0 5**
Mean cell hemoglobin (pg)						
Day 5	20 7 ± 0 2	19 9 ± 0 3	19 8 ± 0 2*	20 1 ± 0 2[3]	20 3 ± 0 1	20 5 ± 0 2
Day 21	19 4 ± 0 1	19 4 ± 0 1	19 4 ± 0 1	19 3 ± 0 1	19 3 ± 0 1	19 2 ± 0 1
Day 90	17 0 ± 0 1	17 0 ± 0 1	17 5 ± 0 1**	17 7 ± 0 1**	17 0 ± 0 1*	17 9 ± 0 2**
Mean cell hemoglobin concentration (g/dL)						
Day 5	34 8 ± 0 1	34 4 ± 0 3	34 4 ± 0 2	34 6 ± 0 2[3]	35 1 ± 0 2	35 2 ± 0 2
Day 21	34 7 ± 0 2	34 6 ± 0 1	34 9 ± 0 1	34 6 ± 0 1	34 6 ± 0 2	34 7 ± 0 1
Day 90	33 9 ± 0 1	34 0 ± 0 2	34 4 ± 0 2	34 4 ± 0 1*	34 2 ± 0 2	34 7 ± 0 2**
Platelets (10^3/μL)						
Day 5	1059 0 ± 24 2	1041 9 ± 29 3	998 0 ± 16 5	1123 9 ± 55 6[3]	1063 0 ± 26 9	1127 2 ± 39 4
Day 21	833 8 ± 21 5	844 6 ± 15 7	860 3 ± 17 4	861 8 ± 20 2	944 5 ± 12 7**	938 3 ± 15 9**
Day 90	653 7 ± 12 7	691 0 ± 9 7*	691 7 ± 18 4	650 4 ± 21 1	821 2 ± 25 9**	795 7 ± 44 7**
Leukocytes (10^3/μL)						
Day 5[2]	3 79 ± 0 48	3 58 ± 0 37	3 57 ± 0 30	3 41 ± 0 30[3]	3 10 ± 0 28	3 21 ± 0 37
Day 21[2]	8 73 ± 0 51	7 56 ± 0 53	6 87 ± 0 36	6 54 ± 0 45	9 03 ± 0 48	10 51 ± 0 57
Day 90[4]	10 03 ± 0 28	10 62 ± 0 47	8 36 ± 0 32	7 52 ± 0 42*	11 16 ± 0 53	10 21 ± 0 52
Segmented neutrophils (10^3/μL)						
Day 5	0 63 ± 0 08	0 53 ± 0 06	0 57 ± 0 08	0 50 ± 0 04[3]	0 49 ± 0 04	0 45 ± 0 09
Day 21	0 85 ± 0 12	0 73 ± 0 08	0 76 ± 0 10	0 57 ± 0 06	1 46 ± 0 24*	1 97 ± 0 20*
Day 90	1 14 ± 0 12	1 18 ± 0 13	1 02 ± 0 10	1 17 ± 0 16	1 81 ± 0 17*	1 20 ± 0 15
Lymphocytes (10^3/μL)						
Day 5	2 94 ± 0 36	2 83 ± 0 27	2 77 ± 0 22	2 67 ± 0 24[3]	2 40 ± 0 22	2 62 ± 0 28
Day 21	7 84 ± 0 52	6 79 ± 0 51	6 12 ± 0 36	5 94 ± 0 44	7 54 ± 0 37	8 52 ± 0 55
Day 90	8 37 ± 0 27	8 87 ± 0 38	6 90 ± 0 25	6 02 ± 0 27*	8 73 ± 0 34	8 48 ± 0 39
Monocytes (10^3/μL)						
Day 5	0 25 ± 0 05	0 16 ± 0 02[3]	0 23 ± 0 05	0 24 ± 0 04[3]	0 17 ± 0 03	0 13 ± 0 03
Day 21	—[5]	—	—	—	—	—
Day 90	0 45 ± 0 06	0 41 ± 0 06	0 39 ± 0 06	0 29 ± 0 04	0 44 ± 0 08	0 45 ± 0 08

Table B1 Hematology Data for F344/N Rats in the 13-Week Feed Study of Trinitrofluorenone (continued)

Analysis	0 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm	16,000 ppm
MALE (continued)						
n	10	10	10	10	10	10
Eosinophils ($10^3/\mu L$)						
Day 5	0 01 ± 0 01	0 00 ± 0 00	0 00 ± 0 00	0 02 ± 0 02[a]	0 02 ± 0 01	0 01 ± 0 01
Day 21	0 04 ± 0 02	0 04 ± 0 02	0 02 ± 0 01	0 03 ± 0 02	0 02 ± 0 01	0 01 ± 0 01
Day 90	0 08 ± 0 01	0 12 ± 0 03	0 06 ± 0 02	0 02 ± 0 01	0 20 ± 0 06	0 08 ± 0 04
FEMALE						
n	10	10	10	10	10	10
Hematocrit (%)						
Day 5	40 6 ± 0 3[a]	40 3 ± 0 5	40 5 ± 0 6	40 4 ± 0 5[a]	39 7 ± 0 5[a]	40 8 ± 0 5
Day 21	46 4 ± 0 5	46 8 ± 0 3	48 0 ± 0 2	46 5 ± 0 3	45 6 ± 0 3	45 2 ± 0 4
Day 90	46 5 ± 0 5	46 8 ± 0 3	46 3 ± 0 4	45 9 ± 0 6	43 0 ± 0 5**	42 7 ± 0 6**
Hemoglobin (g/dL)						
Day 5	13 8 ± 0 1[a]	13 7 ± 0 1	14 0 ± 0 2	13 9 ± 0 2[a]	13 8 ± 0 1[a]	14 0 ± 0 1
Day 21	16 2 ± 0 2	16 2 ± 0 1	16 5 ± 0 1	16 2 ± 0 1	15 8 ± 0 1	15 8 ± 0 1*
Day 90	15 5 ± 0 1	15 5 ± 0 1	15 4 ± 0 1	15 2 ± 0 2	14 3 ± 0 1**	14 3 ± 0 2**
Erythrocytes ($10^6/\mu L$)						
Day 5	7 07 ± 0 07[a]	7 15 ± 0 13	7 17 ± 0 17	7 08 ± 0 13[a]	7 06 ± 0 10[a]	7 16 ± 0 12
Day 21	8 24 ± 0 09	8 33 ± 0 06	8 53 ± 0 06	8 33 ± 0 09	8 26 ± 0 05	8 19 ± 0 07
Day 90	8 68 ± 0 08	8 68 ± 0 06	8 44 ± 0 08	8 42 ± 0 09	7 82 ± 0 09**	7 73 ± 0 10**
Mean cell volume (fL)						
Day 5	57 6 ± 0 4[a]	56 4 ± 0 4	56 6 ± 0 8	57 2 ± 1 0[a]	56 3 ± 0 3[a]	57 2 ± 0 8
Day 21	56 3 ± 0 3	56 2 ± 0 2	56 5 ± 0 3	55 8 ± 0 3	55 2 ± 0 3*	55 1 ± 0 3**
Day 90	53 6 ± 0 2	54 0 ± 0 2	55 0 ± 0 2**	54 5 ± 0 2**	54 8 ± 0 1**	55 2 ± 0 3**
Mean cell hemoglobin (pg)						
Day 5	19 6 ± 0 2[a]	19 2 ± 0 2	19 5 ± 0 4	19 6 ± 0 3[a]	19 5 ± 0 2[a]	19 6 ± 0 3
Day 21	19 7 ± 0 1	19 4 ± 0 1*	19 4 ± 0 1	19 4 ± 0 1	19 2 ± 0 1**	19 2 ± 0 1**
Day 90	17 9 ± 0 1	17 8 ± 0 1	18 2 ± 0 1*	18 0 ± 0 1	18 3 ± 0 1**	18 5 ± 0 1**
Mean cell hemoglobin concentration (g/dL)						
Day 5	34 0 ± 0 2[a]	34 0 ± 0 2	34 5 ± 0 2	34 3 ± 0 2[a]	34 6 ± 0 2[a]	34 4 ± 0 2
Day 21	35 0 ± 0 2	34 5 ± 0 2	34 4 ± 0 2	34 7 ± 0 1	34 8 ± 0 1	34 9 ± 0 3
Day 90	33 3 ± 0 2	33 0 ± 0 2	33 2 ± 0 2	33 1 ± 0 2	33 3 ± 0 2	33 4 ± 0 2
Platelets ($10^3/\mu L$)						
Day 5	984 7 ± 18 6[a]	983 8 ± 13 8	957 0 ± 17 1	1002 4 ± 23 9[a]	973 6 ± 15 8[a]	1072 8 ± 27 1*
Day 21	738 6 ± 10 8	735 8 ± 17 5	799 2 ± 31 4	804 5 ± 25 1	773 5 ± 19 3	851 6 ± 17 2**
Day 90	653 0 ± 15 9	674 2 ± 17 3	673 0 ± 12 4	665 1 ± 18 2	746 3 ± 11 2**	724 1 ± 17 3**
Leukocytes ($10^3/\mu L$)						
Day 5	4 11 ± 0 39[a]	3 45 ± 0 17	2 93 ± 0 19**[a]	3 33 ± 0 41**[a]	2 83 ± 0 30**[a]	3 68 ± 0 54*
Day 21	6 69 ± 0 56	7 98 ± 0 60	6 34 ± 0 53	6 33 ± 0 45	5 93 ± 0 39	7 60 ± 0 64
Day 90	5 87 ± 0 55	5 10 ± 0 49	5 41 ± 0 44	5 63 ± 0 45	5 29 ± 0 59	6 79 ± 0 51
Segmented neutrophils ($10^3/\mu L$)						
Day 5	0 57 ± 0 08[a]	0 50 ± 0 06	0 45 ± 0 06	0 41 ± 0 05[a]	0 34 ± 0 06**[a]	0 38 ± 0 07*
Day 21	0 64 ± 0 08	0 99 ± 0 14	0 67 ± 0 10	0 89 ± 0 09	0 85 ± 0 12	1 04 ± 0 25
Day 90	0 83 ± 0 14	0 69 ± 0 10	0 63 ± 0 11	0 72 ± 0 08	0 72 ± 0 09	0 96 ± 0 12

Table B1 Hematology Data for F344/N Rats in the 13-Week Feed Study of Trinitrofluorenone (continued)

Analysis	0 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm	16,000 ppm
FEMALE (continued)						
n	10	10	10	10	10	10
Lymphocytes ($10^3/\mu L$)						
Day 5	3 52 ± 0 33[3]	2 95 ± 0 15	2 50 ± 0 19**[3]	2 90 ± 0 37*[3]	2 46 ± 0 29*[3]	3 29 ± 0 54*
Day 21	5 63 ± 0 47	6 40 ± 0 51	5 07 ± 0 43	4 86 ± 0 37	4 72 ± 0 29	5 89 ± 0 41
Day 90	4 77 ± 0 44	4 17 ± 0 41	4 63 ± 0 38	4 66 ± 0 35	4 40 ± 0 51	5 50 ± 0 43
Monocytes ($10^3/\mu L$)						
Day 5	—	—	—	—	—	—
Day 21	0 40 ± 0 08	0 53 ± 0 07	0 54 ± 0 11	0 53 ± 0 08	0 34 ± 0 05	0 61 ± 0 12
Day 90	0 20 ± 0 03	0 20 ± 0 03	0 15 ± 0 04	0 24 ± 0 05	0 15 ± 0 04	0 28 ± 0 07
Eosinophils ($10^3/\mu L$)						
Day 5	0 01 ± 0 01[3]	0 00 ± 0 00	0 00 ± 0 00	0 01 ± 0 01[3]	0 00 ± 0 00[3]	0 00 ± 0 00
Day 21	0 04 ± 0 02	0 06 ± 0 03	0 04 ± 0 02	0 07 ± 0 03	0 04 ± 0 02	0 09 ± 0 04
Day 90	0 06 ± 0 02	0 04 ± 0 02	0 02 ± 0 01	0 01 ± 0 01	0 03 ± 0 02	0 09 ± 0 03

[1] Mean ± standard error
[2] Data for Days 5 and 21 are from special study rats killed at Day 21
[3] n=9
[4] Data for Day 90 are from core study rats
[5] Monocyte counts not reported for this time period
* Statistically significantly different (P≤0 05) from the control group by Dunn's test or Shirley's test
** Statistically significantly different (P≤0 01) from the control group by Dunn's test or Shirley's test

Table B2 Clinical Chemistry Data for F344/N Rats in the 13-Week Feed Study of Trinitrofluorenone[1]

Analysis	0 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm	16,000 ppm
MALE						
n	10	10	10	10	10	10
Albumin (g/dL)						
Day 5[2]	3 6 ± 0 1	3 6 ± 0 1	3 7 ± 0 0	3 6 ± 0 1	3 6 ± 0 1	3 6 ± 0 1
Day 21[2]	3 6 ± 0 1	3 6 ± 0 0	3 7 ± 0 0	3 6 ± 0 1	3 4 ± 0 1	3 4 ± 0 1
Day 90[3]	3 8 ± 0 1	4 1 ± 0 1*	4 0 ± 0 1	4 1 ± 0 1	3 9 ± 0 1	3 8 ± 0 2
Alkaline phosphatase (IU/L)						
Day 5	754 ± 19	694 ± 17*	674 ± 13**	667 ± 14**	661 ± 10**	710 ± 21**
Day 21	533 ± 17	502 ± 19	500 ± 12	471 ± 10*	463 ± 13*	399 ± 16**
Day 90	273 ± 12	221 ± 6**	226 ± 4*	213 ± 5	209 ± 14**	254 ± 6
Alanine aminotransferase (IU/L)						
Day 5	50 ± 3	48 ± 1	53 ± 1	49 ± 3	53 ± 3	56 ± 3
Day 21	49 ± 2	45 ± 1	44 ± 1	42 ± 2	48 ± 1	54 ± 2
Day 90	51 ± 1	56 ± 2	51 ± 2	55 ± 2	47 ± 2	55 ± 2
Bile acids (µmol/L)						
Day 5	21 60 ± 2 06	26 80 ± 1 98	30 30 ± 2 37	23 40 ± 1 20	15 40 ± 2 84	11 90 ± 2 35*
Day 21	10 40 ± 0 83	22 00 ± 1 76	22 60 ± 1 82	17 30 ± 2 07	3 70 ± 0 65	1 70 ± 0 37*
Day 90	14 80 ± 2 38	20 10 ± 2 72	16 44 ± 1 85[4]	26 60 ± 2 14*	10 80 ± 2 34	9 56 ± 2 69[4]
Creatine kinase (IU/L)						
Day 5	595 ± 67	556 ± 55	612 ± 50	632 ± 45	515 ± 55	569 ± 61
Day 21	423 ± 52	408 ± 54	426 ± 47	695 ± 144	368 ± 37	460 ± 48
Day 90	260 ± 47	300 ± 28	377 ± 73	522 ± 100	336 ± 78	374 ± 68
Creatinine (mg/dL)						
Day 5	0 44 ± 0 02	0 43 ± 0 01	0 47 ± 0 01	0 46 ± 0 01	0 48 ± 0 02	0 52 ± 0 02**
Day 21	0 63 ± 0 02	0 63 ± 0 02	0 65 ± 0 02	0 66 ± 0 03	0 69 ± 0 02	0 68 ± 0 02
Day 90	0 69 ± 0 02	0 69 ± 0 03	0 65 ± 0 02	0 68 ± 0 03	0 74 ± 0 04	0 75 ± 0 04
Methemoglobin (g/dL)						
Day 5	0 49 ± 0 08	0 48 ± 0 12[3]	0 40 ± 0 06	0 49 ± 0 06[3]	0 51 ± 0 07[3]	0 82 ± 0 16
Day 21	0 40 ± 0 05	0 33 ± 0 03	0 33 ± 0 02	0 37 ± 0 03	0 52 ± 0 04**	0 73 ± 0 04**
Day 90	0 48 ± 0 05	0 39 ± 0 03	0 46 ± 0 04	0 50 ± 0 11	0 82 ± 0 22*	0 68 ± 0 04*[3]
Sorbitol dehydrogenase (IU/L)						
Day 5	5 ± 0	5 ± 0	4 ± 0	5 ± 0	5 ± 0	5 ± 0
Day 21	8 ± 1	8 ± 1	7 ± 1	8 ± 1	7 ± 1	6 ± 1
Day 90	5 ± 1	7 ± 1	7 ± 1[4]	9 ± 1	4 ± 0	4 ± 0[5]
Total protein (g/dL)						
Day 5	6 2 ± 0 1	6 0 ± 0 1	6 2 ± 0 1	6 1 ± 0 1	6 0 ± 0 1	5 8 ± 0 1**
Day 21	6 5 ± 0 1	6 5 ± 0 1	6 3 ± 0 2	6 4 ± 0 1	6 0 ± 0 2*	5 9 ± 0 1**
Day 90	6 8 ± 0 1	7 2 ± 0 1*	6 9 ± 0 1	7 2 ± 0 1	7 0 ± 0 0	7 0 ± 0 2
Urea nitrogen (mg/dL)						
Day 5	18 7 ± 0 5	20 0 ± 0 4*	20 1 ± 0 7	19 6 ± 0 5	21 2 ± 0 7**	21 2 ± 0 9*
Day 21	23 1 ± 0 6	22 4 ± 0 6	23 4 ± 0 7	23 7 ± 0 8	22 2 ± 0 7	21 2 ± 0 9
Day 90	21 5 ± 0 8	21 0 ± 0 6	20 1 ± 0 8	21 6 ± 0 6	23 7 ± 1 9	21 3 ± 0 7

Table B2 Clinical Chemistry Data for F344/N Rats in the 13-Week Feed Study of Trinitrofluorenone (continued)

Analysis	0 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm	16,000 ppm
FEMALE						
n	10	10	10	10	10	10
Albumin (g/dL)						
Day 5	3 8 ± 0 1	3 7 ± 0 1	3 7 ± 0 1	3 7 ± 0 1	3 7 ± 0 1	3 8 ± 0 1
Day 21	3 7 ± 0 2[4]	3 9 ± 0 1	3 9 ± 0 1	3 8 ± 0 2	4 0 ± 0 1	3 9 ± 0 1
Day 90	4 7 ± 0 1	4 5 ± 0 0	4 4 ± 0 0*	4 4 ± 0 0	4 7 ± 0 0	4 6 ± 0 1
Alkaline phosphatase (IU/L)						
Day 5	626 ± 17	561 ± 14*	559 ± 15*	569 ± 12	558 ± 21	581 ± 17*
Day 21	413 ± 9	358 ± 5**	356 ± 9**	349 ± 13**	347 ± 15**	356 ± 14**
Day 90	250 ± 6	231 ± 5	241 ± 13	207 ± 7*	236 ± 7	301 ± 10
Alanine amino transferase3 (IU/L)						
Day 5	39 ± 1	44 ± 1*	43 ± 1*	40 ± 1	47 ± 2**	50 ± 2**
Day 21	39 ± 1	39 ± 1	42 ± 1	40 ± 0	41 ± 1	46 ± 1**
Day 90	39 ± 1	47 ± 1**	42 ± 1	43 ± 1	40 ± 1	46 ± 2
Bile acids (µmol/L)						
Day 5	22 80 ± 3 04	25 80 ± 1 53	22 20 ± 1 94	12 30 ± 0 90**	5 50 ± 1 34**	8 67 ± 2 30**[4]
Day 21	11 56 ± 1 47[4]	13 10 ± 1 03	21 20 ± 2 39	11 70 ± 1 66	4 10 ± 1 00*	2 25 ± 0 59**[5]
Day 90	19 70 ± 2 77	30 70 ± 2 73	28 80 ± 3 14	24 70 ± 2 63	12 70 ± 2 15	1 88 ± 0 67**[5]
Creatine kinase (IU/L)						
Day 5	294 ± 53	313 ± 26	317 ± 44	322 ± 42	257 ± 26	357 ± 51
Day 21	450 ± 32[4]	506 ± 44	608 ± 99	491 ± 43	530 ± 42	612 ± 67
Day 90	298 ± 19	331 ± 26	323 ± 23	451 ± 81	214 ± 20[4]	276 ± 25
Creatinine (mg/dL)						
Day 5	0 45 ± 0 02	0 41 ± 0 02	0 44 ± 0 01	0 46 ± 0 01	0 45 ± 0 02	0 52 ± 0 01**
Day 21	0 52 ± 0 02[4]	0 53 ± 0 02	0 53 ± 0 02[4]	0 58 ± 0 02	0 61 ± 0 02**	0 63 ± 0 01**
Day 90	0 59 ± 0 01	0 56 ± 0 03	0 57 ± 0 02	0 61 ± 0 02	0 65 ± 0 02	0 69 ± 0 02**
Methemoglobin (g/dL)						
Day 5	0 31 ± 0 04[3]	0 34 ± 0 07	0 33 ± 0 10	0 32 ± 0 05[3]	0 35 ± 0 06[3]	0 77 ± 0 24**
Day 21	0 42 ± 0 04	0 36 ± 0 04	0 46 ± 0 07	0 57 ± 0 17	0 66 ± 0 10	0 66 ± 0 02**
Day 90	0 35 ± 0 05	0 31 ± 0 02	0 48 ± 0 12	0 36 ± 0 03	0 55 ± 0 07**	0 61 ± 0 03**
Sorbitol dehydrogenase (IU/L)						
Day 5	5 ± 1	5 ± 0	4 ± 0	5 ± 0	6 ± 0	5 ± 0
Day 21	6 ± 0[4]	5 ± 0	5 ± 0*	5 ± 0	6 ± 0	5 ± 0
Day 90	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0	5 ± 0
Total protein (g/dL)						
Day 5	6 2 ± 0 1	6 0 ± 0 1	6 1 ± 0 1	6 1 ± 0 1	6 0 ± 0 1	6 0 ± 0 1
Day 21	6 5 ± 0 1[4]	6 3 ± 0 1	6 3 ± 0 1	6 3 ± 0 1	6 4 ± 0 1	6 2 ± 0 1
Day 90	7 1 ± 0 1	7 0 ± 0 1	6 9 ± 0 1	6 8 ± 0 1	7 2 ± 0 1	7 0 ± 0 1

Table B2 Clinical Chemistry Data for F344/N Rats in the 13-Week Feed Study of Trinitrofluorenone (continued)

Analysis	0 ppm	1000 ppm	2000 ppm	4000 ppm	8000 ppm	16,000 ppm
FEMALE (continued)						
n	10	10	10	10	10	10
Urea nitrogen (mg/dL)						
Day 5	20.6 ± 0.4	20.4 ± 0.6	22.6 ± 0.8	22.1 ± 0.7	23.0 ± 0.7*	23.0 ± 0.7*
Day 21	19.9 ± 0.7[4]	20.6 ± 0.6	19.6 ± 0.7	19.0 ± 0.7	20.3 ± 0.5	20.2 ± 0.6
Day 90	24.5 ± 0.6	23.8 ± 0.9	20.2 ± 0.5**	22.2 ± 0.8	25.4 ± 0.6	24.9 ± 1.0

[1] Mean ± standard deviation.
[2] Data for Days 5 and 21 are from special study rats kiled at Day 21.
[3] Data for Day 90 are from core study rats.
[4] n=9.
[5] n=8.
* Statistically significantly different (P≤0.05) from the control group by Dunn's test or Shirley's test.
** Statistically significantly different (P≤0.01) from the control group by Dunn's test or 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 Feed Study of Trinitrofluorenone C-2

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

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

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

Table C1 Summary of Reproductive Tissue Evaluations in Male F344/N Rats in the 13-Week Feed Study of Trinitrofluorenone

Study Parameters[1]	0 ppm	4000 ppm	8000 ppm	16,000 ppm
Weights (g)				
Necropsy body weight	365 ± 3	274 ± 6**	312 ± 6**	293 ± 5**
Left testis	1 59 ± 0 02	1 50 ± 0 03	1 37 ± 0 10*	1 09 ± 0 14**
Left epididymis	0 466 ± 0 004	0 408 ± 0 014*	0 428 ± 0 012	0 592 ± 0 046
Left epididymal tail	0 184 ± 0 005	0 163 ± 0 008	0 169 ± 0 007	0 239 ± 0 016**
Spermatozoal measurements				
Motility (%)	88 ± 2	90 ± 2	89 ± 1[2]	0 ± 0**
Concentration (millions)	442 ± 48	520 ± 45	436 ± 25[2]	11 ± —[3]
Spermatid count (mean/10[4] mL suspension)	58 98 ± 2 27	58 70 ± 3 06	58 09 ± 2 59[2]	68 13 ± 5 13[4]
Spermatid heads (10[7]/testis)	11 80 ± 0 45	11 74 ± 0 61	11 62 ± 0 52[2]	13 63 ± 1 03[4]
Spermatid heads (10[7]/g testis)	7 43 ± 0 29	7 88 ± 0 46	7 66 ± 0 27[2]	7 22 ± 0 13[4]

[1] Data presented as mean ± standard error, n=10 for all groups except where noted Spermatozoal measurements were not significant by Dunn's test
[2] n=8
[3] n=1, no statistic calculated, insufficient measurements
[4] n=2
* Statistically significantly different (P≤0 05) from the control group by Dunn's test or Shirley's test
** Statistically significantly different (P≤0 01) from the control group by Shirley's test

Table C2 Summary of Estrous Cycle Characterization in Female F344/N Rats in the 13-Week Feed Study of Trinitrofluorenone

Study Parameters[1]	0 ppm	4000 ppm	8000 ppm	16,000 ppm
Estrous cycle length (days)	5 00 ± 0 13	5 60 ± 0 27	5 00 ± 0 00	5 70 ± 0 41
Estrous stages as % of cycle				
Diestrus	37 5	45 8	38 3	44 2
Proestrus	16 7	15 0	17 5	15 8
Estrus	28 3	20 8	24 2	22 5
Metestrus	17 5	18 3	20 0	16 7
Uncertain diagnosis	0 0	0 0	0 0	0 8

[1] Data presented as mean ± standard error n=10 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 frequency of time spent in the estrous stages

Table C3 Summary of Reproductive Tissue Evaluations in Male B6C3F$_1$ Mice in the 13-Week Feed Study of Trinitrofluorenone

Study Parameters[1]	0 ppm	6250 ppm	12,500 ppm	25,000 ppm
Weights (g)				
Necropsy body weight	32 3 ± 0 9	31 4 ± 0 9	30 1 ± 0 4	30 0 ± 0 6
Left testis	0 115 ± 0 002	0 118 ± 0 003	0 119 ± 0 000	0 113 ± 0 002
Left epididymis	0 044 ± 0 001	0 045 ± 0 003[2]	0 045 ± 0 001	0 044 ± 0 000
Left epididymal tail	0 015 ± 0 001	0 015 ± 0 001[2]	0 014 ± 0 001	0 014 ± 0 001
Spermatozoal measurements				
Motility (%)	87 ± 1	89 ± 1	87 ± 1	86 ± 2
Concentration (millions)	1305 ± 69	1153 ± 39	1260 ± 122	1216 ± 67
Spermatid count (mean/10^4 mL suspension)	64 13 ± 2 38	63 04 ± 2 22	59 42 ± 3 26	58 78 ± 2 65
Spermatid heads (10^7/testis)	2 05 ± 0 08	2 02 ± 0 07	1 90 ± 0 10	1 88 ± 0 08
Spermatid heads (10^7/g testis)	17 97 ± 0 76	17 14 ± 0 40	16 03 ± 0 89	16 60 ± 0 51

[1] Data presented as mean ± standard error For the 0 and 25,000 ppm dose groups, n=10 For the 6250 ppm dose group n=7 for the 12,500 ppm dose group, n=9 Differences in weights and spermatozoal measurements were not significant by Dunn s test or Shirley s test

[2] n=8

Table C4 Summary of Estrous Cycle Characterization in Female B6C3F$_1$ Mice in the 13-Week Feed Study of Trinitrofluorenone

Study Parameters[1]	0 ppm	6250 ppm	12,500 ppm	25,000 ppm[2]
Estrous cycle length (days)	4 05 ± 0 05	4 05 ± 0 05	3 95 ± 0 05	4 00 ± 0 00
Estrous stages as % of cycle				
Diestrus	29 2	28 3	25 0	29 2
Proestrus	21 7	14 2	16 7	18 3
Estrus	29 2	32 5	35 0	34 2
Metestrus	20 0	25 0	23 3	18 3

[1] Data presented as mean ± standard error, n=10 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 frequency of time spent in the estrous stages

[2] For 1/10 animals at this dose, estrous cycle length exceeded 12 days and the data were not included in the mean

APPENDIX D

Genetic Toxicology

Table D1 Mutagenicity of Trinitrofluorenone in *Salmonella typhimurium* D-2

Table D1 Mutagenicity of Trinitrofluorenone in *Salmonella typhimurium*[1]

Strain	Dose (μg/plate)	Revertants/plate[2] - S9 Trial 1	- S9 Trial 2	- S9 Trial 3
TA100				
	0 00	96 ± 4 2	124 ± 8 1	123 ± 5 0
	0 03	135 ± 6 7	127 ± 3 4	162 ± 19 2
	0 10	256 ± 2 4	236 ± 7 3	215 ± 2 7
	0 30	360 ± 21 7	355 ± 13 4	421 ± 9 4
	1 00	474 ± 27 3	736 ± 17 9	862 ± 27 0
	3 00	783 ± 64 6	1051 ± 18 8	1364 ± 18 2
Trial summary		Positive	Positive	Positive
Positive control[3]		402 ± 23 7	466 ± 13 6	433 ± 6 7

Strain	Dose (μg/plate)	+30% hamster S9 Trial 1	+30% hamster S9 Trial 2	+30% rat S9 Trial 1	+30% rat S9 Trial 2
TA100 (continued)					
	0 0	125 ± 14 2	158 ± 11 3	129 ± 11 3	145 ± 5 0
	1 0	149 ± 3 8	175 ± 2 1	111 ± 3 5	
	1 6	172 ± 6 5	193 ± 17 4		
	3 0	172 ± 6 5	193 ± 17 4	110 ± 13 0	179 ± 6 9
	6 0				318 ± 5 0
	10 0	316 ± 9 1	394 ± 11 4	250 ± 11 1	428 ± 17 3
	16 0		559 ± 16 0		625 ± 7 9
	33 0	667 ± 27 1	1020 ± 26 2	899 ± 31 2	1060 ± 65 7
	66 0	914 ± 45 2		1184 ± 85 0	
Trial summary		Positive	Positive	Positive	Positive
Positive control		654 ± 45 6	553 ± 54 3	615 ± 41 0	498 ± 9 8

Table D1 Mutagenicity of Trinitrofluorenone in *Salmonella typhimurium* (continued)

Strain	Dose (μg/plate)	Revertants/plate - S9 Trial 1	Trial 2	Trial 3	Trial 4
TA98					
	0 000	18 ± 1 3	17 ± 1 2	19 ± 0 7	20 ± 2 0
	0 001		238 ± 13 9	50 ± 4 2	42 ± 5 2
	0 003		420 ± 16 9	137 ± 8 7	115 ± 14 1
	0 010		884 ± 6 1		
	0 030	844 ± 7 6			
	0 100	1823 ± 71 4			
	0 300	x			
	1 000	x			
	3 000	x			
Trial summary		Positive	Positive	Positive	Positive
Positive control		495 ± 25 5	561 ± 23 3	785 ± 24 7	785 ± 24 7

Strain	Dose (μg/plate)	+30% hamster S9 Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
TA98 (continued)						
	0 00	32 ± 3 2	28 ± 3 5	26 ± 2 8	24 ± 2 1	32 ± 4 4
	0 03		52 ± 9 8	44 ± 1 3	35 ± 5 4	38 ± 5 0
	0 10		66 ± 0 3	78 ± 9 5	36 ± 2 0	61 ± 0 9
	0 30		115 ± 15 7	101 ± 14 9	70 ± 7 2	88 ± 10 3
	1 00	249 ± 31 7	266 ± 7 8	207 ± 13 9	122 ± 6 2	224 ± 42 7
	1 60		532 ± 50 0	161 ± 29 9	178 ± 11 0	310 ± 13 1
	3 00	774 ± 19 4				
	10 00	1772 ± 63 7				
	33 00	x				
	66 00	x				
Trial summary		Positive	Positive	Positive	Positive	Positive
Positive control		367 ± 30 9	436 ± 2 5	471 ± 65 8	297 ± 19 6	297 ± 19 6

Table D1 Mutagenicity of Trinitrofluorenone in *Salmonella typhimurium* (continued)

Strain	Dose (μg/plate)	Revertants/plate +30% rat S9 Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
TA98 (continued)						
	0 00	31 ± 1 3	133 ± 2 9	41 ± 6 0	44 ± 4 0	47 ± 3 0
	0 03		41 ± 3 5	47 ± 3 8	45 ± 4 7	47 ± 5 0
	0 10		44 ± 3 8	62 ± 7 4	45 ± 2 8	41 ± 5 2
	0 30		67 ± 6 9	76 ± 2 0	53 ± 5 3	67 ± 3 4
	1 00	120 ± 5 2	85 ± 5 8	208 ± 9 1	81 ± 0 6	112 ± 11 6
	1 60		139 ± 3 0	188 ± 12 8	103 ± 10 2	156 ± 26 0
	3 00	678 ± 62 1				
	10 00	1537 ± 57 7				
	33 00	x				
	66 00	x				
Trial summary		Positive	Positive	Positive	Positive	Positive
Positive control		168 ± 7 3	160 ± 8 7	193 ± 10 9	151 ± 4 3	151 ± 4 3

[1] Study performed at SRI, International The detailed protocol is presented in Zeiger *et al* (1988)

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

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