

National Toxicology Program  
Toxicity Report Series  
Number 44

**NTP Technical Report  
on Comparative Toxicity and Carcinogenicity Studies of**

***o*-Nitrotoluene and  
*o*-Toluidine Hydrochloride**

(CAS Nos. 88-72-2 and 636-21-5)

**Administered in Feed  
to Male F344/N Rats**

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**NIH Publication 96-3936  
March 1996**

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

## Note to the Reader

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

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

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This NTP report on the toxicity studies of *o*-nitrotoluene and *o*-toluidine hydrochloride is based primarily on 26-week feed studies that began in March 1992 and ended in June 1992 at Battelle Columbus Operations, Columbus, OH.

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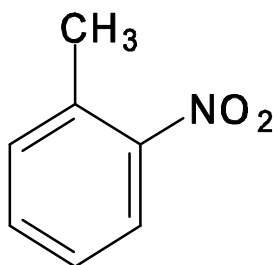
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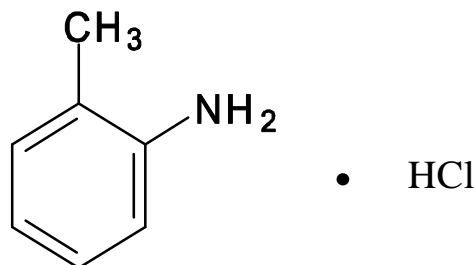
## ABSTRACT

*o*-Nitrotoluene



<b>Molecular Formula</b>	C <sub>7</sub> H <sub>7</sub> NO <sub>2</sub>
<b>CAS Number</b>	88-72-2
<b>Molecular Weight</b>	137.13
<b>Synonyms</b>	2-nitrotoluene <i>o</i> -methylnitrobenzene nitrotoluol nitrophenylmethane

*o*-Toluidine Hydrochloride



<b>Molecular Formula</b>	C <sub>7</sub> H <sub>9</sub> N•HCl
<b>CAS Number</b>	636-21-5
<b>Molecular Weight</b>	143.62
<b>Synonyms</b>	2-toluidine hydrochloride <i>o</i> -aminotoluene hydrochloride methylaniline

*o*-Nitrotoluene and *o*-toluidine hydrochloride are structurally related chemicals that are suspected and demonstrated animal carcinogens, respectively. The metabolic potential of the gastrointestinal flora is considered an important factor in *o*-nitrotoluene-induced toxicity and involves the reduction of the nitro group to the corresponding amine (forming *o*-toluidine). These studies were designed to 1) compare the target organ toxicities of *o*-nitrotoluene and *o*-toluidine hydrochloride administered in feed at approximately equimolar doses (5,000 ppm) to male F344/N rats for 13 or 26 weeks, 2) determine the potential progression or reversibility of toxic or proliferative lesions following chemical withdrawal (stop-exposure) for 13 weeks after 13 weeks of exposure, and 3) examine the effect of antibiotic-altered gastrointestinal flora on the toxicity and/or carcinogenicity of *o*-nitrotoluene.

*o*-Nitrotoluene and *o*-toluidine hydrochloride caused mesothelial hyperplasia and mesothelioma in male rats after 13 or 26 weeks of dietary exposure. The incidence of mesothelioma was greater and the latency was less in rats administered *o*-nitrotoluene than in rats administered *o*-toluidine hydrochloride. Additionally, *o*-nitrotoluene caused testicular degeneration in rats. Effects of *o*-nitrotoluene administration in the liver included progressive, irreversible increases in liver weight and irreversible increases in the incidences of cytoplasmic vacuolization and oval-cell hyperplasia.

Placental glutathione *S*-transferase (PGST)-positive foci of cellular alteration occurred in the liver after 13 weeks of *o*-nitrotoluene exposure, and the number and size (as reflected by the volume fraction) of foci were increased after 26 weeks of continuous exposure. During the recovery period, the number of PGST-positive foci in rats in the stop-exposure group decreased slightly, but the size of the foci continued to increase. After 26 weeks, cholangiocarcinoma occurred in 2 of 20 rats in the stop-exposure group and 1 of 20 rats in the continuous-exposure group administered *o*-nitrotoluene. In contrast, liver effects in rats administered *o*-toluidine hydrochloride consisted of minimal hemosiderin accumulation in Kupffer cells; the incidence of this lesion in the stop-exposure group decreased during the recovery period. *o*-Toluidine hydrochloride caused fewer and much smaller PGST-positive foci than those caused by *o*-nitrotoluene.

*o*-Nitrotoluene caused an accumulation of hyaline droplets in the renal tubule epithelium; this accumulation did not increase in severity with continued exposure and completely regressed during the recovery period. Exposure to *o*-toluidine hydrochloride did not cause hyaline droplet accumulation but did cause an accumulation of hemosiderin pigment in renal tubule epithelium.

This change progressed in severity during the 26-week continuous-exposure study but decreased in severity in the stop-exposure study during the recovery period.

Exposure to *o*-nitrotoluene or *o*-toluidine hydrochloride caused increased incidences of hematopoiesis, hemosiderin accumulation, and capsular fibrosis in the spleen. In rats administered *o*-toluidine, spleen effects were much more prominent and were also reflected by congestion and markedly increased spleen weights. During the recovery period for the stop-exposure groups administered either compound, incidences of hemosiderin accumulation and hematopoiesis were decreased, but the capsular fibrosis did not resolve. Hyperplasia of the transitional epithelium in the urinary bladder was observed only in rats administered *o*-toluidine hydrochloride; this lesion did not increase in severity after 26 weeks of continuous exposure and completely regressed in the stop-exposure group during the recovery period. Alteration of the gastrointestinal flora by daily gavage administration of antibiotics did not affect the pattern or severity of toxicity at any site or the development of mesothelioma in rats exposed to *o*-nitrotoluene, although cholangiocarcinomas that occurred in three rats with the normal flora did not occur in groups with the altered intestinal flora. Subsequent studies determined that the antibiotic regimen used was effective only in reducing the gut population of aerobic microorganisms and had little effect on obligate anaerobes, which are thought to play a major role in nitro group reduction.



In summary, these studies confirmed the target organs and compared the relative toxicity for *o*-nitrotoluene and *o*-toluidine hydrochloride administered to male F344/N rats at equimolar concentrations in feed. With the exception of the spleen toxicity observed with each chemical, but more prominently with *o*-toluidine hydrochloride, morphologic effects of exposure to each of these chemicals in the testis/epididymis, liver, kidney, and urinary bladder were different. The results of these studies demonstrate the somewhat greater relative carcinogenic potential of *o*-nitrotoluene compared to *o*-toluidine hydrochloride after 13 or 26 weeks of administration based on the occurrence of mesothelioma and cholangiocarcinoma. The apparently lower potency of *o*-toluidine hydrochloride relative to *o*-nitrotoluene in the induction of mesothelioma suggests that simple intestinal reduction of the nitro group may not be sufficient for carcinogenic activity in the mesothelium.

### Summary of the Comparative Toxicity and Carcinogenicity Studies of o-Nitrotoluene and o-Toluidine Hydrochloride in Male F344/N Rats

	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidine Hydrochloride (5,000 ppm)
<b>Body weights</b>		Less than controls at all time points	Less than controls at all time points
<b>Lesions</b>			
13 weeks	<u>Kidney</u> : regeneration (3/10)  <u>Spleen</u> : hematopoietic cell proliferation (2/10)	<u>Kidney</u> : hyaline droplet accumulation (20/20); regeneration (16/20) <u>Liver</u> : cytoplasmic vacuolization (20/20); oval cell hyperplasia (20/20) <u>Spleen</u> : hematopoietic cell proliferation (20/20); hemosiderin pigmentation (20/20); capsule, fibrosis (1/20)  <u>Testis/epididymis</u> : degeneration (20/20)	<u>Kidney</u> : pigmentation (20/20)  <u>Liver</u> : hemosiderin pigmentation (20/20); cytoplasmic vacuolization (1/20) <u>Spleen</u> : congestion (20/20); hematopoietic cell proliferation (20/20); hemosiderin pigmentation (20/20); thrombosis (3/20); capsule, fibrosis (20/20) <u>Testis/epididymis</u> : degeneration (1/20) <u>Urinary bladder</u> : transitional epithelium, hyperplasia (10/20)
Stop-exposure	<u>Kidney</u> : protein casts (3/10); regeneration (7/10)  <u>Spleen</u> : hematopoietic cell proliferation (3/10); hemosiderin pigmentation (3/10)	<u>Kidney</u> : protein casts (20/20); regeneration (19/20)  <u>Liver</u> : cytoplasmic vacuolization (20/20); oval cell hyperplasia (20/20); cholangiocarcinoma (2/20) <u>Spleen</u> : hematopoietic cell proliferation (18/20); hemosiderin pigmentation (17/20); capsule, fibrosis (7/20)  <u>Testis/epididymis</u> : degeneration (20/20); mesothelial hyperplasia (2/20); mesothelioma (5/20)	<u>Kidney</u> : protein casts (6/20); pigmentation (20/20); regeneration (10/20) <u>Liver</u> : hemosiderin pigmentation (11/20); cytoplasmic vacuolization (1/20)  <u>Spleen</u> : congestion (20/20); hematopoietic cell proliferation (1/20); hemosiderin pigmentation (18/20); capsule, fibrosis (20/20); capsule, lymphatic angiectasis (15/20) <u>Testis/epididymis</u> : degeneration (2/20); mesothelioma (2/20)

**Summary of the Comparative Toxicity and Carcinogenicity Studies  
of o-Nitrotoluene and o-Toluidine Hydrochloride in Male F344/N Rats (continued)**

	<b>Control (0 ppm)</b>	<b>o-Nitrotoluene (5,000 ppm)</b>	<b>o-Toluidine Hydrochloride (5,000 ppm)</b>
<b>Lesions</b>			
26 weeks	<p><u>Kidney</u>: protein casts (3/10); regeneration (7/10)</p> <p><u>Spleen</u>: hematopoietic cell proliferation (3/10); hemosiderin pigmentation (3/10)</p>	<p><u>Kidney</u>: hyaline droplet accumulation (20/20); protein casts (20/20); regeneration (19/20)</p> <p><u>Liver</u>: cytoplasmic vacuolization (20/20); oval cell hyperplasia (20/20); cholangiocarcinoma (1/20)</p> <p><u>Spleen</u>: hematopoietic cell proliferation (20/20); hemosiderin pigmentation (20/20); capsule, fibrosis (3/20)</p> <p><u>Testis/epididymis</u>: degeneration (20/20); mesothelial hyperplasia (2/20); mesothelioma (7/20)</p>	<p><u>Kidney</u>: protein casts (1/20); pigmentation (20/20); regeneration (5/20)</p> <p><u>Liver</u>: hemosiderin pigmentation (20/20)</p> <p><u>Spleen</u>: congestion (20/20); hematopoietic cell proliferation (20/20); hemosiderin pigmentation (20/20); thrombosis (2/20); capsule, fibrosis (20/20); capsule, lymphatic angiectasis (6/20)</p> <p><u>Testis/epididymis</u>: degeneration (2/20); mesothelial hyperplasia (1/20)</p> <p><u>Urinary bladder</u>: transitional epithelium, hyperplasia (17/20)</p>



# INTRODUCTION

## Physical and Chemical Properties, Production, Use, and Exposure

*o*-Nitrotoluene is synthesized by the nitration of toluene with a mixture of nitric acid and sulfuric acid and subsequent separation by fractional distillation (*Merck Index*, 1983). *o*-Nitrotoluene is a yellowish liquid at room temperature and is virtually insoluble in water but soluble in alcohol, benzene, and ether. This flammable liquid has a specific gravity of 1.16 g/mL at 20° C, a melting point of -10° C, a boiling point of 222° C, and a vapor pressure of 0.15 mm Hg at 20° C (Mackison *et al.*, 1981; *Merck Index*, 1983).

*o*-Nitrotoluene is used as a chemical intermediate and in the synthesis of dyestuffs and explosives (Beard and Noe, 1981). The U.S. production of *o*-nitrotoluene has been estimated to be 28 million pounds in 1981 (Dunlap, 1981) and from 1 to 10 million pounds in 1984 (Chemicals in Commerce Information System, 1984). The annual consumption of *o*-nitrotoluene has been estimated at 10 to 12 million pounds (Howard *et al.*, 1976). More recent figures are not available. Human exposure to *o*-nitrotoluene occurs primarily through occupational sources. According to the National Institute of Occupational Safety and Health (NIOSH, 1990), 4,354 people were exposed to nitrotoluenes in the workplace (the isomer or isomers were not indicated).

*o*-Toluidine hydrochloride is prepared by the amination of toluene with methylhydroxylamine or hydroxylammonium salts in the presence of aluminum trichloride (*Merck Index*, 1983), and then converted to the aniline hydrochloride salt. *o*-Toluidine hydrochloride, a white, crystalline powder, is very soluble in water and ethanol but insoluble in benzene and diethyl ether. The melting point for this compound is 243° C. When heated to decomposition, *o*-toluidine hydrochloride emits toxic fumes of hydrochloric acid and nitrogen oxides (USDHHS, 1991).

*o*-Toluidine and its hydrochloride salt are used in the manufacture of various dyes, including azo pigment, triarylmethane, sulfur, and indigo (USDHHS, 1991). *o*-Toluidine is also used as a photographic dye and as an antioxidant in the manufacture of rubber (Stanford Research Institute, 1976).

In 1983, U.S. production of *o*-toluidine was between 11 and 21 million pounds, and 34 companies were identified as *o*-toluidine suppliers (*Chem Sources USA* 1983). The United States imported 35,700 pounds of *o*-toluidine and 992 pounds of its hydrochloride salt in 1983 (USEPA, 1984). More recent figures are not available.

The primary routes of potential human exposure to *o*-toluidine and its hydrochloride salt are through occupational exposure via inhalation and dermal contact (USDHHS, 1991). The National Occupational Exposure Survey, conducted by NIOSH from 1981 to 1983, estimated that 13,053 workers were potentially exposed to *o*-toluidine in the workplace (NIOSH, 1995).

## Disposition and Metabolism

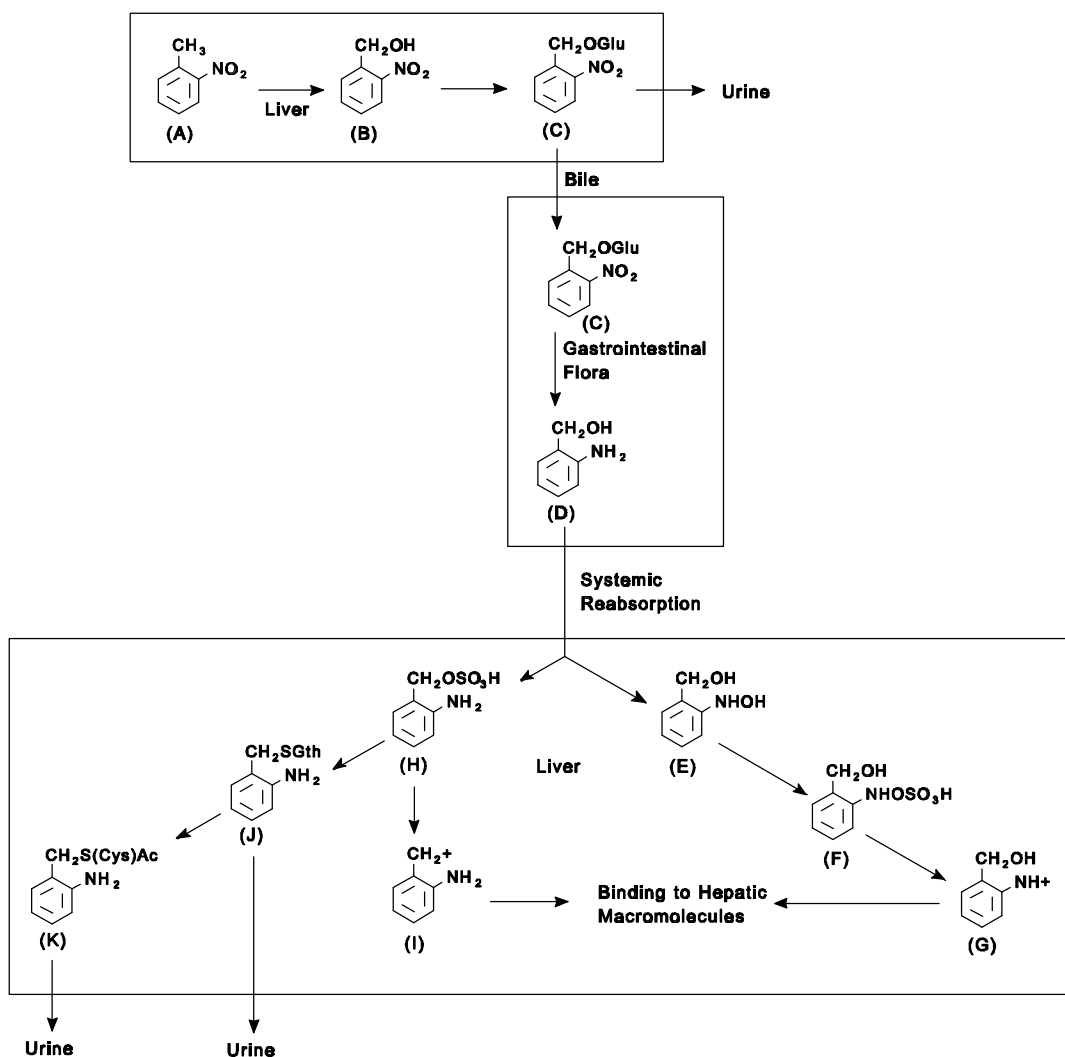
In hepatocytes isolated from F344 rats, *o*-nitrotoluene was converted to nitrobenzyl alcohol via a cytochrome P<sub>450</sub>-dependent process (deBethizy and Rickert, 1984). *o*-Nitrobenzyl alcohol, the principal metabolite of *o*-nitrotoluene, was primarily conjugated with glucuronic acid (28% of the metabolized nitrotoluene); a smaller percentage (3%) of metabolized *o*-nitrotoluene was oxidized to *o*-nitrobenzoic acid.

Following oral administration of 200mg *o*-nitrotoluene per kilogram body weight to male F344 rats, 86% of the dose was eliminated in the urine within 72 hours (Chismet *et al.*, 1984). The major urinary metabolites of *o*-nitrotoluene were *S*-(2-nitrobenzyl)-*N*-acetylcysteine (12%), *o*-nitrobenzyl glucuronide (14%), and a metabolite tentatively identified as a sulfur-containing conjugate of *o*-toluidine (16%).

For male F344 rats, 29% of an oral dose (200mg/kg) of *o*-nitrotoluene was excreted in the bile after 12 hours (Chism and Rickert, 1985). *o*-Nitrobenzyl glucuronide was 78% of the *o*-nitrotoluene metabolites in bile. The proposed scheme for the metabolism of *o*-nitrotoluene is presented in Figure 1. *o*-Nitrotoluene is first converted to *o*-nitrobenzyl alcohol in the liver by a cytochrome-P<sub>450</sub>-dependent process. The subsequent conjugation of *o*-nitrobenzyl alcohol with glucuronic acid is mediated by hepatic UDPglucuronosyltransferases. *o*-Aminobenzyl alcohol is formed from *o*-nitrobenzyl glucuronide by the hydrolytic and reductive activities of gastrointestinal flora. Bacterial nitroreductase activity in the gastrointestinal tract appears to be essential for the genotoxicity/carcinogenicity of many nitroaromatic compounds (Doolittle *et al.*, 1983; Rickert

*et al.*, 1986a; Rickert, 1987; deBethizy and Hayes, 1994). Following systemic reabsorption, the amino group may be oxidized by hepatic microsomal enzymes to the corresponding hydroxylamine. The final activation step for *o*-nitrotoluene appears to be dependent upon sulfotransferase (Rickert *et al.*, 1986b). Either *o*-aminobenzyl alcohol or *o*-hydroxylaminobenzyl alcohol may be conjugated with sulfate. *o*-Aminobenzyl sulfate is expected to be a highly reactive alkylating agent (Chism *et al.*, 1984). *o*-Hydroxylaminobenzyl alcohol is thought to conjugate with sulfate to yield an unstable *N*-sulfate which decomposes to an electrophilic nitrenium ion (Chism *et al.*, 1984; Rickert, 1987; Chism and Rickert, 1989).

Following the subcutaneous administration of a single dose (400mg/kg) of <sup>14</sup>C-radiolabeled *o*-toluidine hydrochloride to male CDF rats, the primary urinary metabolites were identified as the sulfate and glucuronide conjugates of 4-amino-*m*-cresol and *N*-acetyl-4-amino-*m*-cresol (Son *et al.*, 1977). Following a single oral administration of [<sup>14</sup>C]-*o*-toluidine hydrochloride (methyl carbon labeled) (500 mg/kg) to male Sprague-Dawley rats, greater than 92% of the radiolabel was eliminated in the urine within 24 hours (Cheever *et al.*, 1980). Metabolism was primarily on the aromatic ring; greater than 50% of the administered dose was eliminated as phenol conjugates. No metabolites of *o*-toluidine hydrochloride derived from oxidation of the methyl group were detected even though *o*-aminobenzoic acid could have been detected at levels as low as 0.1% of the administered dose. Approximately 40% of the administered dose was excreted as the parent compound.



**FIGURE 1** Proposed Metabolic Scheme for o-Nitrotoluene: (A) o-Nitrotoluene; (B) o-Nitrobenzyl Alcohol; (C) o-Nitrobenzyl Alcohol Glucuronide; (D) o-Aminobenzyl Alcohol; (E) o-Hydroxylaminobenzyl Alcohol; (F) Unstable N-Sulfate; (G) Nitrenium Ion; (H) o-Aminobenzyl Alcohol Sulfate; (I) Carbonium Ion; (J) S-(o-Aminobenzyl) Glutathione; (K) S-(o-Aminobenzyl)-N-acetylcysteine



## Toxicity

Oral LD<sub>50</sub> values for *o*-nitrotoluene in unspecified strains of rats have been reported to be 2,144 mg/kg (Hanavan, 1975 – personal communication, E.I. duPont de Nemours and Co.) and 891 mg/kg (Christensen and Luginbyhl, 1974). An oral LD<sub>0</sub> of 2,462 mg/kg has been reported for an unspecified strain of mice (Christensen and Luginbyhl, 1974). A TC<sub>0</sub> (toxic concentration, low) of 700 µg/m<sup>3</sup> has been reported in humans following inhalation exposure to *o*-nitrotoluene (Sweet, 1983).

The National Toxicology Program (NTP) conducted 13-week feed studies of *o*-nitrotoluene in F344/N rats and B6C3F<sub>1</sub> mice (NTP, 1992; Dunnick *et al.*, 1994). In these studies, groups of 10 male and 10 female animals of each species were exposed for 13 consecutive weeks to *o*-nitrotoluene at concentrations of 0, 650, 1,250, 2,500, 5,000, and 10,000 ppm in NIH-07 diet. These doses delivered approximately 45 to 700 mg of *o*-nitrotoluene per kilogram of body weight to rats and 100 to 700 mg of *o*-nitrotoluene per kilogram of body weight to mice. During the 13-week studies, there were no treatment-related deaths. An exposure-dependent decrease in average feed consumption was evident in all treatment groups of male and female rats and mice. Mean body weights of exposed male and female rats and mice were also lower than those of the controls.

In male and female F344/N rats and B6C3F<sub>1</sub> mice, several organ weights were affected by 13 weeks of *o*-nitrotoluene treatment. In male rats, the livers were grossly enlarged and the testes were reduced in size. Absolute liver and relative liver and kidney weights increased with increasing exposure level in male rats. In addition, statistically significant, exposure-related reductions in testicular and epididymal weights were apparent in the 5,000 and 10,000 ppm groups. Results of clinical pathology evaluations from male and female rats revealed a methemoglobinemia and regenerative anemia which increased in severity with increasing exposure level (NTP, 1992; Dunnick *et al.*, 1994).

Microscopic lesions in male and female mice were restricted to degeneration and metaplasia of the olfactory epithelium in the nasal cavity. Several histopathologic changes were observed in male and female rats. Male rats had hyaline droplet accumulation, testis degeneration, liver vacuolization, oval cell hyperplasia, and mesothelioma of the epididymis (3/10 in the 5,000 ppm

group). Male and female rats had increased incidences of splenic hematopoiesis and pigment accumulation in the red pulp and splenic capsule thickening.

The oral LD<sub>50</sub> of *o*-toluidine hydrochloride was reported as 2,951mg/kg in Osborne-Mendel rats (Lindstrom *et al.*, 1969), 900 mg/kg in Sprague-Dawley rats (Jacobson, 1972), and 15mg/kg in an unspecified strain of mice (IARC, 1982). *o*-Toluidine has reportedly induced methemoglobinemia in mice (Nomura, 1977), rats (Lunkin, 1967), and humans (Ellenhorn and Barceloux, 1988).

In 7-week studies sponsored by the National Cancer Institute (NCI), groups of five male and five female F344 rats and B6C3F<sub>1</sub> mice were administered *o*-toluidine hydrochloride in feed at nine concentrations ranging from 1,000 to 50,000 ppm (NCI, 1979a). Seven weeks of exposure were followed by 1 week of control diet administration. Mortality was observed in male and female rats at the 50,000 ppm level. Exposure-related decreases in group mean body weights were evident in male and female rats and mice. Microscopically, small amounts of renal and splenic pigmentation were observed in male and female rats in the 12,500ppm groups. In male and female mice exposed to 50,000 ppm, pigment deposition was evident in the spleen, kidneys, and liver.

#### CARCINOGENICITY

There have been no carcinogenicity studies performed for any of the mononitrotoluene isomers. The carcinogenicity of dinitrotoluene, a structural analogue of nitrotoluene, has been demonstrated in long-term studies.

In a 2-year dosed-feed study sponsored by the Chemical Industry Institute of Toxicology (CIIT), a technical grade of dinitrotoluene (tDNT) was demonstrated to be carcinogenic in male and female F344 rats (CIIT, 1979). The tDNT was composed primarily of 2,4-dinitrotoluene and 2,6-dinitrotoluene. Rats received an estimated dose of 0, 3.5, 14, or 35mg tDNT per kilogram of body weight per day. After 1 year, all males and 50% of females in the 35mg/kg groups had developed hepatocellular carcinomas. There was a high incidence of hepatocellular carcinoma in the 14 mg/kg group after 2 years. Long-term administration of tDNT also resulted in the formation of cholangiocarcinomas which were presumably derived from the bile duct epithelium.

In a 78-week bioassay sponsored by NCI, male and female F344 rats were administered 0.008% or 0.02% 2,4-dinitrotoluene (96% pure) in feed for 78 weeks (NCI, 1978a). B6C3F<sub>1</sub> mice were similarly administered 0.008% or 0.04% 2,4-dinitrotoluene in feed. Exposure to 2,4-dinitrotoluene was terminated after 78 weeks and animals were maintained on the control diet for an additional 26 weeks (rats) or 13 weeks (mice). Under the conditions of this bioassay, 2,4-dinitrotoluene was not hepatocarcinogenic, although there was an increased incidence of benign skin fibromas in exposed male rats and mammary gland fibroadenomas in female rats given 0.02%. There was no evidence of carcinogenicity in male or female B6C3F<sub>1</sub> mice.

Popp and Leonard (1983) determined, in a series of 1-year dosed feed studies, that 7 or 14 mg/kg 2,6-dinitrotoluene (99.9% pure) was hepatocarcinogenic, while 27 mg/kg 2,4-dinitrotoluene (99.9% pure) was not. Based on these results and the lack of hepatocarcinogenicity for 2,4-dinitrotoluene in the aforementioned NCI study (NCI, 1978a), the reported hepatocarcinogenicity of tDNT (CIIT, 1979) appears to be primarily due to its 2,6-substituted isomer. Studies with the structurally related compound, toluidine (which exists as *ortho*-, *meta*-, and *para*-isomers), also suggest that the *ortho*-substituted compound is a more potent carcinogen than the *meta*- or *para*-substituted isomers (Weisburger *et al.*, 1978).

NCI sponsored 2-year bioassays of *o*-toluidine hydrochloride in F344 rats and B6C3F<sub>1</sub> mice (NCI, 1979a). In NCI studies, groups of 50 male and 50 female rats and mice were given *o*-toluidine hydrochloride in feed at concentrations of 3,000 or 6,000 ppm for rats and 1,000 or 3,000 ppm for mice for 101 to 104 weeks. Relatively high, exposure-related mortality was observed in male and female rats by the end of the study. Mortality in mice did not appear to be significantly affected by treatment. Mean body weights in male and female rats and mice were lower than those of the controls at the end of the studies.

When compared to controls, rats given *o*-toluidine hydrochloride in feed had increased incidences of benign and malignant tumors (sarcomas) of the spleen in males and females, mesotheliomas of the abdominal cavity or scrotum in males (control, 0%; 3,000 ppm, 24%; 6,000 ppm, 12%), and transitional-cell carcinomas of the urinary bladder in females (0%, 20%, 47%) (NCI, 1979a). *o*-Toluidine hydrochloride exposure also resulted in increased incidences of fibromas of the subcutaneous tissue in males (0%, 56%, 55%) and fibroadenomas or adenomas of the mammary gland in females (36%, 40%, 71%).

In mice given *o*-toluidine hydrochloride, there were increased incidences of hemangiosarcomas in males (5%, 4%, 24%) and of hepatocellular carcinomas or adenomas in females (0%, 8%, 26%) (NCI, 1979a).

#### GENETIC TOXICITY

The genetic toxicity data for *o*-nitrotoluene are summarized in NTP Toxicity Report 23 (NTP, 1992). Results from genetic toxicity tests were mixed. Negative results were obtained in a *Salmonella typhimurium* mutation assay (Haworth *et al.*, 1983) and a test for induction of chromosomal aberrations in cultured Chinese hamster ovary cells (Galloway *et al.*, 1987). Positive results were reported for *o*-nitrotoluene in a test for induction of sister chromatid exchanges in cultured Chinese hamster ovary cells (Galloway *et al.*, 1987) and in an assay for induction of unscheduled DNA synthesis in hepatocytes of male rats treated *in vivo* (NTP, 1992). *o*-Nitrotoluene administered intraperitoneally did not induce micronucleated polychromatic erythrocytes in rat or mouse bone marrow (NTP, unpublished). In the intraperitoneal study, the highest dose tested in rats was 2,500 mg/kg (single injection) and in mice the highest dose was three 400 mg/kg injections, administered at 24-hour intervals.

Because activation of *o*-nitrotoluene by intestinal bacteria is necessary to induce a positive unscheduled DNA synthesis response, the *S. typhimurium* assay, performed in the absence of a specialized protocol which employs reductive metabolism, would not be expected to yield a positive response. Also, the metabolic pathways that generate the active metabolite of *o*-nitrotoluene appear to be species and sex specific. Therefore, detection of the mutagenic activity of *o*-nitrotoluene is highly dependent upon the test system and the protocol employed.

The genotoxicity of *o*-toluidine has recently been reviewed in detail (Danford, 1991). *o*-Toluidine was genotoxic in a variety of *in vitro* and *in vivo* assays, although the experimental conditions for detecting genotoxicity were rather stringent, particularly with regard to the metabolic activation system used for the *in vitro* studies (Danford, 1991). Positive results were reported for *o*-toluidine in the following assay systems: bacterial mutation tests (Tanaka *et al.*, 1980; Kada, 1981; Trueman, 1981; Venitt and Crofton, Sleight, 1981; Zeiger and Haworth, 1985), yeast aneuploidy tests (Parry and Sharp, 1981; Parry and Eckardt, 1985), *Drosophila* somatic recombination assays (Vogel, 1985; Würigler *et al.*, 1985), *in vitro* cytogenetics assays (Perry and Thomson, 1981;

Danford, 1985; Darroudi and Natarajan, 1985; Gulati *et al.*, 1985; Ishidate and Sofuni, 1985; Lane *et al.*, 1985; Palitti *et al.*, 1985; Priston and Dean, 1985; vanWent, 1985), and DNA damage assays (Martin and McDermid, 1981; Cesarone *et al.*, 1982; Barrett, 1985; Bradley, 1985; Douglas *et al.*, 1985; Glauert *et al.*, 1985; Martin and Campbell, 1985). Results from these assays, however, were not uniform among all testing laboratories, and appear to be protocol dependent. Experimental parameters such as S9 source and concentration, length of exposure time to *o*-toluidine, dose, and cell line or type were important in determining whether a particular test was positive or negative.

Results of *in vivo* mouse bone marrow micronucleus assays with *o*-toluidine were negative (Salamone *et al.*, 1981; Tsuchimoto and Matter, 1981). However, results of recent NTP investigations suggest that species differences may play an important role in the induction of micronucleated normochromatic erythrocytes (NTP, unpublished). These studies showed that *o*-toluidine induced micronuclei in bone marrow cells of male F344/N rats, but not male B6C3F mice. Rats were given an intraperitoneal injection of 400mg/kg *o*-toluidine at 24-hour intervals for a total of three injections, and bone marrow was examined 24 hours after the final injection. Mice were administered a single injection of 400mg/kg *o*-toluidine, and bone marrow samples were taken 24 hours after dose administration. These treatments were the maximum nonlethal dose that could be administered to each species, as determined by preliminary range finding tests. In another study, *o*-toluidine hydrochloride was shown to induce sister chromatid exchange but not chromosomal aberrations or micronuclei in bone marrow cells of male B6C3F mice (McFee *et al.*, 1989). In this test, 150 to 600mg/kg *o*-toluidine hydrochloride was administered to the mice as a single intraperitoneal injection.

The genetic toxicity test data for *o*-toluidine indicate that the compound has genotoxic activity and detection of this activity is highly dependent on the assay system and protocol employed. The genotoxic activity demonstrated in several assays has not been strong; thus *o*-toluidine would perhaps best be described as a weak mutagen.

## Study Rationale and Design

*o*-Nitrotoluene and *o*-toluidine hydrochloride are high production, structurally related compounds. In a 13-week dosed-feed study, three male rats given 5,000 ppm of *o*-nitrotoluene developed mesothelioma of the epididymis, a neoplasm that had not previously been identified in a 13-week

toxicity study (NTP, 1992; Dunnick *et al.*, 1994). In a 2-year carcinogenicity study with *o*-toluidine hydrochloride, there were increased treatment-related incidences of several tumor types in F344 rats, including mesothelioma of the abdominal cavity or scrotum; potential toxic and carcinogenic effects were not evaluated at 13 weeks (NCI, 1979a).

The NTP studies reported here were designed to confirm the carcinogenic effects of *o*-nitrotoluene and to compare the potential toxicity and carcinogenicity of *o*-nitrotoluene and *o*-toluidine hydrochloride with respect to the development, progression, and regression of treatment-related effects during 13-week or 26-week exposure. Because metabolism and genotoxicity studies of *o*-nitrotoluene have indicated that gastrointestinal flora may play an obligatory role in the metabolic activation (reduction of the nitro group to the amine function) and genotoxicity of this compound, additional groups were included in an attempt to determine the effect of altered gastrointestinal flora on the toxicity or carcinogenicity of *o*-nitrotoluene.

Based on earlier studies, the concentration chosen for these studies, 5,000 ppm, was expected to cause a marked decrease in the body weight gains of rats receiving *o*-nitrotoluene. It was recognized that this lower weight gain might inhibit the expression of neoplasms; however, this dose was effective in producing mesothelioma in the study reported by Dunnick *et al.* (1994) and was therefore chosen for the present comparative study.

# MATERIALS AND METHODS

## Procurement and Characterization of *o*-Nitrotoluene and *o*-Toluidine Hydrochloride

Two lots of *o*-nitrotoluene (Lots 1110CJ and 08506MV) were obtained from the Aldrich Chemical Company (Metuchen, NJ). A single lot of *o*-toluidine hydrochloride (Lot 308828/1 891) was obtained from SAF Bulk Chemicals (Ronkonkoma, NY). Lot 1110CJ of *o*-nitrotoluene was used for chemistry methods development and stability studies. Lot 08506MV of *o*-nitrotoluene and Lot 308828/1 891 of *o*-toluidine hydrochloride were used for the 26-week studies.

Chemical analyses of *o*-nitrotoluene were performed by Midwest Research Institute (MRI; Kansas City, MO). For Lot 1110CJ, infrared, ultraviolet/visible, and nuclear magnetic resonance (NMR) spectra were consistent with the structure of *o*-nitrotoluene and with a literature reference (*Sadtler Standard Spectra*). The results of elemental analyses for carbon, hydrogen, and nitrogen agreed with theoretical values for *o*-nitrotoluene. Karl Fischer analysis indicated  $0.021\% \pm 0.003\%$  water. Functional group titration, by nitro reduction with titanium (III) chloride followed by back titration with standardized ferric ammonium sulfate, indicated a purity of  $99\% \pm 1\%$ . Thin-layer chromatography by two solvent systems indicated a major spot only in each system. Two gas chromatographic systems with flame ionization detection (FID) showed a major peak and no impurities with relative areas equal to or greater than 0.38%.

Lot 08506MV was identified as *o*-nitrotoluene (a clear, pale yellow liquid) by MRI with infrared, ultraviolet, and NMR spectroscopy. A boiling point of 221.6 C and a density ( $d_{3,4}^{23,7}$ ) of 1.156 g/mL ( $n=3$ ) for Lot 08506MV agreed with a literature reference (*Merck Index*, 1983) and with the boiling point and density of Lot 1110CJ. Infrared, ultraviolet/visible, and NMR spectra were consistent with the structure of *o*-nitrotoluene, with literature references (*Sadtler Standard Spectra Aldrich*, 1985), and with the spectra of Lot 1110CJ. Concomitant analyses of Lot 08506MV and Lot 1110CJ by gas chromatography with FID showed a purity of  $99.8\% \pm 0.3\%$  for Lot 08506MV relative to Lot 1110CJ.

An accelerated stability study was performed by MRI on Lot 1110CJ of *o*-nitrotoluene. Gas chromatographic analyses with FID indicated that bulk *o*-nitrotoluene was stable for 2 weeks when stored protected from light at temperatures up to 60 C. At the study laboratory, *o*-nitrotoluene

was stored in sealed amber glass bottles at approximately 25° C. The study laboratory reanalyzed the bulk chemical (Lot 08506MV) by gas chromatography with FID before the start of the studies, during the studies, and again after the studies ended; no degradation of *o*-nitrotoluene was observed.

Chemical analyses of *o*-toluidine hydrochloride were performed by SAF Bulk Chemicals on Lot 308828/1 891. The chemical, a white, crystalline powder with lumps, was identified as *o*-toluidine hydrochloride. An infrared spectrum was consistent with the structure of *o*-toluidine hydrochloride and with a literature reference. The results of elemental analyses for carbon, hydrogen, and nitrogen agreed with theoretical values for *o*-toluidine hydrochloride. Argentometric titration (chloride determination) indicated a purity of 100% for *o*-toluidine hydrochloride. At the study laboratory, *o*-toluidine hydrochloride was stored in sealed amber glass bottles at approximately 25° C. The study laboratory reanalyzed the bulk chemical by high performance liquid chromatography (HPLC) before the start of the studies, during the studies, and again after the studies ended; no degradation of *o*-toluidine hydrochloride was observed.

## Preparation and Analysis of Dose Formulations

*o*-Nitrotoluene or *o*-toluidine hydrochloride was mixed with NIH-07 Open Formula Diet (Zeigler Bros., Inc., Gardners, PA) in meal form. A premix was prepared by adding an accurately weighed amount of *o*-nitrotoluene or *o*-toluidine hydrochloride to a small portion of feed. Additional feed was added until the desired premix weight was reached. Then, the premix was blended with a weighed amount of feed in a Patterson-Kelley twin-shell blender (East Stroudsburg, PA) for 15 minutes, with the intensifier bar on for the first 5 minutes.

MRI performed homogeneity and stability studies on a feed mixture containing 0.5 mg *o*-nitrotoluene (Lot 1110CJ) per gram of feed. *o*-Nitrotoluene was mixed with NIH-07 Open Formula Diet by the method described previously. After feed blending, a sample was taken from each of three blender ports, and the samples were extracted with ethanol and analyzed for *o*-nitrotoluene by HPLC. Homogeneity of the feed mixture was confirmed. Approximately 12% *o*-nitrotoluene was lost during feed preparation.

Samples of the 0.5 mg/g *o*-nitrotoluene-feed mixture were stored in sealed containers in the dark at room temperature, 5° C, or -20° C for 7, 14, or 21 days or in a rat cage, open to air and light,



for 1, 2, 3, 4, or 7 days. All samples were analyzed by HPLC. Feed samples stored in sealed containers in the dark at 5° C were stable for 3 weeks. Feed samples stored in the rat cage for all time periods showed statistically significant losses of *o*-nitrotoluene. Samples analyzed after 1 day in the rat cage showed a loss of 6.3% *o*-nitrotoluene, and, after 7 days of storage, samples showed a loss of 18.7% *o*-nitrotoluene. These losses were in addition to the approximately 12% *o*-nitrotoluene lost during feed preparation.

The study laboratory performed homogeneity and stability studies on a feed mixture containing 5 mg *o*-nitrotoluene/g feed (5,000 ppm) (Lot 08506MV). *o*-Nitrotoluene was mixed with NIH-07 Open Formula Diet by the method described previously. After feed blending, a sample was taken from each of three blender ports, and the samples were extracted with ethanol and analyzed for *o*-nitrotoluene by HPLC. Homogeneity of the feed mixture was confirmed. Approximately 2% *o*-nitrotoluene was lost during feed preparation.

Samples of the 5 mg/g *o*-nitrotoluene-feed mixture were stored in sealed amber glass bottles at -20° C, 5° C, or room temperature for 28 days or in an animal feeder, open to air and light, for 1 or 4 days. All samples were analyzed by gas chromatography with FID. Samples stored in sealed containers at -20° and 5° C, protected from light, were stable for at least 28 days. Samples stored in sealed containers at room temperature, protected from light, showed statistically significant losses at 7, 14, and 28 days. Samples stored in an animal feeder, open to air and light, exhibited a loss of 3.4% after 1 day and 10.0% after 4 days. The loss after 4 days was statistically significant and was likely due to evaporation of *o*-nitrotoluene from the open feeders.

At the study laboratory, *o*-nitrotoluene feed mixtures were stored in sealed containers at approximately 5° C for up to 28 days. On Day 7 of the study, *o*-nitrotoluene-feed mixtures were stored at room temperature because of refrigerator malfunction.

In the animal rooms, clean feed jars with fresh feed were provided to all rats at least weekly. *o*-Nitrotoluene-feed mixtures were prepared once every 2 weeks for the first 14 weeks and once every 3 weeks thereafter. Samples of the *o*-nitrotoluene-feed mixtures prepared on 22 April and 20 May 1992 were taken before dosing and after dosing (from the animal rooms) and analyzed for *o*-nitrotoluene by gas chromatography with FID. The animal room samples of the 22 April and 20 May 1992 mixtures showed losses of 6% and 9% *o*-nitrotoluene, respectively. These losses are

consistent with the results of prestart stability studies, which showed a loss of approximately 10% *o*-nitrotoluene after storage in open containers for 4 days.

The study laboratory performed homogeneity and stability studies on a feed mixture containing 5 mg *o*-toluidine hydrochloride/g feed (5,000 ppm). *o*-Toluidine hydrochloride was mixed with NIH-07 Open Formula Diet by the method described previously. After blending a sample was taken from each of three blender ports, and the samples were extracted with methanol and analyzed for *o*-toluidine hydrochloride by HPLC. Homogeneity of the feed mixture was confirmed. Approximately 9% *o*-toluidine hydrochloride was lost during feed preparation.

Samples of the 5 mg/g *o*-toluidine hydrochloride-feed mixture were stored in sealed containers in the dark at room temperature, -20° C or 5° C for 7, 14, or 28 days or in an animal feeder, open to air and light, for 1 or 4 days. All samples were analyzed by HPLC. Feed samples stored in sealed containers in the dark at -20° or 5° C were stable for 28 days. The feed sample stored at room temperature for 28 days contained 90% of the *o*-toluidine hydrochloride detected in the sample before storage.

At the study laboratory, *o*-toluidine hydrochloride feed mixtures were stored in sealed containers at approximately 5° C, with one exception, for up to 28 days. From Day 1 to Day 7 of the study, the *o*-toluidine hydrochloride feed mixtures were improperly stored at room temperature. Based on the results of the stability study, storage at room temperature was expected to decrease the *o*-toluidine concentration by 5% to 10%.

In the animal rooms, clean feed jars with fresh feed were provided to all rats at least weekly. *o*-Toluidine hydrochloride feed mixtures were prepared once every 3 weeks. Samples of the feed mixtures prepared on 3 March, 14 April, 6 May, 7 July, and 18 August 1992, were collected before dosing and analyzed for *o*-toluidine hydrochloride content by HPLC. The animal room samples collected from feeders 4 or 5 days after preparation on 6 May, 7 July, and 18 August 1992 mixtures showed losses of 0%, 15%, and 35% *o*-toluidine hydrochloride, respectively. For the samples showing 15% and 35% losses, contamination with animal hair and feces contributed to an indeterminate amount of the loss in each sample. Differences in water content between the feed used to prepare the standards and the feed removed from the animal rooms accounted for 3% of the loss in each sample.

## Preparation of Antibiotic Mixture

Tetracycline hydrochloride, neomycin sulfate, and nystatin were obtained from Sigma Chemical Co. (St. Louis, MO). Weighed amounts of each antibiotic (in powdered form) were combined, apportioned into sealed containers in daily allotments, and stored at approximately 0 C. Before dosing, the antibiotic mixture was reconstituted by adding the appropriate volume of deionized water. No chemical analyses of the neat chemicals or dose formulations were required. The efficacy and acute toxicity of the antibiotic mixture were evaluated in non-GLP pilot studies. The results of these studies are presented in Appendix C.

## Toxicity Study Designs

### BASE STUDIES

Male F344/N rats used in these studies were obtained from Charles River Breeding Laboratories (Raleigh, NC). Rats were 36 days old at receipt, were quarantined for 9 days, and were 45 days old at the start of the studies. Blood samples were collected from 10 sentinel rats at the start of the studies and were analyzed for antibody titers to rodent viruses (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b); all results were negative. Additional details concerning the study design are provided in Table 1.

All exposed male rats received 5,000 ppm *o*-nitrotoluene or *o*-toluidine hydrochloride in feed for 13 or 26 weeks. The dose selected for administration of *o*-nitrotoluene was based on the previous 13-week study where a concentration of 5,000 ppm in the diet caused mesothelioma in 3 of 10 rats. At that dose, there was no effect on survival, but body weights were 7% less than those of controls. For the comparison study, rats were administered an approximately equimolar amount (5,000 ppm) of *o*-toluidine hydrochloride. *o*-Nitrotoluene was administered to 60 rats with normal gastrointestinal flora and to 40 rats with altered gastrointestinal flora. *o*-Toluidine hydrochloride was administered to 60 rats with normal gastrointestinal flora. There were two control groups with 20 unexposed rats each, one with normal gastrointestinal flora and one with altered flora. Rats in the altered gastrointestinal flora groups received a single gavage dose of an antibiotic mixture (20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin) in deionized water daily for 6 days before the start of the study and daily for 13 weeks thereafter. This treatment was based on the results of a pilot study (Appendix C) that demonstrated a reduction of aerobic flora greater than 97% compared to controls. On one day during Week 12, rats did not receive the antibiotic

mixture due to a technician oversight. The missed treatment likely had minimal impact on microbial growth, the formation of *o*-nitrotoluene-derived metabolites, or any associated toxicity.

Following the 13-week administration of *o*-nitrotoluene or *o*-toluidine hydrochloride, 10 rats from each control group (normal and altered gastrointestinal flora) and 20 rats from each exposure group (normal and altered gastrointestinal flora) were killed for the 13-week interim evaluation. Following the 13-week administration of *o*-nitrotoluene or *o*-toluidine hydrochloride and the 13-week maintenance period on the control diet, 20 rats from each exposure group (normal and altered gastrointestinal flora) were killed for the stop-exposure evaluation. Following the 26-week administration of *o*-nitrotoluene or *o*-toluidine hydrochloride, 20 rats from each normal gastrointestinal flora exposure group were killed for the 26-week evaluation. Ten rats from each control group (normal and altered gastrointestinal flora) were killed at 26 weeks for the stop exposure and 26-week evaluations.

Rats were housed five per cage, and NIH-07 Open Formula Diet (Zeigler Brothers, Inc., Gardners, PA) in meal form and water (Columbus, OH) were available *ad libitum*. Feed consumption was measured weekly. Animal rooms were maintained at  $72 \pm 3^\circ$  F and  $50\% \pm 15\%$  relative humidity, with at least 10 room air changes per hour. Fluorescent light was provided for 12 hours per day.

Necropsies, with limited tissue collection, were performed on all rats exposed to *o*-nitrotoluene or *o*-toluidine hydrochloride for 13 weeks, 13 weeks with 13 weeks of recovery, or 26 weeks. The epididymis, right kidney, liver, spleen, and right testis of each rat were weighed. Organs and tissues were examined for gross lesions and fixed in 10% neutral buffered formalin. Tissues to be examined microscopically were trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For all paired organs (i.e., adrenal gland, kidney, ovary), samples from each organ were examined. Osmium staining for the presence of fat was performed on formalin-fixed samples of liver from control and *o*-nitrotoluene-exposed rats. Proliferating cell nuclear antigen staining of formalin-fixed, paraffin-embedded samples of cholangiocarcinoma were also performed (Greenwell *et al.*, 1991). Histopathologic evaluations were performed on all rats at the 13-week interim evaluation and the end of the studies. Tissues examined microscopically are listed in Table 1.

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

#### SUPPLEMENTAL EVALUATIONS

At the 13-week interim evaluation, cecal specimens were collected from five rats per group in the control groups (normal and altered gastrointestinal flora) and o-nitrotoluene groups (normal and altered gastrointestinal flora). The samples were diluted with sterile phosphate buffered saline (1:100 on a weight:weight basis) and mixed for 4 minutes with a Model 400 Stomacher Lab Blender. Three serial dilutions were made for each sample and plated in triplicate on MacConkey pour plates. The plates were incubated aerobically at 35 to 37° C for 18 to 24 hours. Plates containing 30 to 300 colonies were counted manually. If no plates fell within this range, counts were estimated by standard microbiological procedures. Counts were reported as colony-forming units per gram of sample analyzed.

Additional sections of liver were prepared and stained to determine and quantitate placental glutathione S-transferase (PGST)-positive foci of cellular alteration (Hsu *et al.*, 1981). Livers from 9 or 10 rats in the 13-week interim and 26-week continuous-exposure groups were evaluated for PGST-positive foci. Image analyses of PGST-positive foci were performed to determine the number of foci per cubic centimeter of liver and the volume fraction (expressed as a percentage) of liver occupied by PGST-positive foci (Tonet *et al.*, 1995).

**TABLE 1 Experimental Design and Materials and Methods in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride**

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**EXPERIMENTAL DESIGN****Study Laboratory**

Battelle Columbus Operations, Columbus, OH

**Strain and Species**

Male F344/N rats

**Animal Source**

Charles River Breeding Laboratories, Raleigh, NC

**Size of Study Groups**

13-week interim:

Normal gastrointestinal flora groups: 10 control rats; 20 rats (o-nitrotoluene); 20 rats (o-toluidine hydrochloride)

Altered gastrointestinal flora groups: 10 control rats; 20 rats (o-nitrotoluene)

Stop-exposure:

Normal gastrointestinal flora groups: 20 rats (o-nitrotoluene); 20 rats (o-toluidine hydrochloride)

Altered gastrointestinal flora groups: 10 control rats; 20 rats (o-nitrotoluene); 20 rats (o-toluidine hydrochloride)

26-week study:

Normal gastrointestinal flora groups: 10 control rats; 20 rats (o-nitrotoluene); 20 rats (o-toluidine hydrochloride)

**Route of Administration**

Feed

**Exposure Concentrations/ Duration**

13-Week interim groups:

0 ppm or 5,000 ppm o-nitrotoluene or o-toluidine hydrochloride for 13 weeks

Stop-exposure groups:

0 ppm or 5,000 ppm o-nitrotoluene or o-toluidine hydrochloride for first 13 weeks; 0 ppm for final 13 weeks

26-Week continuous-exposure groups:

0 ppm or 5,000 ppm o-nitrotoluene or o-toluidine hydrochloride for 26 weeks

**Date of First Exposure**

6 March 1992

**Date of Last Exposure/ Necropsy**

13-Week interim: 8 June 1992

Study termination: 8 September 1992 (stop-exposure study groups), 9 September 1992 (26-week continuous-exposure groups)

**Type and Frequency of Observation**

Rats were observed twice daily. Body weights and clinical signs were recorded weekly and at necropsy. Feed consumption was measured weekly.

**Necropsy and Histologic Examinations**

Complete necropsies were performed on all rats exposed to o-nitrotoluene or o-toluidine hydrochloride for 13 weeks, 13 weeks with 13 weeks of recovery, or 26 weeks. The right kidney, liver, spleen, right testis, and epididymis of all control rats and 10 of 20 rats from each exposure group were weighed. Histopathologic evaluations were performed on all rats at the 13-week interim evaluation and at the end of the studies. The following tissues were examined in all groups: epididymides (three portions of each), gross lesions, liver, right kidney, spleen, and testes. The urinary bladder was also examined in rats administered o-toluidine hydrochloride for 13 or 26 weeks.

**Supplemental Evaluations**

At the 13-week interim, cecal specimens were collected from five rats per group in the control groups (normal and altered gastrointestinal flora) and o-nitrotoluene groups (normal and altered gastrointestinal flora); microbial colony counts were taken for each specimen.

At the 13-week interim and the end of the 26-week continuous and stop-exposure periods, liver samples were collected from 9 or 10 rats per group in the control groups (normal and altered gastrointestinal flora) and o-nitrotoluene groups (normal and altered gastrointestinal flora); tissue was stained for placental glutathione S-transferase-positive foci.

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**TABLE 1 Experimental Design and Materials and Methods in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride (continued)**

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**ANIMAL MAINTENANCE****Time Held Before Studies**

9 days

**Age When Studies Began**

45 days

**Age When Killed**

19 weeks (13-week interim); 32 weeks (stop-exposure and 26-week continuous-exposure)

**Method of Animal Distribution**

Animals were distributed randomly into groups of approximately equal initial mean body weight.

**Diet**NIH-07 Open Formula Diet (Zeigler Bros., Inc., Gardners, PA) in meal form and water (City of Columbus), available *ad libitum*.**Animal Room Environment**Rats were housed five per cage. In the animal room, the temperature was maintained at  $72^{\circ} \pm 3^{\circ}$  F and relative humidity at  $50\% \pm 15\%$ , with at least 10 room air changes per hour. Fluorescent light was provided for 12 hours per day.

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**Statistical Methods**

## ANALYSIS OF LESION INCIDENCES

The Fisher exact test (Armitage, 1971; Gart *et al.*, 1979), a procedure based on the overall proportion of lesion-bearing animals, was used to evaluate histopathologic lesion data.

## ANALYSIS OF CONTINUOUS VARIABLES

Organ and body weight data, which have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955). The number of PGST-positive foci per cubic centimeter of liver and the volume fraction of liver occupied by PGST-positive foci were analyzed with Wilcoxon's rank sum test (Conover, 1971).

Before analysis, extreme values identified by the outlier test of Dixon and Massey (1951) were examined by NTP personnel. Implausible values, extreme values from animals that were suspected of being sick due to causes other than treatment, and values that the study laboratory indicated as being inadequate due to technical problems were eliminated from the analysis.

## Quality Assurance

The animal studies of *o*-nitrotoluene and *o*-toluidine hydrochloride were performed in compliance with United States FDA Good Laboratory Practices regulations (21 CFR, Part 58). The Quality Assurance Unit of Battelle Columbus Operations performed audits and inspections of protocols, procedures, data, and reports throughout the course of the studies.



# RESULTS

## 26-Week Feed Studies in Male F344/N Rats

All rats in the 13-week interim, stop-exposure, and 26-week continuous-exposure groups survived until the scheduled evaluations (Table 2). Average daily fed consumption was 12% to 18% lower than that by controls for rats in the *o*-nitrotoluene-exposed groups and 5% to 9% lower than that by controls for rats in the *o*-toluidine hydrochloride-exposed groups (Table 3). Feed consumption was similar in the corresponding control and exposed rats in the normal and altered gastrointestinal flora groups. Mean body weight gains of rats in all exposed groups were lower than those of the controls after 13 or 26 weeks of exposure. At the 13-week interim, mean body weight gains were 42% (altered flora) and 44% (normal flora) lower for rats exposed to *o*-nitrotoluene and 21% lower for rats exposed to *o*-toluidine hydrochloride than those of the respective controls (Table 2 and Figure 2). In the 26-week continuous-exposure groups, mean body weight gains were 53% lower for rats exposed to *o*-nitrotoluene and 27% lower for rats exposed to *o*-toluidine hydrochloride than the control value (Table 2 and Figure 3). Rats in the stop-exposure groups had slightly greater mean body weight gains than rats continuously exposed for 26 weeks but still weighed less than the controls (Table 2 and Figure 4). There were no treatment-related clinical signs in the 13-week or 26-week stop- or continuous-exposure groups.

**Table 2 Survival and Body Weights of Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride**

Group	Survival <sup>1</sup>	Mean Body Weight (grams)			Final Weight Relative to Controls <sup>2</sup> (%)
		Initial	Final	Change	
<b>13-WEEK INTERIM</b>					
<b>Normal Gastrointestinal Flora</b>					
Control (0 ppm)	10/10	157	331	175	
o-Nitrotoluene (5,000 ppm)	10/10	163	261	98	79
o-Toluidine HCl (5,000 ppm)	10/10	155	293	139	89
<b>Altered Gastrointestinal Flora</b>					
Control (0 ppm)	10/10	157	314	158	
o-Nitrotoluene (5,000 ppm)	10/10	153	245	92	78
<b>STOP-EXPOSURE</b>					
<b>Normal Gastrointestinal Flora</b>					
Control (0 ppm)	10/10	159	382	224	
o-Nitrotoluene (5,000 ppm)	10/10	159	297	139	78
o-Toluidine HCl (5,000 ppm)	10/10	155	342	187	89
<b>Altered Gastrointestinal Flora</b>					
Control (0 ppm)	10/10	158	379	221	
o-Nitrotoluene (5,000 ppm)	10/10	159	273	115	72
<b>26-WEEK CONTINUOUS-EXPOSURE</b>					
<b>Normal Gastrointestinal Flora</b>					
Control (0 ppm)	10/10	159	382	224	
o-Nitrotoluene (5,000 ppm)	10/10	159	265	106	69
o-Toluidine HCl (5,000 ppm)	10/10	151	314	163	82

<sup>1</sup> Number surviving at 13 or 26 weeks/number of animals per exposure group.

<sup>2</sup> (Exposure group mean/control group mean) x 100.

**Table 3 Feed and Estimated Compound Consumption by Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride**

Group	Feed Consumption (g/day) <sup>1</sup>	Compound Consumption (mg/kg/day) <sup>2</sup>
<b>13-WEEK INTERIM</b>		
<b>Normal Gastrointestinal Flora</b>		
Control (0 ppm)	14.9	
o-Nitrotoluene (5,000 ppm)	12.2	293
o-Toluidine HCl (5,000 ppm)	13.5	301
<b>Altered Gastrointestinal Flora</b>		
Control (0 ppm)	14.3	
o-Nitrotoluene (5,000 ppm)	12.0	296
<b>STOP-EXPOSURE</b>		
<b>Normal Gastrointestinal Flora</b>		
Control (0 ppm)	14.7	
o-Nitrotoluene (5,000 ppm)	12.9	293
o-Toluidine HCl (5,000 ppm)	13.9	304
<b>Altered Gastrointestinal Flora</b>		
Control (0 ppm)	14.5	
o-Nitrotoluene (5,000 ppm)	12.7	293
<b>26-WEEK CONTINUOUS-EXPOSURE</b>		
<b>Normal Gastrointestinal Flora</b>		
Control (0 ppm)	14.7	
o-Nitrotoluene (5,000 ppm)	12.3	292
o-Toluidine HCl (5,000 ppm)	13.4	285

<sup>1</sup> Average of group mean feed consumption values measured at Weeks 1-14 for rats in the 13-week interim evaluation or Weeks 1-27 for rats in the stop-exposure evaluation and 26-week study.

<sup>2</sup> Average of group mean compound consumption values measured at Weeks 1-14 for rats in the 13-week interim evaluation or Weeks 2-27 for rats in the stop-exposure evaluation and 26-week study.

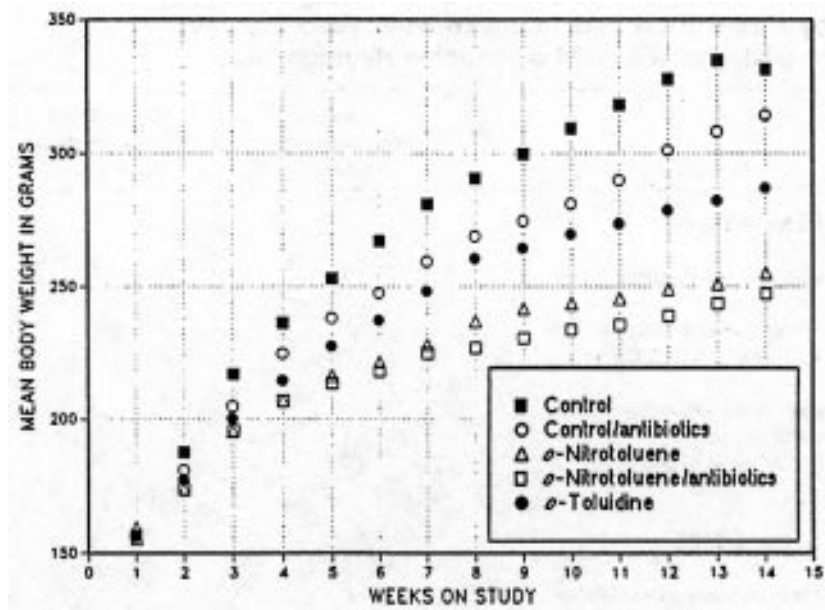


FIGURE 2 Body Weights of Male F344/N Rats Exposed to *o*-Nitrotoluene or *o*-Toluidine Hydrochloride in Feed for 13 Weeks

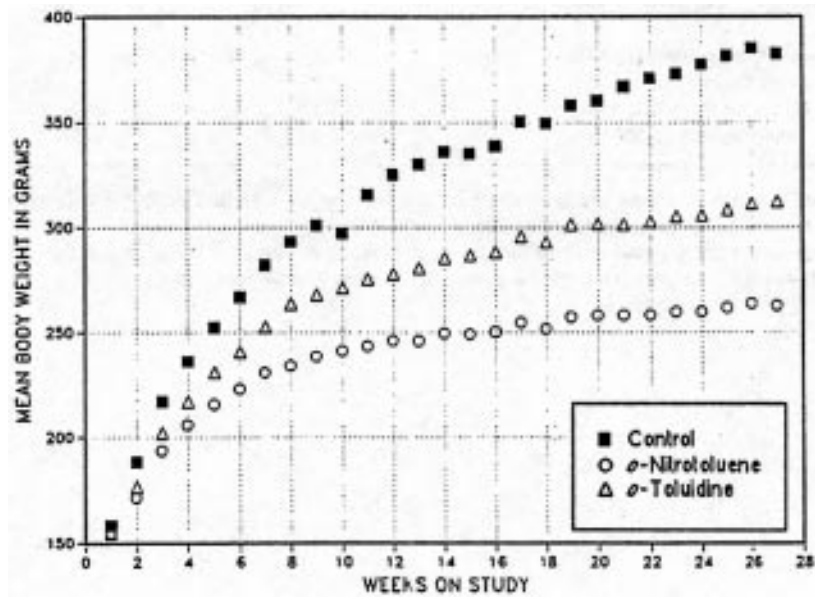
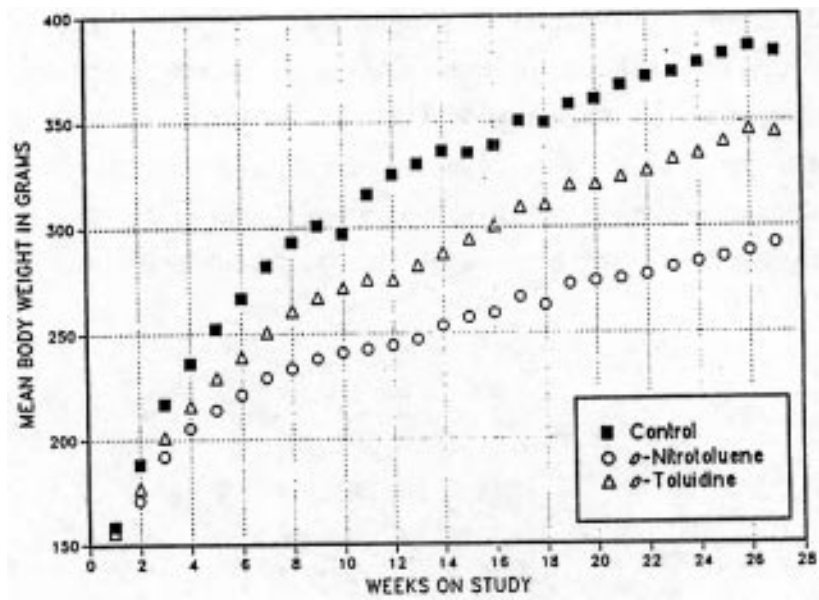


FIGURE 3 Body Weights of Male F344/N Rats Exposed to *o*-Nitrotoluene or *o*-Toluidine Hydrochloride in Feed for 26 Weeks

### Normal Gastrointestinal Flora



### Altered Gastrointestinal Flora

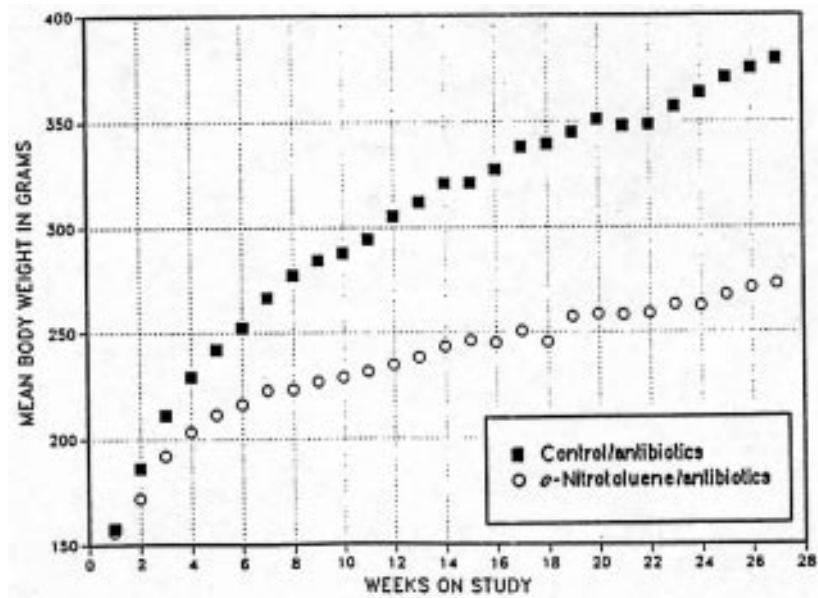


FIGURE 4 Body Weights of Male F344/N Rats Exposed to o-Nitrotoluene or o-Toluidine Hydrochloride in Feed for 13 Weeks and Allowed to Recover for 13 Weeks

Chemical-related effects were present in the testis and epididymis, liver, kidney, and spleen of rats exposed to *o*-nitrotoluene or *o*-toluidine hydrochloride and in the urinary bladder of rats exposed to *o*-toluidine hydrochloride. Because of the variation in the character, severity, and potential for progression or reversal of these lesions, the effects of *o*-nitrotoluene and *o*-toluidine hydrochloride for each target organ in the 13-week interim and stop-exposure groups (normal and altered gastrointestinal flora) and 26-week continuous-exposure group are described separately. Organ weight changes presented in Table 4 are discussed with the chemical-related histopathology findings for each organ.

**TABLE 4 Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride<sup>1</sup>**

	Normal Gastrointestinal Flora			Altered Gastrointestinal Flora <sup>2</sup>	
	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidine (5,000 ppm)	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)
<b>13-WEEK INTERIM</b>					
n	10	10	10	10	10
Necropsy body wt	345 ± 5	261 ± 2**	298 ± 4**	318 ± 5	247 ± 4**
Right kidney					
Absolute	1.130 ± 0.016	1.058 ± 0.023	1.043 ± 0.026*	1.034 ± 0.023	1.032 ± 0.022
Relative	3.27 ± 0.03	4.05 ± 0.06**	3.50 ± 0.05**	3.25 ± 0.03	4.19 ± 0.08**
Liver					
Absolute	12.056 ± 0.235	13.844 ± 0.316**	12.671 ± 0.206	10.140 ± 0.237	13.201 ± 0.400**
Relative	34.91 ± 0.31	53.08 ± 1.07**	42.57 ± 0.36**	31.86 ± 0.36	53.53 ± 1.55**
Spleen					
Absolute	0.730 ± 0.010	0.789 ± 0.021	2.212 ± 0.056**	0.648 ± 0.010	0.729 ± 0.015**
Relative	2.12 ± 0.03	3.02 ± 0.06**	7.43 ± 0.16**	2.04 ± 0.02	2.96 ± 0.08**
Right testis					
Absolute	1.593 ± 0.027	1.186 ± 0.028**	1.508 ± 0.020*	1.536 ± 0.030	1.114 ± 0.025**
Relative	4.61 ± 0.03	4.55 ± 0.08	5.07 ± 0.07**	4.83 ± 0.07	4.51 ± 0.08*
Epididymis					
Absolute	0.477 ± 0.006	0.331 ± 0.013**	0.420 ± 0.009**	0.469 ± 0.011	0.321 ± 0.007**
Relative	1.38 ± 0.02	1.27 ± 0.04*	1.41 ± 0.03	1.48 ± 0.05	1.30 ± 0.03**
<b>STOP-EXPOSURE<sup>3</sup></b>					
n	10	10	10	10	10
Necropsy body wt	389 ± 5	304 ± 6**	351 ± 9**	388 ± 5	281 ± 3**
Right kidney					
Absolute	1.379 ± 0.036	1.326 ± 0.027	1.255 ± 0.047*	1.274 ± 0.029	1.371 ± 0.022*
Relative	3.55 ± 0.08	4.36 ± 0.04**	3.56 ± 0.05	3.29 ± 0.07	4.88 ± 0.07**
Liver					
Absolute	14.405 ± 0.257	18.186 ± 0.301**	13.910 ± 0.532	13.929 ± 0.390	19.089 ± 0.416**
Relative	37.09 ± 0.69	59.89 ± 1.08**	39.58 ± 1.13	35.88 ± 0.75	67.98 ± 1.59**
Spleen					
Absolute	0.806 ± 0.017	0.818 ± 0.017	1.130 ± 0.048**	0.739 ± 0.020	0.748 ± 0.012
Relative	2.08 ± 0.05	2.70 ± 0.06**	3.23 ± 0.16**	1.90 ± 0.04	2.66 ± 0.05**
Right testis					
Absolute	1.615 ± 0.027	0.893 ± 0.077**	1.496 ± 0.025	1.585 ± 0.017	0.821 ± 0.090**
Relative	4.16 ± 0.06	2.93 ± 0.25**	4.28 ± 0.12	4.09 ± 0.07	2.91 ± 0.31**
Epididymis					
Absolute	0.484 ± 0.006	0.288 ± 0.018** <sup>4</sup>	0.432 ± 0.014	0.469 ± 0.008	0.254 ± 0.020**
Relative	1.25 ± 0.01	0.95 ± 0.06** <sup>4</sup>	1.23 ± 0.02	1.21 ± 0.02	0.90 ± 0.07**

**TABLE 4 Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride (continued)**

	Normal Gastrointestinal Flora		
	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidine (5,000 ppm)
<b>26-WEEK CONTINUOUS-EXPOSURE</b>			
n	10	10	10
Necropsy body wt	389 ± 5	269 ± 4**	316 ± 3**
Right kidney			
Absolute	1.379 ± 0.036	1.304 ± 0.029	1.257 ± 0.024*
Relative	3.55 ± 0.08	4.86 ± 0.11**	3.99 ± 0.09**
Liver			
Absolute	14.405 ± 0.257	20.054 ± 0.385**	14.426 ± 0.315
Relative	37.09 ± 0.69	74.68 ± 1.21**	45.71 ± 0.88**
Spleen			
Absolute	0.806 ± 0.017	0.877 ± 0.026	2.845 ± 0.097**
Relative	2.08 ± 0.05	3.27 ± 0.10**	9.01 ± 0.26**
Right testis			
Absolute	1.615 ± 0.027	0.740 ± 0.055**	1.528 ± 0.020
Relative	4.16 ± 0.06	2.76 ± 0.21**	4.84 ± 0.05**
Epididymis			
Absolute	0.484 ± 0.006	0.244 ± 0.017**	0.435 ± 0.007**
Relative	1.25 ± 0.01	0.91 ± 0.06**	1.38 ± 0.02

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

<sup>2</sup> Rats received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 14 consecutive weeks.

<sup>3</sup> Rats were exposed to o-nitrotoluene or o-toluidine hydrochloride in feed for 13 weeks and allowed to recover for 13 weeks.

<sup>4</sup> n=9.

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

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



## TESTIS AND EPIDIDYMIS

*o*-Nitrotoluene The absolute and relative weights of the right testis and epididymis of rats from all exposed groups at 13 and 26 weeks (normal and altered gastrointestinal flora) were generally significantly less than those of controls. At 26 weeks, the testes of all rats in the stop- and continuous-exposure groups were grossly reduced in size relative to those of the controls. The right testis and epididymis weights of rats in the stop-exposure groups (normal and altered gastrointestinal flora) continued to decrease during the recovery period and were similar to those of rats treated with continuous exposure (Table 4). Lower testis weights and grossly smaller testes than those of controls were a reflection of mild testicular degeneration seen microscopically (Table 5). Degeneration consisted of a loss of spermatogenic epithelium and a reduction in the diameter of the seminiferous tubules. In some tubules, there was an almost complete absence of germinal epithelium and the remaining cells were primarily Sertoli cells; dense aggregates of mineral deposition were seen in some tubules. The distribution of degeneration was multifocal to diffuse in both testes. In the 26-week continuous-exposure group, the average severity of degeneration was greater than that seen in the 13-week interim group. In the stop-exposure group, there was no morphological evidence for resolution of degeneration and the average severity of this lesion was slightly greater than that present in the 13-week interim group. Interstitial cell hyperplasia was present in a few rats from the stop- and continuous-exposure groups at 26 weeks. This consisted of a minimal focal increase in the amount of interstitial cells between degenerative seminiferous tubules.

Mesothelial cell hyperplasia and/or mesothelioma occurred on the mesothelial surface of the tunica vaginalis on the testis and epididymis of rats in the stop- and continuous-exposure groups. Mesothelioma occurred in 2 of 20 rats from the 13-week interim group (altered gastrointestinal flora). By 26 weeks, an increased incidence of mesothelioma occurred in rats from the continuous-exposure group (7/20) and the stop-exposure groups with normal (5/20) or altered (8/20) gastrointestinal flora. Focal hyperplasia of the epididymal mesothelium was also seen in five rats from these three groups examined at 26 weeks (Table 5). Hyperplasia was a focal or multifocal proliferation of one to two layers of basophilic-staining cuboidal cells on the mesothelial surface of the epididymis. Mesotheliomas had broad-based attachment to the tunica vaginalis and consisted of cuboidal or polyhedral-shaped cells forming solid areas with papillary or villous projections that extended into the peritoneal space. A dense, fibrous core was present in most

neoplasms, and mitotic figures were common features of these neoplasms (Plates 1, 2, 3, and 4). There was no indication that these neoplasms had metastasized to distant sites.

*o-Toluidine Hydrochloride:* There were no gross lesions in the 13-week interim and 26-week stop- and continuous-exposure groups. In *allo*-toluidine hydrochloride groups, absolute testis and epididymis weights were slightly less than those of the controls and were considered secondary to body-weight reduction (Table 4). Degeneration of seminiferous tubules was a unilateral testicular lesion present in 5% to 10% of rats from each exposed group (Table 5). At 26 weeks, 2 of 20 rats had epididymal mesothelioma (stop-exposure group) and one rat had epididymal mesothelial cell hyperplasia (continuous-exposure group). The morphology of these lesions was the same as that in rats exposed to *o*-nitrotoluene.

**TABLE 5 Selected Lesions of the Testis and Epididymis in Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride<sup>1</sup>**

	Normal Gastrointestinal Flora			Altered Gastrointestinal Flora <sup>2</sup>	
	Control <sup>3</sup> (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidi <sup>ne</sup> (5,000 ppm)	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)
<b>13-WEEK INTERIM</b>					
Testis <sup>4</sup>	10	20	20	10	20
Degeneration <sup>5</sup>	0	20 ** (2.0)	1 (1.0)	0	20 ** (1.6)
Epididymis	10	20	20	10	20
Mesothelial hyperplasia	0	0	0	0	2 (1.0)
Mesothelioma	0	0	0	0	2
<b>STOP-EXPOSURE<sup>6</sup></b>					
Testis	10	20	20	10	20
Degeneration	0	20 ** (2.4)	2 (4.0)	0	20 ** (3.1)
Interstitial cell, hyperplasia	0	11 ** (1.0)	0	0	7 * (1.0)
Tunic, mesothelioma	0	2	0	0	4
Epididymis	10	20	20	10	20
Mesothelial hyperplasia	0	2 (1.0)	0	0	1 (2.0)
Mesothelioma	0	4	2	0	8 *
Testis or epididymis	10	20	20	10	20
Mesothelioma	0	5	2	0	8 *
<b>26-WEEK CONTINUOUS-EXPOSURE</b>					
Testis	10	20	20		
Degeneration	0	20 ** (2.9)	2 (2.5)		
Interstitial cell, hyperplasia	0	3 (1.0)	0		
Bilateral, hemangioma	0	1	0		
Tunic, mesothelioma	0	2	0		
Epididymis	10	20	20		
Mesothelial hyperplasia	0	2 (1.0)	1 (1.0)		
Mesothelioma	0	7 *	0		

<sup>1</sup> For nonneoplastic lesions, average severity (in parentheses) is based on the number of animals with lesions: 1=minimal, 2=mild, 3=moderate, and 4=marked.

<sup>2</sup> Rats received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 14 consecutive weeks.

<sup>3</sup> For statistical analysis, control rats in the normal and altered gastrointestinal flora groups were pooled.

<sup>4</sup> Number of animals with organ examined microscopically.

<sup>5</sup> Number of animals with lesion.

<sup>6</sup> Rats were exposed to o-nitrotoluene or o-toluidine hydrochloride in feed for 13 weeks and allowed to recover for 13 weeks.

\* Significantly different ( $P \leq 0.05$ ) from the control group by Fisher's exact test.

\*\* Significantly different ( $P \leq 0.01$ ) from the control group by Fisher's exact test.

## LIVER

*o*-Nitrotoluene: At the 13-week interim evaluation, rats in both *o*-nitrotoluene-exposed groups (normal and altered gastrointestinal flora) had grossly enlarged livers and significantly greater liver weights than the controls (Table 4). At 26 weeks, the increased size of the liver was apparent grossly, and liver weights had increased with continuous exposure. The liver weights in the two stop-exposure groups (normal and altered gastrointestinal flora) also continued to increase during the 13-week recovery period, and the increases were similar to those seen with continuous exposure at the end of 26 weeks.

Oval cell hyperplasia and cytoplasmic vacuolization were present in the livers of all exposed rats (Table 6). Oval cell hyperplasia consisted of a minimal increase in the number of cells with a scant amount of visible cytoplasm and oval-shaped nuclei. Proliferation of these oval cells was most prominent in the periportal region of hepatic lobules. This change was of minimal severity, was present in all exposed groups (normal and altered gastrointestinal flora), and did not increase in severity in the continuous-exposure group or regress in the stop-exposure group. Cytoplasmic vacuolization consisted of hepatocytes containing one to several vacuoles of varying sizes. This was generally a diffuse change but was more prominent in the centrilobular to midzonal area of the hepatic lobule. Vacuoles stained positive for the presence of lipid. The severity of this change was minimal to mild at the 13-week interim (normal and altered gastrointestinal flora groups) and increased to a moderate severity at 26 weeks. In the stop-exposure groups, this lesion did not regress and was similar in severity to that seen in the continuous-exposure group (Table 6) (Plates 5 and 6).

Foci of cellular alteration based on placental glutathione *S*-transferase (PGST)-positive staining of hepatocytes occurred with *o*-nitrotoluene exposure by 13 weeks and increased in number and size (as reflected by volume fraction) with continuous exposure for 26 weeks (Table 7). In the stop-exposure groups (normal and altered gastrointestinal flora) at the end of the recovery period, the number of foci was less than that in the corresponding 13-week interim groups but remained significantly greater than that in controls. The size of the foci continued to increase in *o*-nitrotoluene-exposed rats in both normal and altered gastrointestinal flora groups during the recovery period. Areas representing PGST-positive foci generally could not be recognized in the routine hematoxylin/eosin-stained sections. Although a few PGST-positive foci corresponded to

areas of cytoplasmic vacuolization in hepatocytes, most of the cells affected by vacuolization did not stain positive for PGST.

At 26 weeks, three focal lesions were identified by gross examination of the liver at necropsy. Two rats from the stop-exposure group (normal gastrointestinal flora) had a mass (1 centimeter in the greatest dimension) located in the right posterior lobe. A third rat from the continuous-exposure group had a pale focal area (4 mm by 4 mm) in the caudal lobe. Microscopically, these expansile masses were identified as cholangiocarcinomas that consisted of large, irregularly shaped bile ducts separated by dense, fibrous connective tissue. The ducts were lined by pleomorphic columnar to low cuboidal, hyperchromatic epithelium; many of these ductular lining cells stained positive for proliferating cell nuclear antigen. The lumen of some of these atypical ducts contained a mucous exudate. Cholangiocarcinomas were not seen in the altered gastrointestinal flora groups (Plates 7 and 8).

*o-Toluidine Hydrochloride:* There were no gross findings and no treatment-related effects on liver weights. Accumulation of hemosiderin pigment in Kupffer cell cytoplasm was present in all *o*-toluidine hydrochloride groups at 13 and 26 weeks and was a minimal, reversible change (Table 6). At each time point, the incidence of this lesion was significantly greater than that in the controls.

At 26 weeks, the number of PGST-positive foci was significantly greater in the *o*-toluidine hydrochloride exposure group than in controls. However, the number was significantly lower than that seen with 26 weeks of exposure to *o*-nitrotoluene (Table 7), and the volume fraction was significantly less than that seen with *o*-nitrotoluene.

**TABLE 6 Selected Lesions of the Liver in Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride<sup>1</sup>**

	Normal Gastrointestinal Flora			Altered Gastrointestinal Flora <sup>2</sup>	
	Control <sup>3</sup> (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidi <sup>ne</sup> (5,000 ppm)	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)
<b>13-WEEK INTERIM</b>					
Liver <sup>4</sup>	10	20	20	10	20
Pigmentation, hemosiderin <sup>5</sup>	0	0	20 ** (1.0)	0	0
Vacuolization, cytoplasmic	0	20 ** (1.8)	1 (1.0)	0	20 ** (1.5)
Bile duct, hyperplasia	2 (1.0)	0	1 (1.0)	0	0
Oval cell, hyperplasia	0	20 ** (1.0)	0	0	20 ** (1.0)
<b>STOP-EXPOSURE<sup>6</sup></b>					
Liver	10	20	20	10	20
Pigmentation, hemosiderin	0	0	11 ** (1.0)	0	0
Vacuolization, cytoplasmic	0	20 ** (2.9)	1 (1.0)	0	20 ** (3.0)
Bile duct, hyperplasia	4 (1.0)	0 **	5 (1.0)	4 (1.0)	0 **
Oval cell, hyperplasia	0	20 ** (1.0)	0	0	20 ** (1.0)
Cholangiocarcinoma	0	2	0	0	0
<b>26-WEEK CONTINUOUS-EXPOSURE</b>					
Liver	10	20	20		
Pigmentation, hemosiderin	0	0	20 ** (1.1)		
Vacuolization, cytoplasmic	0	20 ** (3.0)	0		
Bile duct, hyperplasia	4 (1.0)	0 **	10 (1.1)		
Oval cell, hyperplasia	0	20 ** (1.0)	0		
Cholangiocarcinoma	0	1	0		

<sup>1</sup> For nonneoplastic lesions, average severity (in parentheses) is based on the number of animals with lesions: 1=minimal, 2=mild, 3=moderate, and 4=marked.

<sup>2</sup> Rats received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 14 consecutive weeks.

<sup>3</sup> For statistical analysis, control rats in the normal and altered gastrointestinal flora groups were pooled.

<sup>4</sup> Number of animals with liver examined microscopically.

<sup>5</sup> Number of animals with lesion.

<sup>6</sup> Rats were exposed to o-nitrotoluene or o-toluidine hydrochloride in feed for 13 weeks and allowed to recover for 13 weeks.

\*\* Significantly different ( $P \leq 0.01$ ) from the control group by Fisher's exact test.

**TABLE 7 Placental Glutathione S-Transferase-Positive Foci in the Liver of Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride<sup>1</sup>**

	Normal Gastrointestinal Flora			Altered Gastrointestinal Flora <sup>2</sup>	
	Control <sup>3</sup> (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidi <sup>ne</sup> (5,000 ppm)	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)
<b>13-WEEK INTERIM</b>					
Foci/cm <sup>3</sup> tissue	6 ± 6	445 ± 78**	— <sup>4</sup>	0 ± 0	609 ± 176**
Mean volume (mm <sup>3</sup> )	0.002 ± 0.000	0.049 ± 0.006**	—	0.000 ± 0.000	0.034 ± 0.013**
<b>STOP-EXPOSURE<sup>5</sup></b>					
Foci/cm <sup>3</sup> tissue	17 ± 2	181 ± 43**▲▲	—	10 ± 10	458 ± 148** <sup>6</sup>
Mean volume (mm <sup>3</sup> )	0.000 ± 0.000	0.085 ± 0.023**	—	0.000 ± 0.000	0.115 ± 0.031**▲
<b>26-WEEK CONTINUOUS-EXPOSURE</b>					
Foci/cm <sup>3</sup> tissue	17 ± 2	961 ± 91**	145 ± 61**	—	—
Mean volume (mm <sup>3</sup> )	0.000 ± 0.000	0.446 ± 0.101**	0.017 ± 0.004**	—	—

<sup>1</sup> Ten rats were evaluated from each group at each time point. Data are given as mean ± standard error.

<sup>2</sup> Rats received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 14 consecutive weeks.

<sup>3</sup> For statistical analysis, control rats in the normal and altered gastrointestinal flora groups were pooled.

<sup>4</sup> Not examined or not applicable.

<sup>5</sup> Rats were exposed to o-nitrotoluene or o-toluidine hydrochloride in feed for 13 weeks and allowed to recover for 13 weeks.

<sup>6</sup> n=9.

\*\* Significantly different ( $P \leq 0.01$ ) from the normal or altered gastrointestinal flora control groups by Wilcoxon's rank sum test.

▲ Significantly different ( $P \leq 0.05$ ) from exposed rats evaluated at 13 weeks by Wilcoxon's rank sum test.

▲▲ Significantly different ( $P \leq 0.01$ ) from exposed rats evaluated at 13 weeks by Wilcoxon's rank sum test.

## KIDNEY AND URINARY BLADDER

*o*-Nitrotoluene: There were no treatment-related gross lesions or consistent effects on kidney weights in the *o*-nitrotoluene-exposed groups (Table 4). Slight increases in relative kidney weights were attributed to decreased mean bodyweights in exposed groups. At 13 weeks, there was an accumulation of hyaline droplets in the renal tubule of exposed rats in the normal or altered gastrointestinal flora groups. The incidence, but not the severity, of renal tubule regeneration was increased only in rats exposed to *o*-nitrotoluene in the normal flora group in the 13-week interim study (Table 8). In the stop-exposure groups (normal and altered gastrointestinal flora), the accumulation of hyaline droplets completely regressed, but both stop-exposure normal and altered gastrointestinal flora groups exposed to *o*-nitrotoluene had a similar slight increase in the incidence and severity of lesions including foci of renal tubule epithelial regeneration and dilated renal tubules containing eosinophilic-staining protein casts. With continued exposure to *o*-nitrotoluene for 26 weeks, the hyaline droplet accumulation was still present but was not increased in severity. The incidence and severity of other renal lesions typically associated with nephropathy (regeneration and protein casts) were greater than those in the controls but did not differ from those seen in the stop-exposure groups.

No gross or microscopic effects were identified in the urinary bladder of rats from any *o*-nitrotoluene-exposure group.

*o*-Toluidine Hydrochloride: There were no gross kidney lesions in any *o*-toluidine hydrochloride-exposed group; kidney weights of exposed rats were lower than those of the controls, but this difference was considered secondary to overall reductions in body weights of exposed rats (Table 4). Microscopic lesions in exposed rats were limited to increased hemosiderin pigment accumulation in the cytoplasm of renal tubule epithelium (Table 8). Hemosiderin accumulation was of mild severity in the 13-week interim and 26-week continuous-exposure groups. The severity of this lesion was slightly reduced in the stop-exposure group.

There were no gross lesions in the urinary bladders of rats from any *o*-toluidine hydrochloride-exposed group. Mild diffuse hyperplasia of the uroepithelium was present in *o*-toluidine hydrochloride-exposed rats in the 13-week interim and 26-week continuous-exposure group (Table 8). The average severity of this lesion did not increase with longer exposure. Hyperplasia was not observed in rats from the stop-exposure groups, indicating that the lesion was reversible (Plates 9 and 10).



**TABLE 8 Selected Lesions of the Kidney and Urinary Bladder in Male F344/N Rats in the 26-Week Feed Studies of *o*-Nitrotoluene and *o*-Toluidine Hydrochloride<sup>1</sup>**

	Normal Gastrointestinal Flora			Altered Gastrointestinal Flora <sup>2</sup>	
	Control <sup>3</sup> (0 ppm)	<i>o</i> -Nitrotoluene (5,000 ppm)	<i>o</i> -Toluidi <sup>ne</sup> (5,000 ppm)	Control (0 ppm)	<i>o</i> -Nitrotoluene (5,000 ppm)
<b>13-WEEK INTERIM</b>					
Kidney <sup>4</sup>	10	20	20	10	20
Hyaline droplet accumulation <sup>5</sup>	0	20 ** (2.0)	0	0	20 ** (1.1)
Pigmentation	0	0	20 ** (2.0)	0	0
Regeneration	3 (1.0)	16 * (1.0)	7 (1.0)	3 (1.0)	2 (1.0)
Urinary bladder	10	20		10	20
Inflammation	0	0	2 (2.0)	0	0
Transitional epithelium, hyperplasia	0	0	10 ** (2.2)	0	0
<b>STOP-EXPOSURE<sup>6</sup></b>					
Kidney	10	20	20	10	20
Hyaline droplet accumulation	0	0	0	0	0
Casts, protein (1.1)	3 (1.0)	20 ** (1.2)	6 (1.0)	0	20 **
Pigmentation	0	0	20 ** (1.1)	0	0
Regeneration (1.8)	7 (1.0)	19 (1.8)	10 (1.0)	6 (1.0)	20 **
<b>26-WEEK CONTINUOUS-EXPOSURE</b>					
Kidney	10	20	20		
Hyaline droplet accumulation	0	20 ** (1.6)	0		
Casts, protein	3 (1.0)	20 ** (1.0)	1 (1.0)		
Pigmentation	0	0	20 ** (2.2)		
Regeneration	7 (1.0)	19 (1.1)	5 (1.0)		
Urinary bladder	10	20	18		
Transitional epithelium, hyperplasia	0	0	17 ** (2.0)		

<sup>1</sup> For nonneoplastic lesions, average severity (in parentheses) is based on the number of animals with lesions: 1=minimal, 2=mild, 3=moderate, and 4=marked.  
<sup>2</sup> Rats received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 14 consecutive weeks.  
<sup>3</sup> For statistical analysis, control rats in the normal and altered gastrointestinal flora groups were pooled.  
<sup>4</sup> Number of animals with organ examined microscopically.  
<sup>5</sup> Number of animals with lesion.  
<sup>6</sup> Rats were exposed to *o*-nitrotoluene or *o*-toluidine hydrochloride in feed for 13 weeks and allowed to recover for 13 weeks.  
\* Significantly different ( $P \leq 0.05$ ) from the control group by Fisher's exact test.  
\*\* Significantly different ( $P \leq 0.01$ ) from the control group by Fisher's exact test.

## SPLEEN

*o*-Nitrotoluene: In the 13-week interim groups (normal and altered gastrointestinal flora) exposed to *o*-nitrotoluene, absolute and relative spleen weights were generally slightly but significantly greater than those of the controls (Table 4). In the 26-week continuous- and stop-exposure groups, spleen weights were slightly greater than those of the controls; however, only the relative spleen weights were significantly greater than the control value.

There were no grossly visible changes in the spleen. Microscopic findings were limited to increased incidences and severity of hematopoiesis and hemosiderin accumulation in the splenic red pulp of rats in the 13-week interim and 26-week continuous-exposure groups relative to the controls (Table 9). Minimal focal areas of fibrosis in the spleen capsule were also present. After 26 weeks of continuous *o*-nitrotoluene exposure, hematopoiesis and hemosiderin accumulation were slightly more severe than at 13 weeks. There was no evidence of a reduction in the severity or incidence of the spleen effects in the stop-exposure group.

*o*-Toluidine Hydrochloride: At the 13-week interim evaluation, the spleens of *o*-toluidine hydrochloride-exposed rats were grossly enlarged, and the absolute and relative spleen weights were significantly greater than those of the controls (Table 4). After 26 weeks of continuous *o*-toluidine hydrochloride exposure, the spleens were also enlarged, but the spleen weights were only slightly greater than those seen at the 13-week interim evaluation. In the *o*-toluidine hydrochloride stop-exposure groups, the spleens did not appear grossly enlarged, but the absolute and relative spleen weights were significantly greater than those of the controls (Table 4).

The spleens of all *o*-toluidine hydrochloride-exposed rats were distinguished grossly from the spleens of the control group by the presence of white, granular plaques on the capsular surface. Small (2 mm diameter) fluid-filled cysts were attached to the capsule in five rats. Focal, thickened areas of capsular fibrosis seen microscopically corresponded to the irregular plaques observed grossly on the capsular surface (Table 9). Within the areas of fibrosis were infiltrates of lymphocytes and hematopoietic cells; the more extensively dilated lymphatic vessels in the capsule (capsular angiectasis) corresponded to the cysts observed grossly. Microscopic findings in the continuous- and stop-exposure groups included increases in hematopoietic cell proliferation and hemosiderin concentration above the background amounts seen in the spleens of control rats. The amount of blood in sinusoids (congestion) of the red pulp in exposed rats was also greater than that in the controls (Table 9) (Plates 11 and 12).

With the exception of a slight increase in the severity of capsular fibrosis, the spleen changes that occurred with 26 weeks of *o*-toluidine hydrochloride exposure were similar to those seen at 3 weeks. In the stop-exposure groups, a slight recovery of the exposure-related spleen lesions was suggested by the reduction in the severity of congestion and by the lower incidence of hematopoiesis.

**TABLE 9 Selected Lesions of the Spleen in Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride<sup>1</sup>**

	Normal Gastrointestinal Flora			Altered Gastrointestinal Flora <sup>2</sup>	
	Control <sup>3</sup> (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidi <sup>ne</sup> (5,000 ppm)	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)
<b>13-WEEK INTERIM</b>					
Spleen <sup>4</sup>	10	20	20	10	20
Congestion <sup>5</sup>	0	0	20 ** (2.0)	0	0
Hematopoietic cell proliferation	2 (1.0)	20 ** (1.4)	20 ** (2.0)	0	20 ** (1.8)
Pigmentation, hemosiderin	0	20 ** (1.9)	20 ** (1.6)	0	20 ** (1.6)
Thrombosis	0	0	3 (1.0)	0	0
Capsule, fibrosis	0	1 (1.0)	20 ** (1.9)	0	1 (1.0)
<b>STOP-EXPOSURE<sup>6</sup></b>					
Spleen	10	20	20	10	19
Congestion	0	0	20 ** (1.0)	0	0
Hematopoietic cell proliferation	3 (1.0)	18 ** (1.3)	1 (1.0)	1 (1.0)	19 ** (1.8)
Pigmentation, hemosiderin	3 (1.0)	17 ** (1.4)	18 ** (1.0)	5 (1.0)	19 ** (1.4)
Capsule, fibrosis	0	7 * (1.0)	20 ** (2.2)	0	1 (1.0)
Capsule, lymphatic, angiectasis	0	0	15 ** (1.7)	0	0
<b>26-WEEK CONTINUOUS-EXPOSURE</b>					
Spleen	10	20	20		
Congestion	0	0	20 ** (2.0)		
Hematopoietic cell proliferation	3 (1.0)	20 ** (1.8)	20 ** (1.9)		
Pigmentation, hemosiderin	3 (1.0)	20 ** (2.5)	20 ** (1.4)		
Thrombosis	0	0	2 (1.0)		
Capsule, fibrosis	0	3 (1.0)	20 ** (2.7)		
Capsule, lymphatic, angiectasis	0	0	6 (1.3)		

<sup>1</sup> For nonneoplastic lesions, average severity (in parentheses) is based on the number of animals with lesions: 1=minimal, 2=mild, 3=moderate, and 4=marked.

<sup>2</sup> Rats received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 14 consecutive weeks.

<sup>3</sup> For statistical analysis, control rats in the normal and altered gastrointestinal flora groups were pooled.

<sup>4</sup> Number of animals with spleen examined microscopically.

<sup>5</sup> Number of animals with lesion.

<sup>6</sup> Rats were exposed to o-nitrotoluene or o-toluidine hydrochloride in feed for 13 weeks and allowed to recover for 13 weeks.

\* Significantly different ( $P \leq 0.05$ , o-nitrotoluene;  $P=0.065$ , o-toluidine hydrochloride) from the control group by Fisher's exact test.

\*\* Significantly different ( $P \leq 0.01$ ) from the control group by Fisher's exact test.

The results from the microbial analysis of cecal samples obtained at necropsy from the 13-week interim groups are presented in Table 10. Daily administration of antibiotics by gavage over 14 weeks resulted in a significant (P=0.041) reduction in gastrointestinal flora counts, as established under aerobic incubation conditions. The slight reductions in gastrointestinal flora counts with *o*-nitrotoluene exposure in the normal gastrointestinal flora group or the combination effect of *o*-nitrotoluene with antibiotics in the altered gastrointestinal flora group were not statistically significant. However, this suggests that *o*-nitrotoluene in the feed may have contributed slightly to the inhibitory effect of antibiotic administration on bacterial growth in the intestine. The effect of antibiotics on cecal flora counts is similar to that seen in the pilot study (Appendix C) concluded after 7 days of antibiotic dosing.

**TABLE 10 Gastrointestinal Flora Counts from Selected Male F344/N Rats at the 13-Week Interim Evaluation in the 26-Week Studies of *o*-Nitrotoluene and *o*-Toluidine Hydrochloride<sup>1</sup>**

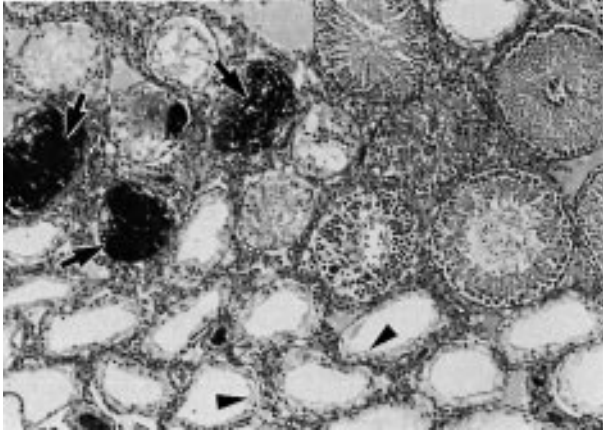
	<u>Gastrointestinal Flora Counts</u>	
	Individual	Group Average
<b>Normal Gastrointestinal Flora Groups</b>		
Control	9.0 x 10 <sup>2</sup>	2.6 x 10 <sup>5</sup>
5.5 x 10 <sup>4</sup>		
1.7 x 10 <sup>5</sup>		
9.5 x 10 <sup>5</sup>		
1.3 x 10 <sup>5</sup>		
<i>o</i> -Nitrotoluene (5,000 ppm)	1.3 x 10 <sup>4</sup> 4.9 x 10 <sup>4</sup>	6.8 x 10 <sup>4</sup>
1.0 x 10 <sup>5</sup>		
3.6 x 10 <sup>4</sup>		
1.4 x 10 <sup>5</sup>		
<b>Altered Gastrointestinal Flora Groups<sup>2</sup></b>		
Control	1.5 x 10 <sup>3</sup>	2.9 x 10 <sup>4</sup> *
7.0 x 10 <sup>2</sup>		
1.3 x 10 <sup>4</sup>		
1.2 x 10 <sup>3</sup>		
1.3 x 10 <sup>5</sup>		
<i>o</i> -Nitrotoluene (5,000 ppm)	1.5 x 10 <sup>4</sup> 7.7 x 10 <sup>3</sup>	6.2 x 10 <sup>3</sup>
7.8 x 10 <sup>3</sup>		
3.3 x 10 <sup>2</sup>		
1.0 x 10 <sup>2</sup>		

<sup>1</sup> Cecal samples were collected from five randomly selected rats per group. Counts are given in colony-forming units per gram (CFU/g).

<sup>2</sup> Rats received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 14 consecutive weeks.

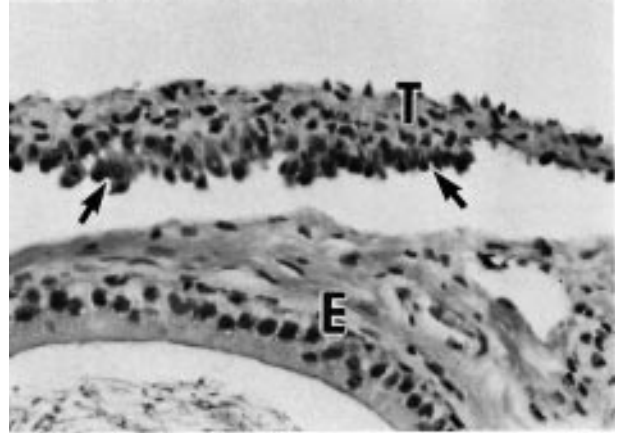
\* Significantly different (P=0.041) from normal flora control based on a two-way analysis of variance on log-transformed flora counts.





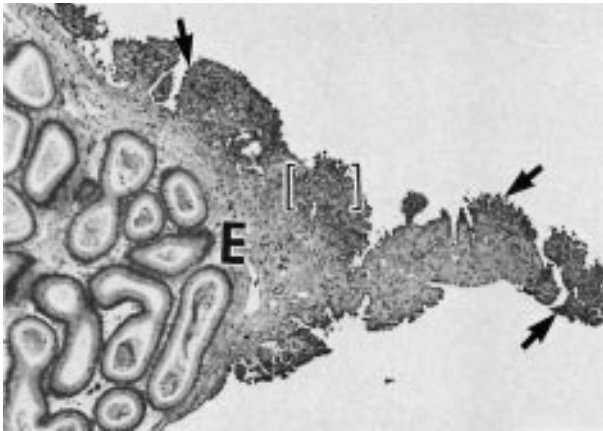
**PLATE 1**

Testis of a male rat administered 5,000 ppm *o*-nitrotoluene in feed for 13 weeks. Seminiferous tubules show focal mineralization (arrows) and degeneration (arrowheads) with the loss of germinal epithelium. Less severely affected tubules are seen in the upper right portion of the plate. H&E, 60 $\times$ .



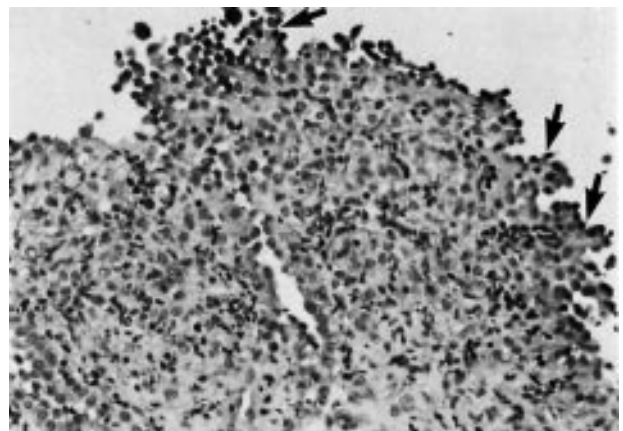
**PLATE 2**

Portion of the epididymis (E) and tunica vaginalis (T) of a male rat administered 5,000 ppm *o*-nitrotoluene in feed for 13 weeks. Note the presence of focal hyperplasia (arrows) of the mesothelium. H&E, 300 $\times$ .



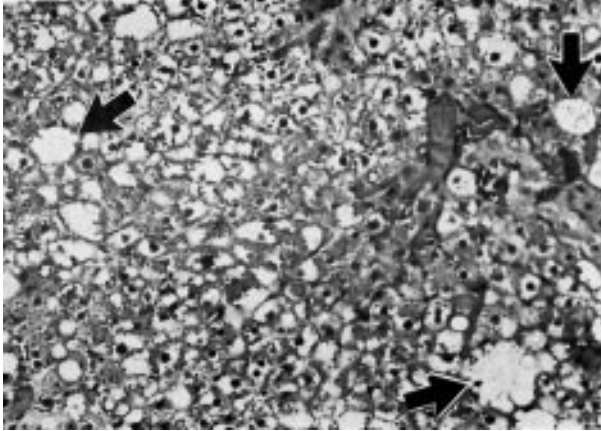
**PLATE 3**

Epididymis of a male rat administered 5,000 ppm *o*-nitrotoluene for 13 weeks and evaluated at 26 weeks. A mesothelioma (arrows) is attached to the surface of the epididymis (E). Detail of the area in brackets is shown in Plate 4. H&E, 40 $\times$ .



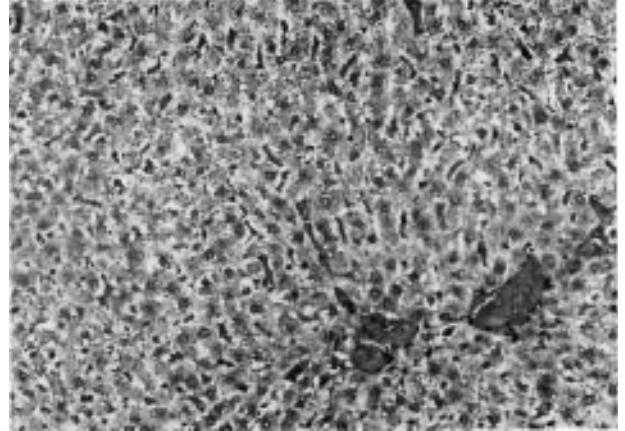
**PLATE 4**

Higher magnification of bracketed area of the epididymis in Plate 3. Two blunt, papillary masses extend from the surface of the epididymis. Polyhedral-shaped neoplastic cells form an irregular villous pattern (arrows) on the surface of the neoplasm. H&E, 185 $\times$ .



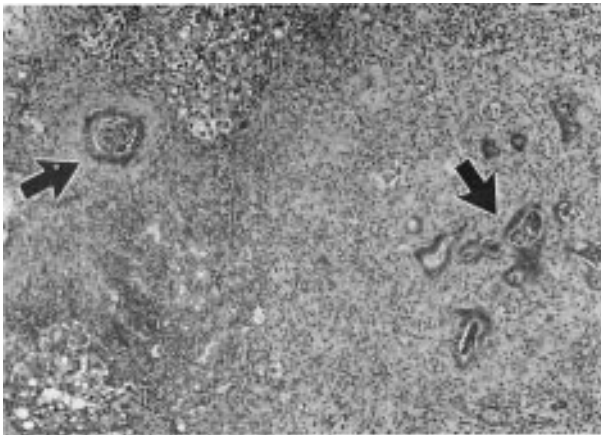
**PLATE 5**

Liver of a male rat administered 5,000 ppm *o*-nitrotoluene in feed for 26 weeks. Prominent cytoplasmic vacuolization (arrows) can be seen in enlarged hepatocytes. H&E, 150 $\times$ .



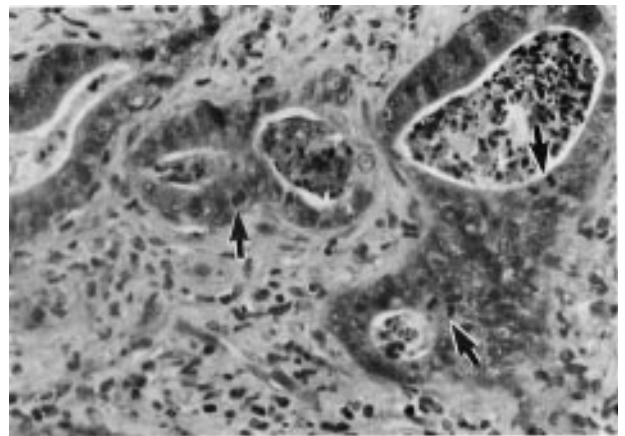
**PLATE 6**

Liver of a control male rat for comparison with Plate 5. H&E, 150 $\times$ .



**PLATE 7**

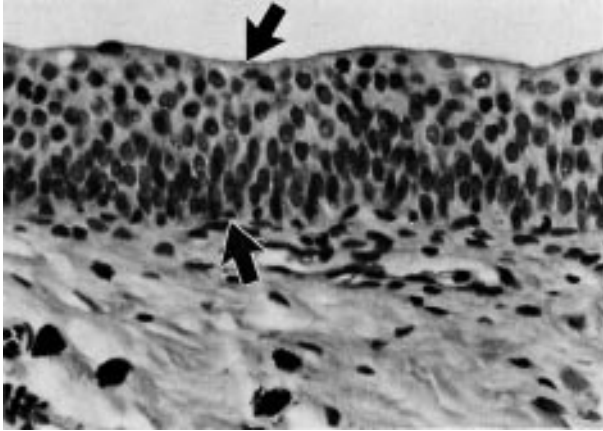
Cholangiocarcinoma in the liver of a male rat administered 5,000 ppm *o*-nitrotoluene in feed for 13 weeks and evaluated at 26 weeks. An expansile mass of fibrous tissue and atypical biliary ducts (arrows) have displaced hepatic lobules. H&E, 60 $\times$ .



**PLATE 8**

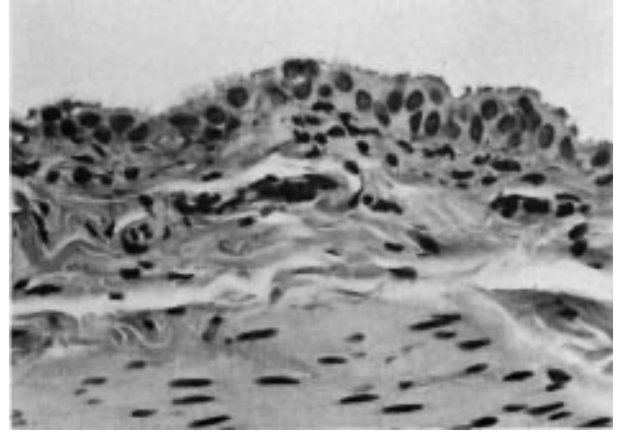
Higher magnification of the biliary epithelium in Plate 7. Ductal structures containing numerous mitotic figures are surrounded by a fibrous stroma (arrows). H&E, 300 $\times$ .





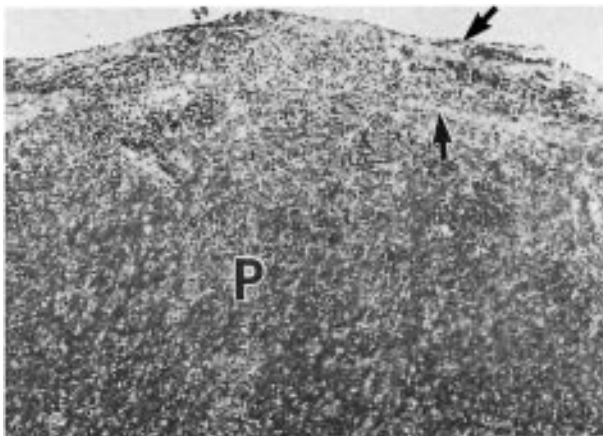
**PLATE 9**

Urinary bladder of a male rat administered 5,000 ppm *o*-toluidine hydrochloride in feed for 26 weeks. There is hyperplasia characterized by an increased thickness of the transitional epithelium (between arrows). H&E, 300 $\times$ .



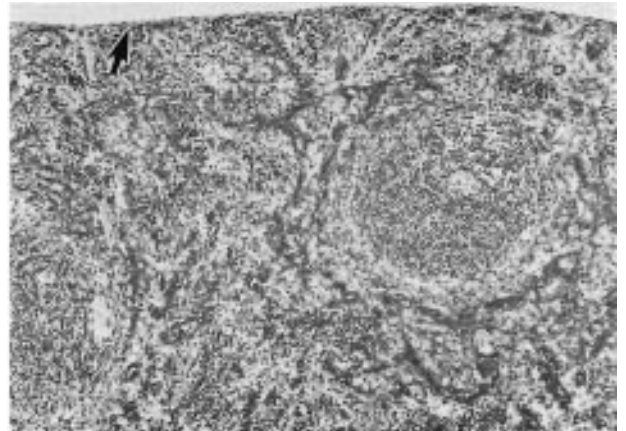
**PLATE 10**

Urinary bladder of a control male rat. Compare the thickness of the transitional epithelial layer to that in Plate 9. H&E, 300 $\times$ .



**PLATE 11**

Spleen of a male rat administered 5,000 ppm *o*-toluidine hydrochloride in feed for 26 weeks. The spleen capsule is thickened (arrows) and the red pulp shows increased congestion and hematopoiesis (P). H&E, 75 $\times$ .



**PLATE 12**

Spleen of a control male rat. Compare the spleen capsule thickness (arrow) and the red pulp with those in Plate 11. Note the distinction between the red and white pulp is more evident because of the less extensive congestion and hematopoiesis than that seen in Plate 11. H&E, 75 $\times$ .

## DISCUSSION

The 13- and 26-week studies of two structurally similar chemicals, *o*-nitrotoluene and *o*-toluidine hydrochloride, in F344/N rats demonstrated several differences in carcinogenic potency and in the site, severity, and potential for regression or progression of treatment-related toxic effects. Bacterial reduction of the nitro group to the amine by the gastrointestinal flora is considered to be an important step in metabolism of nitroaromatic chemicals including *o*-nitrotoluene (Doolittle *et al.*, 1983; Rickert *et al.*, 1986a). For this reason, groups of rats treated with antibiotics were included in these studies in an attempt to determine the effect of a reduced gastrointestinal flora on the carcinogenic potential of *o*-nitrotoluene, although this treatment regimen was subsequently demonstrated to be less effective than desired. Because of the numerous differences observed between *o*-nitrotoluene and *o*-toluidine hydrochloride with respect to their carcinogenicity and toxicity in rats, the effects of chemical exposure and altered intestinal flora are discussed separately.

### MESOTHELIOMA

Mesothelial cells form a single layer of epithelium on a delicate fibrous stroma that lines the peritoneal and pleural cavities. In the F344 rat, spontaneous and treatment-related proliferative lesions (hyperplasia and mesothelioma) of the peritoneal mesothelial cells are most commonly observed on the peritoneal surface lining the testis, epididymis, and scrotal sac (Hall, 1990). Although more malignant and invasive forms of mesothelioma may be disseminated throughout the abdominal cavity and metastasize to lymph nodes in the thorax, the early or smaller neoplasms are typically limited to the surface of the epididymis and testis. In a previous 2-year study of *o*-toluidine hydrochloride, mesothelioma occurred in 24% of male rats exposed to a dietary concentration of 3,000 ppm (NCI, 1979a). It could not be determined if mesothelioma occurred in shorter studies used to set doses for the 2-year study. *o*-Nitrotoluene also caused a small number of mesotheliomas in male rats in a 13-week study (NTP, 1992). Because of this common target-tissue effect and its structural similarity to *o*-nitrotoluene, groups of rats were exposed to approximately equimolar amounts of *o*-nitrotoluene or *o*-toluidine hydrochloride in this study to compare the relative potency of these chemicals with respect to the onset and development of proliferative lesions in the mesothelium. The studies reported here confirm a chemical-related increase in the incidence of mesothelioma in male rats after 13 or 26 weeks of exposure to *o*-nitrotoluene and the development of a smaller number of mesotheliomas in rats exposed to

*o*-toluidine hydrochloride. After only 13 weeks of exposure to *o*-nitrotoluene, mesothelioma or mesothelial hyperplasia occurred in a few rats. However, in the 26-week stop- and continuous-exposure studies, 20 of 60 rats (33%) exposed to *o*-nitrotoluene developed mesothelioma.

Mesothelioma has not previously occurred in control rats from 13-week studies, but is a spontaneously occurring neoplasm in 1% of control male rats from 2-year studies (NTP, 1992). Mesothelial hyperplasia, a potential preneoplastic lesion, also occurred in rats exposed to *o*-nitrotoluene but not in the controls. The incidences and morphology of the mesotheliomas were similar in rats from the stop- and continuous-exposure groups. The development of hyperplasia and mesothelioma in both the stop- and continuous-exposure studies indicates that a relatively short-term exposure to *o*-nitrotoluene or *o*-toluidine hydrochloride is sufficient to produce this carcinogenic effect.

#### TESTIS

Significantly lower testis weights and greater incidences of degeneration of the seminiferous tubule germinal epithelium than those in the controls occurred in all rats exposed to *o*-nitrotoluene. These effects increased in severity with continuous exposure and showed no evidence of recovery in the stop-exposure groups. In some rats with the testicular degeneration, a minimal hyperplasia of the interstitial (Leydig) cells was also present. These testicular effects were not a feature of *o*-toluidine hydrochloride exposure. One or two rats in each *o*-toluidine hydrochloride-exposed group had unilateral testicular degeneration. However, unilateral degeneration of the testes is generally considered to be a nonspecific finding and has been seen occasionally in control F344 rats from 13-week studies (Dixon *et al.*, 1995). The relationship of testicular degeneration and the development of mesothelioma on the adjacent serosa is unclear. Testicular degeneration is a common treatment-related effect with chemical exposure in rats and, in many instances, this lesion has not been associated with an increase in mesothelioma. Glycidol, a multisite, multisex multispecies carcinogen (NTP, 1990), produced degeneration of the testes and mesothelioma. However, cytembera (NTP, 1981) produced a high incidence of mesothelioma but did not cause testicular degeneration.

## LIVER

After 13 weeks of exposure to *o*-nitrotoluene, liver weights were slightly greater than those of the controls, and morphologic alterations included cytoplasmic vacuolization and oval cell hyperplasia. In both the stop- and continuous-exposure groups, the liver weights of rats exposed to *o*-nitrotoluene continued to increase relative to those of the controls. In the stop- and continuous-exposure groups, cytoplasmic vacuolization increased in severity and oval cell hyperplasia persisted. Oval cell hyperplasia is a chemical-induced, putative preneoplastic lesion that is seen in rats that subsequently develop cholangiofibrosis and cholangiocarcinoma (Maronpot *et al.*, 1991). Chemicals causing oval cell hyperplasia may also increase the incidence of hepatocellular adenoma and carcinoma (Maronpot *et al.*, 1991; Stenbäck *et al.*, 1994). Oval cells are precursor cells in cholangiocellular and undifferentiated liver tumors of rats (Steinberg *et al.*, 1994). Oval cell hyperplasia was seen after 13 and 26 weeks of exposure to *o*-nitrotoluene and did not regress in the stop-exposure groups. With *o*-nitrotoluene exposure, three rats developed focal masses considered to be cholangiocarcinoma. In the rat, cholangiofibrosis, cholangiofibroma, and cholangiocarcinoma represent a progression of chemical-induced lesions often preceded by oval cell hyperplasia (Bannash and Gössner 1994). Morphologic features of cholangiofibrosis and the benign and malignant cholangial tumors are similar. However, stop-exposure and transplantation studies have shown the persistence and potentially progressive nature of these chemical-induced proliferative lesions (Maronpot *et al.*, 1991).

In addition to oval cell hyperplasia, cytoplasmic vacuolization with associated hepatocyte and liver enlargement and PGST-positive foci of cellular alteration were significantly increased in *o*-nitrotoluene-exposed groups at 13 and 26 weeks. PGST-positive foci are considered to be a preneoplastic change in the liver (Sato *et al.*, 1984; Satoh *et al.*, 1985). PGST-positive foci have been induced by a variety of unrelated carcinogenic chemicals including 2-acetylaminofluorene (Stenbäck *et al.*, 1994) and estrogen (Ogawa *et al.*, 1995). With hepatocarcinogens PGST-positive staining is seen in individual cells, foci, and neoplasms (Hendrich *et al.*, 1987; Dragan *et al.*, 1994). When chemical exposure to *o*-nitrotoluene is stopped, the number of foci decreases during the 13-week recovery period but the volume fraction increases. After 26 weeks of continuous exposure to *o*-nitrotoluene, the number and volume fraction of foci were approximately 2-fold and 10-fold greater, respectively, compared to those in the 13-week exposure group. In the stop-exposure groups (normal and altered gastrointestinal flora) the number of foci decreased to approximately half of that seen at 13 weeks, but the volume fraction of foci continued

to increase during the recovery period. This partial regression of foci of alteration, persistence of oval cell hyperplasia, and the presence of a small number of cholangiocellular neoplasms at 6 weeks suggests the potential for a strong carcinogenic response in the liver of rats administered *o*-nitrotoluene over a longer period of time. In previous 2-year studies of dinitrotoluene, a chemical structurally related to *o*-nitrotoluene, there were increases in hepatocellular and cholangiocarcinoma (Rickert *et al.*, 1987).

In contrast to *o*-nitrotoluene exposure, the only liver effect seen with *o*-toluidine hydrochloride exposure was a minimal accumulation of hemosiderin pigment in the cytoplasm of the Kupffer cells lining the hepatic sinusoids. This was considered to be secondary to the increased erythrocyte destruction. Immunohistochemical staining of liver sections demonstrated an increase in PGST-positive foci in the *o*-toluidine hydrochloride group relative to controls after 26 weeks of continuous exposure. However, the number and size of these foci were significantly smaller than that seen in the liver of rats exposed to *o*-nitrotoluene. In a 2-year study of *o*-toluidine hydrochloride there was no evidence of a carcinogenic effect in the liver of rats (NCI, 1979a).

#### KIDNEY AND URINARY BLADDER

Hyaline droplet accumulation was present in the renal tubule epithelium and appeared morphologically similar in rats continuously exposed to *o*-nitrotoluene for 13 or 26 weeks. The absence of this finding in the stop-exposure groups demonstrates the reversibility of this effect. However, other kidney lesions (renal tubule epithelium regeneration and protein casts) typically associated with spontaneous nephropathy of rats were slightly increased in incidence and severity in the stop-exposure groups compared to the controls, suggesting incomplete resolution of renal toxicity. Previous studies have shown that the hyaline droplet formation in the kidney of male rats exposed to *o*-nitrotoluene is the result of a dose-related increase in the concentration of  $\alpha_{2u}$ -globulin in the kidney (Dunnick *et al.*, 1994). A variety of structurally dissimilar chemicals also causing an increase in  $\alpha_{2u}$ -globulin are typically associated with an increase in the severity of lesions associated with spontaneous nephropathy; with chronic exposure, an increased incidence of renal tubule neoplasms is sometimes observed (Baetcke *et al.*, 1991). The only renal effect of *o*-toluidine hydrochloride was an increase in hemosiderin pigment in tubule epithelium. The incidence of pigment accumulation increased with exposure duration and decreased in the stop-exposure study. This pigment accumulation was most likely secondary to the increased

erythrocyte destruction reflected by the spleen changes in these rats. In 2-year studies of *o*-toluidine hydrochloride, there was no carcinogenic effect in the kidney (NCI, 1979a).

There were no treatment-related effects in the urinary bladder of rats exposed to *o*-nitrotoluene. In the urinary bladder of rats continuously exposed to *o*-toluidine hydrochloride for 13 or 26 weeks, there was a mild, diffuse hyperplasia of the transitional epithelium. There was no evidence of treatment-related urinary calculus formation. Complete regression of the hyperplasia occurred in the stop-exposure group. In the 2-year study, continuous exposure to *o*-toluidine hydrochloride caused an increase in hyperplasia and neoplasia of the bladder in male and female rats. Other structurally similar chemicals including *o*-nitroanisole (NTP, 1993) 4-chloro-*o*-phenylenediamine (NCI, 1978b), 4-amino-2-nitrophenol (NCI, 1978c), *o*-anisidine (NCI, 1978d), and *p*-cresidine (NCI, 1979b) also caused hyperplasia and neoplasia of the urinary bladder epithelium in 2-year studies.

#### SPLEEN

Increased spleen weights and incidences of hematopoiesis and hemosiderin accumulation were observed in rats exposed to *o*-nitrotoluene. These findings are consistent with results from previous studies where *o*-nitrotoluene has been shown to increase methemoglobin levels and cause a mild regenerative anemia (NTP, 1992). In the present study, there was little difference in the severity of these effects between the 13- and 26-week continuous-exposure groups. The spleen toxicity seen with *o*-toluidine hydrochloride exposure was more severe than that caused by *o*-nitrotoluene. Spleens appeared grossly enlarged and discolored, and spleen weights were two- to threefold greater than those of the controls. These findings corresponded to the increased incidences of congestion, hemosiderin, hematopoiesis and capsular fibrosis seen microscopically. Studies of other aromatic amines (Chhabra *et al.*, 1990; NTP, 1993) have shown similar effects on the hematopoietic system. Spleen changes (congestion, hemosiderin accumulation, fibrosis, and increased hematopoiesis) have been attributed to the denaturation of hemoglobin, Heinz body formation, and increased erythrocyte destruction in the spleen. *o*-Toluidine hydrochloride binds with hemoglobin (DeBord *et al.*, 1992) and causes anemia and methemoglobin formation in rats (IARC, 1982). In the 2-year study of *o*-toluidine hydrochloride, there was a treatment-related increase in the incidence of sarcomas of the spleen (NCI, 1979a). This increased incidence of sarcomas of the spleen has also been seen in 2-year studies of other aromatic amines (Goodman *et al.*, 1984; Chhabra *et al.*, 1991).

## ALTERED GASTROINTESTINAL FLORA DIET

There was little evidence that the attempt to reduce gastrointestinal bacteria affected the carcinogenic potential or the pattern and severity of *o*-nitrotoluene toxicity in male rats. The incidence of mesothelioma and the severity and pattern of toxic lesions in the liver (increased weight, cytoplasmic vacuolization, oval cell hyperplasia, PGST-positive foci), kidney (hyaline droplet accumulation), testes (degeneration), and spleen (hematopoiesis, hemosiderin, capsular fibrosis) were generally similar in the *o*-nitrotoluene groups (normal and altered intestinal flora). The only effect that may be related to the altered gastrointestinal flora diet is the absence of cholangiocarcinomas, which were seen in 3 of 40 rats administered *o*-nitrotoluene in the normal diet. However, the altered gastrointestinal flora did not prevent increased liver weight or hepatic toxicity, reflected by the presence of cytoplasmic vacuolization, PGST-positive foci, and oval cell hyperplasia. Oval cell hyperplasia is considered to be a morphologic change preceding and associated with the development of cholangial neoplasms. Based on these findings, the short duration of the study, and the small number of these liver neoplasms seen in the normal gastrointestinal flora group, it is unlikely that the attempt to alter gastrointestinal flora prevented the development of cholangiocarcinoma.

Previous short-term studies have shown the importance of the gastrointestinal flora in producing adverse effects of *o*-nitrotoluene in the liver of rats (Doolittle *et al.*, 1983; Rickert *et al.*, 1987). Gavage administration of *o*-nitrotoluene caused unscheduled DNA synthesis in primary hepatocyte cultures from male rats (Doolittle *et al.*, 1983). This effect of *o*-nitrotoluene was not seen in similarly treated, bacteria-free rats. Gardner and Renwick (1978) demonstrated a reduction in the bacterially mediated reduction of nitrobenzoic acids in rats receiving a mixture of antibiotics similar to that used in the current studies, but with the bacteriostat bacitracin rather than the antifungal agent nystatin. Although a significant alteration and reduction of the gastrointestinal flora was observed with daily administration of antibiotics in this study, this was only demonstrated for aerobes, and it was subsequently pointed out during a review of an earlier draft of this document that the obligate anaerobes may have been less affected by the antibiotics used. This would have resulted in an insufficient alteration of flora to affect the hydrolytic and reductive activity of the intestinal bacteria on the glucuronidated forms of the *o*-nitrotoluene secreted in the bile. Also, although bacterial counts from cecal contents of dosed rats were significantly reduced at 13 weeks compared to the controls, the numbers of bacteria in the altered gastrointestinal flora diet group were higher than those observed after one week of antibiotic treatment (Appendix C),

and this increase in resistant bacteria may have been sufficient to adequately reduce the chemical conjugates in the intestine.

In an effort to further determine the efficacy of the antibiotic mixture used to reduce gastrointestinal flora, the pilot study described in Appendix C was repeated, and cultures were plated and enumerated under conditions appropriate for determining both aerobic and anaerobic bacteria. The results of the second pilot study extended the findings of the initial study by demonstrating a shift in the types of aerobic bacteria present, with a decline in enteric Gram negative rods but an increase in several other bacteria, including *Streptococcus* sp. The second pilot study also demonstrated a complete lack of efficacy in reducing the gastrointestinal load of obligate anaerobic bacteria. Therefore, no conclusions concerning the involvement of gastrointestinal flora in the pathogenesis of *o*-nitrotoluene-induced lesions could be drawn from these studies.

In summary, the results of these studies have confirmed the carcinogenicity of *o*-nitrotoluene based on the development of a high incidence of mesotheliomas and a small number of cholangiocarcinomas in male rats after only 13 or 26 weeks of exposure. The increased incidence of mesothelioma indicates the relative greater carcinogenic potential or decrease in tumor latency for *o*-nitrotoluene compared to an equivalent dose and exposure time for *o*-toluidine hydrochloride. While the chemicals are similar in structure, basic differences exist in the way *o*-nitrotoluene and *o*-toluidine hydrochloride are metabolized, e.g., methyl group oxidation (*o*-nitrotoluene) versus aromatic ring oxidation (*o*-toluidine hydrochloride). It may be that the metabolite responsible for the increased incidence of mesotheliomas may be a relatively minor metabolite of *o*-toluidine hydrochloride and a major one for *o*-nitrotoluene. Although metabolites of both compounds are excreted primarily in the urine, the renal effects seen with *o*-nitrotoluene and the urinary bladder hyperplasia with *o*-toluidine hydrochloride suggest differences in the toxicity of the urinary metabolites of each chemical. These findings, plus the presence of testicular degeneration, hepatotoxicity, and cholangiocarcinoma only in *o*-nitrotoluene groups and the relatively greater spleen toxicity seen in the *o*-toluidine hydrochloride groups, indicate that the important toxic and carcinogenic effects of *o*-nitrotoluene may not be attributed primarily to the intestinal tract reduction of the nitro group to the amine.





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## APPENDIX A

### Summary of Lesions

<b>Table A</b>	Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Male F344/N Rats in the 26-Week Feed Studies of <i>o</i> -Nitrotoluene and <i>o</i> -Toluidine Hydrochloride .....	A-2
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**TABLE A Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride<sup>1</sup>**

	Normal Gastrointestinal Flora			Altered Gastrointestinal Flora <sup>2</sup>	
	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidine (5,000 ppm)	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)
<b>DISPOSITION SUMMARY</b>					
Animals initially in study	20	60	60	20	40
13-week interim evaluation	10	20	20	10	20
Stop-exposure evaluation <sup>3</sup>		20	20	10	20
Survivors					
Terminal sacrifice	10	20	20		
Animals examined microscopically	20	60	60	20	40
<b>13-WEEK INTERIM</b>					
<b>Alimentary System</b>					
Liver	(10)	(20)	(20)	(10)	(20)
Hepatodiaphragmatic nodule	1 (10%)	1 (5%)	1 (5%)		
Inflammation	9 (90%)	20 (100%)	20 (100%)	10 (100%)	20 (100%)
Pigmentation, hemosiderin			20 (100%)		
Vacuolization, cytoplasmic		20 (100%)	1 (5%)		20 (100%)
Bile duct, hyperplasia	2 (20%)		1 (5%)		
Oval cell, hyperplasia		20 (100%)			20 (100%)
Mesentery	(1)				
Fat, necrosis	1 (100%)				
<b>Cardiovascular System</b>					
None					
<b>Endocrine System</b>					
None					
<b>General Body System</b>					
None					
<b>Genital System</b>					
Epididymis	(10)	(20)	(20)	(10)	(20)
Hyperplasia					2 (10%)
Inflammation	1 (10%)		1 (5%)		1 (5%)
Mesothelioma malignant					2 (10%)
Testes	(10)	(20)	(20)	(10)	(20)
Degeneration		20 (100%)	1 (5%)		20 (100%)
<b>Hematopoietic System</b>					
Spleen	(10)	(20)	(20)	(10)	(20)
Congestion			20 (100%)		
Hematopoietic cell proliferation	2 (20%)	20 (100%)	20 (100%)		20 (100%)
Pigmentation, hemosiderin		20 (100%)	20 (100%)		20 (100%)
Thrombosis			3 (15%)		
Capsule, fibrosis		1 (5%)	20 (100%)		1 (5%)

**TABLE A Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Male F344/N Rats in the 26-Week Feed Studies of *o*-Nitrotoluene and *o*-Toluidine Hydrochloride (continued)**

	Normal Gastrointestinal Flora			Altered Gastrointestinal Flora	
	Control (0 ppm)	<i>o</i> -Nitrotoluene (5,000 ppm)	<i>o</i> -Toluidine (5,000 ppm)	Control (0 ppm)	<i>o</i> -Nitrotoluene (5,000 ppm)
<b>13-WEEK INTERIM (continued)</b>					
<b>Integumentary System</b>					
None					
<b>Musculoskeletal System</b>					
None					
<b>Nervous System</b>					
None					
<b>Respiratory System</b>					
None					
<b>Special Senses System</b>					
None					
<b>Urinary System</b>					
Kidney	(10)	(20)	(20)	(10)	(20)
Accumulation, hyaline droplet		20 (100%)			20 (100%)
Inflammation			1 (5%)		
Pigmentation			20 (100%)		
Regeneration	3 (30%)	16 (80%)	7 (35%)	3 (30%)	2 (10%)
Urinary bladder	(10)	(20)	(20)	(10)	(20)
Inflammation			2 (10%)		
Transitional epithelium, hyperplasia			10 (50%)		
<b>Systemic Lesions</b>					
Multiple organs <sup>4</sup>	(10)	(20)	(20)	(10)	(20)
Mesothelioma malignant					2 (10%)
<b>NEOPLASM SUMMARY</b>					
Total animals with primary neoplasms <sup>5</sup>					2
Total primary neoplasms					2
Total animals with malignant neoplasms					2
Total malignant neoplasms					2

**TABLE A Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride (continued)**

	Normal Gastrointestinal Flora			Altered Gastrointestinal Flora	
	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidine (5,000 ppm)	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)
<b>STOP-EXPOSURE</b>					
<b>Alimentary System</b>					
Liver	(10)	(20)	(20)	(10)	(20)
Basophilic focus		1 (5%)			
Hepatodiaphragmatic nodule		1 (5%)			
Inflammation	10 (100%)	20 (100%)	20 (100%)	10 (100%)	20 (100%)
Pigmentation, hemosiderin			11 (55%)		
Vacuolization, cytoplasmic		20 (100%)	1 (5%)		20 (100%)
Bile duct, hyperplasia	4 (40%)		5 (25%)	4 (40%)	
Oval cell, hyperplasia		20 (100%)			20 (100%)
Cholangiocarcinoma		2 (10%)			
<b>Cardiovascular System</b>					
None					
<b>Endocrine System</b>					
None					
<b>General Body System</b>					
None					
<b>Genital System</b>					
Epididymis	(10)	(20)	(20)	(10)	(20)
Hyperplasia		2 (10%)			1 (5%)
Inflammation				1 (10%)	1 (5%)
Mesothelioma malignant		4 (20%)	2 (10%)		8 (40%)
Testes	(10)	(20)	(20)	(10)	(20)
Degeneration		20 (100%)	2 (10%)		20 (100%)
Interstitial cell, hyperplasia		11 (55%)			7 (35%)
Tunic, mesothelioma malignant		2 (10%)			4 (20%)
<b>Hematopoietic System</b>					
Spleen	(10)	(20)	(20)	(10)	(19)
Congestion			20 (100%)		
Hematopoietic cell proliferation	3 (30%)	18 (90%)	1 (5%)	1 (10%)	19 (100%)
Pigmentation, hemosiderin	3 (30%)	17 (85%)	18 (90%)	5 (50%)	19 (100%)
Capsule, fibrosis		7 (35%)	20 (100%)		1 (5%)
Capsule, lymphatic, angiectasis			15 (75%)		
<b>Integumentary System</b>					
None					

**TABLE A Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Male F344/N Rats in the 26-Week Feed Studies of *o*-Nitrotoluene and *o*-Toluidine Hydrochloride (continued)**

	Normal Gastrointestinal Flora			Altered Gastrointestinal Flora	
	Control (0 ppm)	<i>o</i> -Nitrotoluene (5,000 ppm)	<i>o</i> -Toluidine (5,000 ppm)	Control (0 ppm)	<i>o</i> -Nitrotoluene (5,000 ppm)
<b>STOP-EXPOSURE (continued)</b>					
<b>Musculoskeletal System</b>					
None					
<b>Nervous System</b>					
None					
<b>Respiratory System</b>					
None					
<b>Special Senses System</b>					
None					
<b>Urinary System</b>					
Kidney	(10)	(20)	(20)	(10)	(20)
Casts protein	3 (30%)	20 (100%)	6 (30%)		20 (100%)
Inflammation	2 (20%)		1 (5%)	1 (10%)	3 (15%)
Mineralization				1 (10%)	
Pigmentation			20 (100%)		
Regeneration	7 (70%)	19 (95%)	10 (50%)	6 (60%)	20 (100%)
Urinary bladder	(10)	(20)	(20)	(10)	(20)
<b>Systemic Lesions</b>					
Multiple organs	(10)	(20)	(20)	(10)	(20)
Mesothelioma malignant		5 (25%)	2 (10%)		8 (40%)
<b>NEOPLASM SUMMARY</b>					
Total animals with primary neoplasms		6	2		8
Total primary neoplasms		7	2		8
Total animals with malignant neoplasms		6	2		8
Total malignant neoplasms		7	2		8

**TABLE A Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride (continued)**

	Normal Gastrointestinal Flora		
	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidine (5,000 ppm)
<b>26-WEEK STUDY</b>			
<b>Alimentary System</b>			
Liver	(10)	(20)	(20)
Inflammation	10 (100%)	20 (100%)	20 (100%)
Mixed cell focus		1 (5%)	
Pigmentation, hemosiderin			20 (100%)
Vacuolization, cytoplasmic		20 (100%)	
Bile duct, hyperplasia	4 (40%)		10 (50%)
Oval cell, hyperplasia		20 (100%)	
Cholangiocarcinoma		1 (5%)	
<b>Cardiovascular System</b>			
None			
<b>Endocrine System</b>			
None			
<b>General Body System</b>			
None			
<b>Genital System</b>			
Epididymis	(10)	(20)	(20)
Hyperplasia		2 (10%)	1 (5%)
Mesothelioma malignant		7 (35%)	
Testes	(10)	(20)	(20)
Degeneration		20 (100%)	2 (10%)
Inflammation			1 (5%)
Interstitial cell, hyperplasia		3 (15%)	
Bilateral, hemangioma		1 (5%)	
Tunic, mesothelioma malignant		2 (10%)	
<b>Hematopoietic System</b>			
Spleen	(10)	(20)	(20)
Congestion			20 (100%)
Hematopoietic cell proliferation	3 (30%)	20 (100%)	20 (100%)
Pigmentation, hemosiderin	3 (30%)	20 (100%)	20 (100%)
Thrombosis			2 (10%)
Capsule, fibrosis		3 (15%)	20 (100%)
Capsule, lymphatic, angiectasis			6 (30%)
<b>Integumentary System</b>			
None			

**TABLE A Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Male F344/N Rats in the 26-Week Feed Studies of *o*-Nitrotoluene and *o*-Toluidine Hydrochloride (continued)**

	Normal Gastrointestinal Flora		
	Control (0 ppm)	<i>o</i> -Nitrotoluene (5,000 ppm)	<i>o</i> -Toluidine (5,000 ppm)
<b>26-WEEK STUDY (continued)</b>			
<b>Musculoskeletal System</b>			
None			
<b>Nervous System</b>			
None			
<b>Respiratory System</b>			
None			
<b>Special Senses System</b>			
None			
<b>Urinary System</b>			
Kidney	(10)	(20)	(20)
Accumulation, hyaline droplet		20 (100%)	
Casts protein	3 (30%)	20 (100%)	1 (5%)
Inflammation	2 (20%)	2 (10%)	
Pigmentation			20 (100%)
Regeneration	7 (70%)	19 (95%)	5 (25%)
Urinary bladder	(10)	(20)	(18)
Transitional epithelium, hyperplasia			17 (94%)
<b>Systemic Lesions</b>			
Multiple organs	(10)	(20)	(20)
Mesothelioma malignant		7 (35%)	
<b>NEOPLASM SUMMARY</b>			
Total animals with primary neoplasms		9	
Total primary neoplasms		9	
Total animals with benign neoplasms		1	
Total benign neoplasms		1	
Total animals with malignant neoplasms		8	
Total malignant neoplasms		8	

<sup>1</sup> Number of animals examined microscopically at site and number of animals with lesion.  
<sup>2</sup> Rats received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 14 consecutive weeks. Altered gastrointestinal flora groups were only included in the 13-week interim and stop-exposure evaluations.  
<sup>3</sup> Rats were exposed to *o*-nitrotoluene or *o*-toluidine hydrochloride in feed for 13 weeks and allowed to recover for 13 weeks.  
<sup>4</sup> Number of animals with any tissue examined microscopically.  
<sup>5</sup> Primary neoplasms: all neoplasms except metastatic neoplasms.



## APPENDIX B

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

<b>Table B</b>	Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male F344/N Rats in the 26-Week Feed Studies of <i>o</i> -Nitrotoluene and <i>o</i> -Toluidine Hydrochloride . . . . .	B-2
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**TABLE B Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride<sup>1</sup>**

	Normal Gastrointestinal Flora			Altered Gastrointestinal Flora <sup>2</sup>	
	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidine (5,000 ppm)	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)
<b>13-WEEK INTERIM</b>					
n	10	10	10	10	10
Necropsy body wt	345 ± 5	261 ± 2**	298 ± 4**	318 ± 5	247 ± 4**
Right kidney					
Absolute	1.130 ± 0.016	1.058 ± 0.023	1.043 ± 0.026*	1.034 ± 0.023	1.032 ± 0.022
Relative	3.27 ± 0.03	4.05 ± 0.06**	3.50 ± 0.05**	3.25 ± 0.03	4.19 ± 0.08**
Liver					
Absolute	12.056 ± 0.235	13.844 ± 0.316**	12.671 ± 0.206	10.140 ± 0.237	13.201 ± 0.400**
Relative	34.91 ± 0.31	53.08 ± 1.07**	42.57 ± 0.36**	31.86 ± 0.36	53.53 ± 1.55**
Spleen					
Absolute	0.730 ± 0.010	0.789 ± 0.021	2.212 ± 0.056**	0.648 ± 0.010	0.729 ± 0.015**
Relative	2.12 ± 0.03	3.02 ± 0.06**	7.43 ± 0.16**	2.04 ± 0.02	2.96 ± 0.08**
Right testis					
Absolute	1.593 ± 0.027	1.186 ± 0.028**	1.508 ± 0.020*	1.536 ± 0.030	1.114 ± 0.025**
Relative	4.61 ± 0.03	4.55 ± 0.08	5.07 ± 0.07**	4.83 ± 0.07	4.51 ± 0.08*
Epididymis					
Absolute	0.477 ± 0.006	0.331 ± 0.013**	0.420 ± 0.009**	0.469 ± 0.011	0.321 ± 0.007**
Relative	1.38 ± 0.02	1.27 ± 0.04*	1.41 ± 0.03	1.48 ± 0.05	1.30 ± 0.03**
<b>STOP-EXPOSURE<sup>3</sup></b>					
n	10	10	10	10	10
Necropsy body wt	389 ± 5	304 ± 6**	351 ± 9**	388 ± 5	281 ± 3**
Right kidney					
Absolute	1.379 ± 0.036	1.326 ± 0.027	1.255 ± 0.047*	1.274 ± 0.029	1.371 ± 0.022*
Relative	3.55 ± 0.08	4.36 ± 0.04**	3.56 ± 0.05	3.29 ± 0.07	4.88 ± 0.07**
Liver					
Absolute	14.405 ± 0.257	18.186 ± 0.301**	13.910 ± 0.532	13.929 ± 0.390	19.089 ± 0.416**
Relative	37.09 ± 0.69	59.89 ± 1.08**	39.58 ± 1.13	35.88 ± 0.75	67.98 ± 1.59**
Spleen					
Absolute	0.806 ± 0.017	0.818 ± 0.017	1.130 ± 0.048**	0.739 ± 0.020	0.748 ± 0.012
Relative	2.08 ± 0.05	2.70 ± 0.06**	3.23 ± 0.16**	1.90 ± 0.04	2.66 ± 0.05**
Right testis					
Absolute	1.615 ± 0.027	0.893 ± 0.077**	1.496 ± 0.025	1.585 ± 0.017	0.821 ± 0.090**
Relative	4.16 ± 0.06	2.93 ± 0.25**	4.28 ± 0.12	4.09 ± 0.07	2.91 ± 0.31**
Epididymis					
Absolute	0.484 ± 0.006	0.288 ± 0.018** <sup>4</sup>	0.432 ± 0.014	0.469 ± 0.008	0.254 ± 0.020**
Relative	1.25 ± 0.01	0.95 ± 0.06** <sup>4</sup>	1.23 ± 0.02	1.21 ± 0.02	0.90 ± 0.07**

**TABLE B Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male F344/N Rats in the 26-Week Feed Studies of o-Nitrotoluene and o-Toluidine Hydrochloride (continued)**

	Normal Gastrointestinal Flora		
	Control (0 ppm)	o-Nitrotoluene (5,000 ppm)	o-Toluidine (5,000 ppm)
<b>26-WEEK STUDY</b>			
n	10	10	10
Necropsy body wt	389 ± 5	269 ± 4**	316 ± 3**
Right kidney			
Absolute	1.379 ± 0.036	1.304 ± 0.029	1.257 ± 0.024*
Relative	3.55 ± 0.08	4.86 ± 0.11**	3.99 ± 0.09**
Liver			
Absolute	14.405 ± 0.257	20.054 ± 0.385**	14.426 ± 0.315
Relative	37.09 ± 0.69	74.68 ± 1.21**	45.71 ± 0.88**
Spleen			
Absolute	0.806 ± 0.017	0.877 ± 0.026	2.845 ± 0.097**
Relative	2.08 ± 0.05	3.27 ± 0.10**	9.01 ± 0.26**
Right testis			
Absolute	1.615 ± 0.027	0.740 ± 0.055**	1.528 ± 0.020
Relative	4.16 ± 0.06	2.76 ± 0.21**	4.84 ± 0.05**
Epididymis			
Absolute	0.484 ± 0.006	0.244 ± 0.017**	0.435 ± 0.007**
Relative	1.25 ± 0.01	0.91 ± 0.06**	1.38 ± 0.02

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

<sup>2</sup> Rats received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 14 consecutive weeks. Altered gastrointestinal flora groups were only included in the 13-week interim and stop-exposure evaluations.

<sup>3</sup> Rats were exposed to o-nitrotoluene or o-toluidine hydrochloride in feed for 13 weeks and allowed to recover for 13 weeks.

<sup>4</sup> n=9.

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

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



## APPENDIX C

### **Efficacy and Acute Toxicity of Antibiotic Mixture (Pilot Studies)**

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# EFFICACY AND ACUTE TOXICITY OF ANTIBIOTIC MIXTURE (PILOT STUDIES)

## Materials and Methods

These studies were conducted to evaluate the efficacy and acute toxicity of an antibiotic mixture containing tetracycline hydrochloride, neomycin sulfate, and nystatin. In each pilot study, male F344/N rats given the antibiotic mixture by gavage in water daily for 7 days were evaluated for total gastrointestinal flora counts, body weights, and signs of acute toxicity. Due to the preliminary nature of this work, these studies were not conducted under strict compliance with Good Laboratory Practice regulations.

In the preliminary pilot study, the antibiotic mixture was prepared approximately 1 week before the beginning of the study. Tetracycline hydrochloride, neomycin sulfate, and nystatin were obtained from Sigma Chemical Co. (St. Louis, MO). Weighed amounts of each antibiotic (in powdered form) were combined, apportioned into sealed containers in daily allotments, and stored at approximately 0 ° C. Before dosing, the antibiotic mixture was reconstituted by adding the appropriate amount of deionized water. No chemical analyses of the neat chemicals or dose formulations were required.

Thirty male F344/N rats were obtained from Charles River Laboratories (Kingston, NY). The rats were approximately 4 to 6 weeks old at receipt and were quarantined for 10 days before the beginning of the study. Blood samples were collected from three rats and the sera were analyzed for antibody titers to rodent viruses; all results were negative. Rats were weighed and then randomly distributed into five groups. Four groups contained five rats each and were designated for antibiotic administration and fecal sample collection. The fifth group contained two rats designated for the pre-study collection of cecal contents. Each group of rats was housed in a separate cage. Drinking water and NIH-07 Open Formula Diet (Zeigler Brothers, Inc., Gardners, PA) in meal form were available *ad libitum*.

Rats designated for fecal sample collection were given a single daily gavage dose of the antibiotic mixture (20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin) for 7 consecutive days. One rat was replaced on Day 1 due to a suspected gavage error. Rats were observed twice daily for clinical signs of morbidity and mortality. Body weights were recorded 2 days prior to dosing (for randomization), on Day 1 (before dosing), and on Day 7. Fecal samples were collected on Day 1 (before dosing) and on Day 7 (approximately 4 hours after dosing). Approximately 1 hour before sample collection, rats in each of the four groups were placed in cages (one group per cage) without hardwood bedding. After sample collection, rats were returned to cages with hardwood bedding. At the end of the study, rats were killed by CO<sub>2</sub> asphyxiation.

Rats designated for the pre-study collection of cecal contents were killed by CO<sub>2</sub> asphyxiation on Day 1, and cecal contents were collected and combined. On Day 7, cecal contents were collected and combined from two rats randomly selected from the pool of 20 rats given the antibiotic mixture. All fecal and cecal specimens were placed in autoclaved, screw-top test tubes and transported to Battelle's Microbiology Department on ice. The samples were diluted with sterile phosphate buffered saline (1:100 on a weight:weight basis) and mixed for 4 minutes with a Model 400 Stomacher Lab Blender. Three serial dilutions were made for each sample. The samples were plated in triplicate with MacConkey pour plates and incubated aerobically at 35 ° to 37 ° C for 18 to 24 hours. Plates containing 30 to 300 colonies were counted manually. If no plates fell within this range, counts were estimated using standard microbiological procedures. Counts were reported as colony-forming units per gram of sample analyzed.

In response to scientific criticism, a second pilot study was conducted after the completion of the 13- and 26-week base studies to measure changes in obligate anaerobes as well as total gastrointestinal flora counts in cecal samples. Twenty-one male F344 rats were received from Taconic Laboratories (Germantown, NY). The rats were approximately 4 to 6 weeks old at receipt and were quarantined for 12 days. Blood samples were collected from five rats and the sera were analyzed for antibody titers to rodent viruses; all results were negative. Rats were weighed and then randomly distributed into two groups of five rats each; one group was an untreated control group and the second group received the same antibiotics combination as rats in the preliminary pilot study. The rats were maintained under conditions similar to those in the preliminary pilot study.

Four hours after dosing on Day 7, cecal contents were collected from control and dosed rats. The samples from individual rats were diluted with sterile phosphate buffered saline. Three serial dilutions were made for each sample. The samples were plated in triplicate; aerobic counts were determined with MacConkey agar plates and blood agar plates (trypticase soy agar plus 5% sheep blood) and anaerobic counts were determined with *Brucella* agar plates (*Brucella* agar plus 5% sheep blood, hemin, and Vitamin K1). The plates were incubated at 35 ° to 37 ° C for 24 to 48 hours (aerobic incubation) or for 48 to 72 hours (anaerobic incubation). Plates containing 25 to 250 colonies were counted manually. If no plates fell within this range, counts were estimated using standard microbiological procedures. Individual colony types were counted and characterized by colony morphology and Gram stain results; presumptive bacterial identifications were based on growth on selective media, colony morphology, Gram reaction, distinguishing characteristics, and aerotolerance versus anaerobic requirements.

## Results

No deaths occurred in the studies, and rats displayed no overt signs of toxicity as a result of antibiotic administration. Group mean body weights and absolute mean body weight gains (relative to Day 1) were increased in all four treated groups in the preliminary pilot study (Table C1); the final mean body weight of dosed rats in the second pilot study was slightly lower than that of the controls, due to the low body weight gain of one rat (Table C1).

Following the 7-day antibiotic administration period in the preliminary pilot study, total intestinal microflora were reduced in two of the four fecal samples analyzed (97.68% and 99.63% reductions) (Table C2). The high microflora counts observed on Day 7 in two of the fecal samples may have been due to coliform contamination from the cage floor or from the animals. The greatest reduction in intestinal microflora was observed in cecal contents (99.96%).

In the second pilot study, the total microflora counts of dosed rats were similar to those of the controls; however, shifts in aerobic and anaerobic bacterial types were observed (Table C3). Gamma-hemolytic-presumptive *Streptococcus* sp. were increased in dosed rats; presumptive *Staphylococcus* sp. and enteric Gram-negative rods (i.e., *Escherichia* sp., *Citrobacter* sp., *Enterobacter* sp., *Klebsiella* sp.) were decreased in dosed rats. One *Bacillus* isolate observed in the controls ( $10^8$  CFU/g) was not present in dosed rats, although the range of types and corresponding colony counts of *Bacillus* were greater in cecal samples from dosed rats. One yeast and two fungal isolates were cultured at low levels in dosed rats, but none occurred in the controls.

Obligate anaerobes were less affected by antibiotic treatment than aerobes. More diverse colony types of Gram-negative anaerobes (i.e., *Bacteroides* sp., *Prevotella* sp., *Fusobacterium* sp.) were observed in dosed rats than in the controls, but these anaerobes grew in a wider range in control rats ( $10^6$ - $10^{10}$  CFU/g) than in dosed rats ( $10^8$ - $10^9$  CFU/g). Gram-positive anaerobes (i.e., *Clostridium* sp., *Propionibacterium* sp., *Peptostreptococcus* sp.) was detected primarily at  $10^8$  CFU/g in controls and  $10^9$  CFU/g in dosed rats.

Based on the results of the preliminary pilot study, rats in the altered flora groups in the 26-week studies were given a daily gavage treatment of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin in deionized water. To evaluate the effectiveness of the antibiotic mixture in the 26-week studies, cecal samples were collected at the 13-week interim evaluation from five rats in each of the following groups: altered flora control, normal flora control, altered flora o-nitrotoluene, and normal flora o-nitrotoluene. There is no indication in the literature or in reported safety assessment studies of any carcinogenic potential of the individual antibiotics used in these studies (PDR, 1992).

**TABLE C1 Survival and Body Weights of Male F344/N Rats Following 7 Days of Antibiotic Administration<sup>1</sup>**

	Group	Survival <sup>2</sup>	Body Weight		
			Initial	Final	Change
<b>Study 1</b>					
	1	5/5	141 ± 4	162 ± 9	21
	2	5/5	139 ± 3	165 ± 8	26
	3	5/5	138 ± 5	162 ± 6	24
	4	5/5	139 ± 5	165 ± 6	26
<b>Study 2</b>					
	Control	5/5	103 ± 3	131 ± 3	28
	Dosed	5/5	103 ± 3	127 ± 3	24

<sup>1</sup> Rats in each group received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 7 consecutive days. Body weights are given in grams (mean ± standard deviation).

<sup>2</sup> Number surviving at 7 days/number of animals per group.

**TABLE C2 Gastrointestinal Flora Counts of Male F344/N Rats Following 7 Days of Antibiotic Administration<sup>1</sup>**

	Gastrointestinal Flora Counts		Percent Reduction <sup>3</sup>	
	Day 1 <sup>2</sup>	Day 7		
<b>Fecal Sample<sup>4</sup></b>				
	1	3.7 x 10 <sup>6</sup> †	8.6 x 10 <sup>4</sup>	97.68
	2	2.5 x 10 <sup>5</sup>	1.3 x 10 <sup>5</sup>	48.00
	3	4.4 x 10 <sup>5</sup>	1.6 x 10 <sup>3</sup> ‡	99.64
	4	1.4 x 10 <sup>6</sup>	8.3 x 10 <sup>5</sup>	40.71
<b>Cecal Contents<sup>5</sup></b>				
		6.4 x 10 <sup>6</sup> †	2.3 x 10 <sup>3</sup> †	99.96

<sup>1</sup> Rats, except for two rats designated for pre-study collection of cecal contents, received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 7 consecutive days. Counts are given in colony-forming units per gram (CFU/g).

<sup>2</sup> Samples were taken before antibiotic administration.

<sup>3</sup> [(Day 1 count–Day 7 count)/Day 1 count] x 100.

<sup>4</sup> Each fecal sample was taken from one cage containing five rats that received the antibiotic mixture.

<sup>5</sup> Cecal contents were collected on Day 1 from two rats not given the antibiotic mixture and on Day 7 from two rats randomly selected from the pool of 20 rats given the antibiotic mixture. At each time point, cecal contents were combined before analysis.

† Count was estimated using standard microbiological procedures and expressed using scientific notation.

‡ Small sample.



**TABLE C3 Total Aerobic and Anaerobic Plate Counts of Male F344/N Rats Following 7 Days of Antibiotic Administration<sup>1</sup>**

Animal Number	Plate Count (CFU/g)		
	MAC	BAP <sup>2</sup>	BRU
<b>Control Group</b>			
1	$1.9 \times 10^5$	$2.3 \times 10^8$	$6.6 \times 10^9$
2	$7.5 \times 10^4$	$6.0 \times 10^8$	$3.0 \times 10^9$
3	$6.0 \times 10^4$	$4.7 \times 10^8$	$4.7 \times 10^9$
4	$4.5 \times 10^4$	$1.4 \times 10^8$	$2.3 \times 10^9$
5	$1.6 \times 10^5$	$8.6 \times 10^8$	$>5.7 \times 10^{11}$
<b>Dosed Group</b>			
6	<100	$1.0 \times 10^{10}$	$2.7 \times 10^9$
7	<100	$7.9 \times 10^9$	$3.5 \times 10^9$
8	<100	$1.0 \times 10^{10}$	$3.6 \times 10^9$
9	<100	$9.1 \times 10^9$	$3.2 \times 10^9$
10	$1.5 \times 10^2$	$1.1 \times 10^{10}$	$3.7 \times 10^9$

<sup>1</sup> Rats in each group received a single gavage dose of 20 mg tetracycline hydrochloride, 40 mg neomycin sulfate, and 4 mg nystatin daily for 7 consecutive days. CFU = colony-forming units; MAC = MacConkey agar; BAP = trypticase soy agar plus 5% sheep blood; BRU = *Brucella* agar plus 5% sheep blood, hemin, and Vitamin K1. MAC and BAP plates were used to determine aerobic microflora counts; BRU plates were used to determine anaerobic microflora counts.

<sup>2</sup> Additional dilutions were required to obtain colony counts for one control rat (animal number 5) and all dosed rats.

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<b>Toxicity Report Number</b>	<b>Chemical</b>	<b>Route of Exposure</b>	<b>Publication Number</b>
1	Hexachloro-1,3-butadiene	Dosed Feed	91-3120
2	<i>n</i> -Hexane	Inhalation	91-3121
3	Acetone	Drinking Water	91-3122
4	1,2-Dichloroethane	Drinking Water, Gavage	91-3123
5	Cobalt Sulfate Heptahydrate	Inhalation	91-3124
6	Pentachlorobenzene	Dosed Feed	91-3125
7	1,2,4,5-Tetrachlorobenzene	Dosed Feed	91-3126
8	D & C Yellow No. 11	Dosed Feed	91-3127
9	<i>o</i> -Cresol <i>m</i> -Cresol <i>p</i> -Cresol	Dosed Feed	92-3128
10	Ethylbenzene	Inhalation	92-3129
11	Antimony Potassium Tartrate	Drinking Water, I.P. Inject.	92-3130
12	Castor Oil	Dosed Feed	92-3131
13	Trinitrofluorenone	Dermal, Dosed Feed	92-3132
14	<i>p</i> -Chloro- $\alpha,\alpha,\alpha$ -Trifluorotoluene	Gavage (corn oil, a-CD)	92-3133
15	<i>t</i> -Butyl Perbenzoate	Gavage	92-3134
16	Glyphosate	Dosed Feed	92-3135
17	Black Newsprint Ink	Dermal	92-3340
18	Methyl Ethyl Ketone Peroxide	Dermal	92-3341
19	Formic Acid	Inhalation	92-3342
20	Diethanolamine	Drinking Water, Dermal	92-3343
21	2-Hydroxy-4-Methoxybenzophenone	Dosed Feed, Drinking Water	92-3344
22	N, N-Dimethylformamide	Inhalation	93-3345
23	<i>o</i> -Nitrotoluene <i>m</i> -Nitrotoluene <i>p</i> -Nitrotoluene	Dosed Feed	92-3346
24	1,6-Hexanediamine	Inhalation	93-3347
25	Glutaraldehyde	Inhalation	93-3348
26	Ethylene Glycol Ethers	Drinking Water	93-3349
27	Riddelliine	Gavage	94-3350
28	Tetrachlorophthalic Anhydride	Gavage	93-3351
29	Cupric Sulfate	Drinking Water, Dosed Feed	93-3352
30	Dibutyl Phthalate	Feed	95-3353
31	Isoprene	Inhalation	95-3354

**NTP TECHNICAL REPORTS ON TOXICITY STUDIES  
PRINTED AS OF FEBRUARY 1996 (continued)**

<b>Toxicity Report Number</b>	<b>Chemical</b>	<b>Route of Exposure</b>	<b>Publication Number</b>
32	Methylene Bis(thiocyanate)	Gavage	94-3381
33	2-Chloronitrobenzene 4-Chloronitrobenzene	Inhalation	93-3382
35	Chemical Mixture of 25 Groundwater Contaminants	Drinking Water	93-3384
36	Pesticide/Fertilizer Mixtures	Drinking Water	93-3385
37	Sodium Cyanide	Drinking Water	94-3386
38	Sodium Selenate Sodium Selenite	Drinking Water	94-3387
39	Cadmium Oxide	Inhalation	95-3388
40	$\beta$ -Bromo- $\beta$ -nitrostyrene	Gavage	94-3389
42	1,3-Diphenylguanidine	Feed	95-3933
45	Halogenated Ethanes	Gavage	96-3935