

NTP TECHNICAL REPORT
ON THE
TOXICOLOGY AND CARCINOGENESIS
STUDIES OF *o*-NITROTOLUENE

(CAS NO. 88-72-2)

IN F344/N RATS AND B6C3F₁ MICE

(FEED STUDIES)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

May 2002

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U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

FOREWORD

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

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

The studies described in this Technical Report were performed under the direction of the NIEHS and were conducted in compliance with NTP laboratory health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals. The prechronic and chronic studies were conducted in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations, and all aspects of the chronic studies were subjected to retrospective quality assurance audits before being presented for public review.

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

Details about ongoing and completed NTP studies are available at the NTP's World Wide Web site: <http://ntp-server.niehs.nih.gov>. Abstracts of all NTP Technical Reports and full versions of the most recent reports and other publications are available from the NIEHS' Environmental Health Information Service (EHIS) <http://ehis.niehs.nih.gov> (800-315-3010 or 919-541-3841). In addition, printed copies of these reports are available from EHIS as supplies last. A listing of all the NTP Reports printed since 1982 appears on the inside back cover.

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SUMMARY

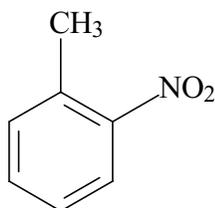
Background: Approximately 30 million pounds of *ortho*-nitrotoluene are used annually in the United States in the production of agricultural and rubber chemicals and dyes. We studied the effects of *o*-nitrotoluene on male and female rats and mice to identify potential toxic or carcinogenic hazards to humans.

Methods: We gave feed containing *o*-nitrotoluene to 12 groups of 60 animals for 2 years. Male and female rats received 625, 1,250, or 2,000 parts per million (ppm) of *o*-nitrotoluene in their feed (equivalent to 0.0625%, 0.125%, or 0.2%); male and female mice received 1,250, 2,500, or 5,000 ppm (0.125%, 0.25%, or 0.5%). Groups of animals receiving untreated feed served as controls. Tissues from more than 40 sites were examined for every animal.

Results: All of the male rats and male mice receiving the highest concentrations died before the end of the study, mainly from chemically induced tumors. Survival rates were also lower in females receiving *o*-nitrotoluene because of chemically induced tumors. Male rats had high incidences of malignant mesotheliomas and skin fibromas or fibrosarcomas; incidences of liver and lung tumors also increased. Most female rats receiving *o*-nitrotoluene developed mammary gland fibroadenomas; skin fibromas and liver adenomas were also seen. Virtually all male mice exposed to the two highest concentrations of *o*-nitrotoluene developed hemangiosarcomas (circulatory system cancers) and died early. Carcinomas of the large intestine were seen in male mice receiving lower doses. Female mice receiving *o*-nitrotoluene also developed lethal hemangiosarcomas, liver adenomas and carcinomas, and large intestine carcinomas.

Conclusions: We conclude that *o*-nitrotoluene was clearly carcinogenic in male and female rats and mice, causing a variety of malignant tumors.

ABSTRACT



o-NITROTOLUENE

CAS No. 88-72-2

Chemical Formula: C₇H₇NO₂ Molecular Weight: 137.14

Synonyms: 2-Methylnitrobenzene; *o*-methylnitrobenzene; 2-nitrotoluene; 2-nitrotoluol

o-Nitrotoluene is used to synthesize agricultural and rubber chemicals, azo and sulfur dyes, and dyes for cotton, wool, silk, leather, and paper. *o*-Nitrotoluene was nominated for study by NIOSH and the NTP based on its considerable human exposure as well as the absence of long-term studies of carcinogenicity in rodents. Male and female F344/N rats and B6C3F₁ mice were exposed to *o*-nitrotoluene (greater than 99% pure) in feed for 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium*, cultured Chinese hamster ovary cells, rat and mouse bone marrow cells, and mouse peripheral blood erythrocytes.

2-YEAR STUDY IN RATS

In the core study, groups of 60 male and 60 female rats were fed diets containing 625, 1,250, or 2,000 ppm *o*-nitrotoluene (equivalent to average daily doses of approximately 25, 50, or 90 mg *o*-nitrotoluene/kg body weight to males and 30, 60, or 100 mg/kg to females) for 105 weeks. In a 3-month stop-exposure study, groups of 70 male rats were fed diets containing 2,000 or 5,000 ppm *o*-nitrotoluene (equivalent to average daily

doses of approximately 125 or 315 mg/kg) for 13 weeks followed by undosed feed for the remainder of the study. A group of 70 male rats receiving undosed feed served as a control group for both male rat studies; 60 female rats receiving undosed feed were the control group for the female core study. Ten control males and 10 males from each stop-exposure group were sacrificed at 3 months.

Survival, Body Weights, and Feed Consumption

All 2,000 ppm core study, all 5,000 ppm stop-exposure, and all but three core study 1,250 ppm male rats died before the end of the studies. Survival of 625 ppm core study and 2,000 ppm stop-exposure males and of 2,000 ppm females was significantly less than that of the controls. Mean body weights of all exposed groups of males except the 625 ppm group were generally less than those of the controls throughout the study. Mean body weights of 2,000 ppm females were less than those of the controls during year 2 of the study. Feed consumption by exposed groups of rats was similar to that by the controls.

Biomarkers of Exposure

Three urinary metabolites were followed during the study as biomarkers of exposure. The ratios of *o*-nitrobenzoic acid to creatinine and of *o*-nitrobenzylmercapturic acid to creatinine determined at 2 weeks and at 3, 12, and 18 months were linearly related to exposure concentration in males and females. The ratio of *o*-aminobenzoic acid to creatinine was not related to exposure concentration.

Pathology Findings

The incidences of malignant mesothelioma in male rats occurred with positive trends in both the core and stop-exposure studies and were significantly greater in exposed groups than in the controls. Incidences of subcutaneous skin neoplasms (fibroma, fibrosarcoma, and lipoma) were increased in exposed groups of males, while the incidences of fibroma or fibrosarcoma (combined) were increased in exposed females. In all exposed groups of males and females except 2,000 ppm core study males, the incidences of mammary gland fibroadenoma were significantly increased. The incidences of mammary gland hyperplasia were significantly increased in 625 and 1,250 ppm females.

Increased incidences of mesothelioma, skin neoplasms, and mammary gland fibroadenoma in the stop-exposure males indicated that 3 months of dosing were sufficient to produce a carcinogenic effect.

Liver weights of 5,000 ppm stop-exposure males were significantly greater than those of the controls at 3 months. The incidences of hepatocellular adenoma in 2,000 ppm core study males and females and of hepatocellular adenoma or carcinoma (combined) in 2,000 ppm core study and 5,000 ppm stop-exposure males were significantly increased. Cholangiocarcinoma occurred in three 5,000 ppm stop-exposure males, and a single hepatocholangiocarcinoma occurred in a 625 ppm male and in a 2,000 ppm core study male. Nonneoplastic lesions of the liver included eosinophilic, mixed cell, and clear cell foci in exposed groups of males and females and mixed cell infiltrate in exposed males and basophilic focus in exposed females.

The incidences of alveolar/bronchiolar adenoma and alveolar/bronchiolar adenoma or carcinoma (combined) were significantly increased in 5,000 ppm stop-exposure males, as were alveolar/bronchiolar hyperplasia in most exposed groups of males and females.

The incidences of hematopoietic cell proliferation of the spleen and of hyperplasia of the mandibular lymph node (females) and bone marrow were increased in exposed groups of males at 3 months and/or 2 years and in exposed groups of females at 2 years.

The incidences of mononuclear cell leukemia were significantly decreased in all groups of males exposed to 1,250 ppm or greater and in all exposed groups of females; the incidence of testicular interstitial cell adenoma was significantly decreased in 5,000 ppm stop-exposure males.

2-YEAR STUDY IN MICE

Groups of 60 male and 60 female mice were fed diets containing 0, 1,250, 2,500, or 5,000 ppm *o*-nitrotoluene (equivalent to average daily doses of approximately 165, 360, or 700 mg/kg to males and 150, 320, or 710 mg/kg to females) for 105 weeks.

Survival, Body Weights, and Feed Consumption

All 2,500 and 5,000 ppm males died before the end of the study. Survival of 1,250 ppm males and 5,000 ppm females was significantly less than that of the controls. Mean body weights of exposed males and 5,000 ppm females were generally less than those of the controls throughout the study, and those of 2,500 ppm females were less during the second year of the study. Feed consumption by 5,000 ppm males was less than that by the controls.

Biomarkers of Exposure

Three urinary metabolites were followed during the study as biomarkers of exposure. The ratios of *o*-nitrobenzoic acid to creatinine determined at 2 weeks and at 3, 12, and 18 months were linearly related to exposure concentration in males and females. The concentrations of *o*-nitrobenzylmercapturic acid and *o*-aminobenzoic acid were below the limit of quantitation at most time points.

Pathology Findings

The incidences of hemangiosarcoma in all exposed groups of males and in 5,000 ppm females were significantly greater than those in the controls. Large intestine (cecum) carcinomas were observed in all exposed groups except 5,000 ppm males.

The incidences of hepatocellular neoplasms were significantly increased in 2,500 and 5,000 ppm females. Nonneoplastic liver lesions including eosinophilic and basophilic foci and minimal to mild necrosis were enhanced in exposed males and females. Also present were focal hepatocyte syncytial alteration in exposed males and hepatocyte necrosis and focal hepatocyte cytoplasmic vacuolization in 5,000 ppm females.

Renal tubule pigmentation occurred more frequently in exposed groups of males and in 5,000 ppm females than in the controls. Olfactory epithelial degeneration occurred in every male and female mouse exposed to 2,500 or 5,000 ppm, and the severity of this lesion increased with increasing exposure concentration.

GENETIC TOXICOLOGY

o-Nitrotoluene was not mutagenic in any of several strains of *S. typhimurium*, with or without metabolic activation enzymes (S9). Sister chromatid exchanges were significantly increased in cultured Chinese hamster ovary cells following exposure to *o*-nitrotoluene in the presence of S9; an equivocal response was seen without S9. *o*-Nitrotoluene did not induce chromosomal aberrations in cultured Chinese hamster ovary cells, with or without S9. *o*-Nitrotoluene did not induce a significant increase in the frequency of micronuclei in bone marrow polychromatic erythrocytes of male rats or male mice when administered by intraperitoneal injection. Results of a peripheral blood micronucleus test were equivocal

for male mice and negative for female mice administered *o*-nitrotoluene in feed for 13 weeks.

CONCLUSIONS

Under the conditions of these studies, there was *clear evidence of carcinogenic activity** of *o*-nitrotoluene in male rats based on increased incidences of malignant mesothelioma, subcutaneous skin neoplasms, mammary gland fibroadenoma, and liver neoplasms. The increased incidences of lung neoplasms in male rats were also considered to be exposure related. There was *clear evidence of carcinogenic activity* of *o*-nitrotoluene in female rats based on increased incidences of subcutaneous skin neoplasms and mammary gland fibroadenoma. The increased incidence of hepatocellular adenoma in female rats was also considered to be exposure related. There was *clear evidence of carcinogenic activity* of *o*-nitrotoluene in male and female mice based on increased incidences of hemangiosarcoma, carcinoma of the large intestine (cecum), and hepatocellular neoplasms (females only).

Exposure to *o*-nitrotoluene caused increased incidences of nonneoplastic lesions of the mammary gland (females only), liver, bone marrow, spleen, lung, and mandibular lymph node (females only) in male and female rats and of the liver, kidney, and nose in male and female mice.

Decreased incidences of mononuclear cell leukemia occurred in exposed groups of rats; the incidence of testicular interstitial cell adenoma was decreased in exposed male rats.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 13. A summary of the Technical Reports Review Subcommittee comments and public discussion on this Technical Report appears on page 15.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of o-Nitrotoluene

	Male F344/N Rats	Female F344/N Rats	Male B6C3F ₁ Mice	Female B6C3F ₁ Mice
Concentrations in feed	0, 625, 1,250, or 2,000 ppm and 2,000 and 5,000 ppm stop-exposure	0, 625, 1,250, or 2,000 ppm	0, 1,250, 2,500, or 5,000 ppm	0, 1,250, 2,500, or 5,000 ppm
Body weights	1,250, 2,000, and 2,000 and 5,000 ppm stop-exposure groups less than the control group	2,000 ppm group less than the control group	Exposed groups less than the control group	2,500 and 5,000 ppm groups less than the control group
Survival rates	39/60, 18/60, 3/60, 0/60, 11/60, 0/60	47/60, 47/60, 39/60, 33/60	52/60, 34/60, 0/60, 0/60	52/60, 46/60, 47/60, 5/60
Nonneoplastic effects	<p><u>Liver</u>: eosinophilic focus (7/60, 18/60, 29/60, 24/60, 15/60, 13/60); mixed cell focus (5/60, 7/60, 12/60, 6/60, 12/60, 8/60); clear cell focus (29/60, 29/60, 34/60, 31/60, 30/60, 34/60); mixed cell cellular infiltration (1/60, 5/60, 11/60, 20/60, 15/60, 33/60)</p> <p><u>Bone marrow</u>: hyperplasia (2/60, 25/60, 43/60, 45/60, 37/60, 33/60)</p> <p><u>Spleen</u>: hematopoietic cell proliferation (7/60, 33/60, 38/60, 47/60, 36/60, 35/60)</p> <p><u>Lung</u>: alveolar epithelial hyperplasia (2/60, 8/60, 3/60, 7/60, 15/60, 29/60)</p>	<p><u>Mammary gland</u>: hyperplasia (14/60, 36/60, 30/60, 19/60)</p> <p><u>Liver</u>: eosinophilic focus (5/60, 12/59, 25/60, 32/60); mixed cell focus (6/60, 9/59, 11/60, 28/60); clear cell focus (16/60, 30/59, 28/60, 33/60); basophilic focus (51/60, 56/59, 60/60, 54/60)</p> <p><u>Bone marrow</u>: hyperplasia (2/60, 7/60, 15/60, 24/60)</p> <p><u>Spleen</u>: hematopoietic cell proliferation (22/60, 38/59, 48/60, 48/59)</p> <p><u>Lung</u>: alveolar epithelial hyperplasia (6/60, 14/60, 16/60, 9/60)</p> <p><u>Lymph node (mandibular)</u>: lymphoid hyperplasia (3/60, 5/60, 6/59, 15/59)</p>	<p><u>Liver</u>: eosinophilic focus (3/60, 14/59, 1/57, 1/60); basophilic focus (0/60, 6/59, 4/57, 0/60); necrosis (1/60, 15/59, 27/57, 30/60); focal hepatocyte syncytial alteration (16/60, 26/59, 43/57, 39/60)</p> <p><u>Kidney</u>: renal tubule pigmentation (1/58, 6/59, 32/58, 35/60)</p> <p><u>Nose</u>: olfactory epithelial degeneration (0/60, 36/60, 60/60, 60/60)</p>	<p><u>Liver</u>: eosinophilic focus (2/60, 3/59, 6/59, 28/60); basophilic focus (1/60, 6/59, 2/59, 6/60); necrosis (3/60, 0/59, 2/59, 13/60); focal hepatocyte necrosis (0/60, 0/59, 0/59, 6/60); focal hepatocyte cytoplasmic vacuolization (1/60, 2/59, 2/59, 9/60)</p> <p><u>Kidney</u>: renal tubule pigmentation (0/59, 1/56, 3/58, 35/59)</p> <p><u>Nose</u>: olfactory epithelial degeneration (0/60, 28/60, 59/59, 57/57)</p>

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of *o*-Nitrotoluene

	Male F344/N Rats	Female F344/N Rats	Male B6C3F₁ Mice	Female B6C3F₁ Mice
Neoplastic effects	<p><u>Mesothelium:</u> malignant mesothelioma (2/60, 20/60, 29/60, 44/60, 44/60, 54/60)</p> <p><u>Skin (subcutaneous):</u> lipoma (0/60, 4/60, 13/60, 13/60, 10/60, 12/60); fibroma (5/60, 46/60, 52/60, 59/60, 45/60, 52/60); fibrosarcoma (0/60, 7/60, 17/60, 20/60, 8/60, 12/60); fibroma or fibrosarcoma (5/60, 47/60, 55/60, 59/60, 47/60, 53/60)</p> <p><u>Mammary gland:</u> fibroadenoma (0/60, 7/60, 10/60, 2/60, 13/60, 20/60)</p> <p><u>Liver:</u> hepatocellular adenoma (2/60, 3/60, 3/60, 7/60, 3/60, 4/60); hepatocellular adenoma or carcinoma (3/60, 3/60, 3/60, 8/60, 3/60, 6/60); cholangiocarcinoma (0/60, 0/60, 0/60, 0/60, 0/60, 3/60); hepato-cholangiocarcinoma (0/60, 1/60, 0/60, 1/60, 0/60, 0/60)</p> <p><u>Lung:</u> alveolar/ bronchiolar adenoma (1/60, 5/60, 1/60, 2/60, 3/60, 8/60); alveolar/ bronchiolar adenoma or carcinoma (2/60, 5/60, 1/60, 2/60, 3/60, 11/60)</p>	<p><u>Skin (subcutaneous):</u> fibroma (3/60, 3/60, 18/60, 20/60); fibroma or fibrosarcoma (3/60, 3/60, 21/60, 22/60)</p> <p><u>Mammary gland:</u> fibroadenoma (23/60, 47/60, 52/60, 56/60)</p> <p><u>Liver:</u> hepatocellular adenoma (1/60, 0/59, 1/60, 6/60)</p>	<p><u>Circulatory system:</u> hemangiosarcoma (4/60, 17/60, 55/60, 60/60)</p> <p><u>Large intestine (cecum):</u> carcinoma (0/60, 12/60, 9/60, 0/60)</p>	<p><u>Circulatory system:</u> hemangiosarcoma (0/60, 2/60, 3/60, 50/60)</p> <p><u>Large intestine (cecum):</u> carcinoma (0/60, 1/60, 4/60, 3/60)</p> <p><u>Liver:</u> hepatocellular adenoma (7/60, 5/59, 19/59, 29/60); hepatocellular carcinoma (2/60, 4/59, 6/59, 16/60); hepatocellular adenoma or carcinoma (9/60, 9/59, 24/59, 39/60)</p>
Decreased incidences	<p><u>Mononuclear cell leukemia:</u> (30/60, 21/60, 3/60, 3/60, 13/60, 1/60)</p> <p><u>Testis:</u> Interstitial cell adenoma (55/60, 53/60, 51/60, 46/60, 50/60, 27/60)</p>	<p><u>Mononuclear cell leukemia:</u> (21/60, 6/60, 4/60, 5/60)</p>	None	None
Level of evidence of carcinogenic activity	Clear evidence	Clear evidence	Clear evidence	Clear evidence

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of o-Nitrotoluene

Genetic toxicology

<i>Salmonella typhimurium</i> gene mutations:	Negative in strains TA98, TA100, TA1535, and TA1537 with and without S9
Sister chromatid exchanges	
Cultured Chinese hamster ovary cells <i>in vitro</i> :	Positive with S9; equivocal without S9
Chromosomal aberrations	
Cultured Chinese hamster ovary cells <i>in vitro</i> :	Negative with and without S9
Micronucleated erythrocytes	
Rat bone marrow <i>in vivo</i> :	Negative in two protocols
Mouse bone marrow <i>in vivo</i> :	Negative
Mouse peripheral blood <i>in vivo</i> :	Equivocal in male mice; negative in female mice

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results (**clear evidence and some evidence**); one category for uncertain findings (**equivocal evidence**); one category for no observable effects (**no evidence**); and one category for experiments that cannot be evaluated because of major flaws (**inadequate study**). These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

For studies showing multiple chemical-related neoplastic effects that if considered individually would be assigned to different levels of evidence categories, the following convention has been adopted to convey completely the study results. In a study with clear evidence of carcinogenic activity at some tissue sites, other responses that alone might be deemed some evidence are indicated as “were also related” to chemical exposure. In studies with clear or some evidence of carcinogenic activity, other responses that alone might be termed equivocal evidence are indicated as “may have been” related to chemical exposure.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

**NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE**

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Technical Report on *o*-nitrotoluene on May 3, 2001 are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing the NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On May 3, 2001, the draft Technical Report on the toxicology and carcinogenesis studies of *o*-nitrotoluene received public review by the National Toxicology Program's Board of Scientific Counselors' Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. J.K. Dunnick, NIEHS, introduced the toxicology and carcinogenesis studies of *o*-nitrotoluene by discussing the uses of the chemical and the rationale for the study, describing the experimental design, reporting on survival and body weight effects, and commenting on chemical-related neoplastic and nonneoplastic lesions in male and female rats and mice. The proposed conclusions for the 2-year studies of *o*-nitrotoluene were *clear evidence* of carcinogenic activity in male and female F344/N rats and B6C3F₁ mice.

Dr. R.C. Sills, NIEHS, presented results of analyses of mutations in *ras* oncogenes and *p53* tumor suppressor genes extracted from hemangiosarcomas found in mice exposed to *o*-nitrotoluene and in control mice from previous NTP studies. While the mutation rate in *ras* oncogenes was similar in hemangiosarcomas from exposed and control mice, mutations occurred in almost all the *p53* tumor suppressor genes from *o*-nitrotoluene-induced hemangiosarcomas, but only rarely in spontaneously occurring neoplasms. Dr. T.R. Devereux, NIEHS, showed results of analyses for mutations that disrupt the regulation of β -catenin from the same set of hemangiosarcoma tissue samples. About half of the *o*-nitrotoluene-induced hemangiosarcomas had such mutations, while spontaneously occurring hemangiosarcomas had none.

Dr. Medinsky, a principal reviewer, was unable to attend the meeting and her comments were read into the record by Dr. M.S. Wolfe, NIEHS. Dr. Medinsky agreed with the proposed conclusions. She asked whether the test chemical was stable in the feed and if the feed was adversely affected by irradiation. Dr. Dunnick replied that the chemical was stored refrigerated and mixed with feed every 2 or 3 days to minimize loss due to instability. Dr. G.N. Rao, NIEHS, explained that to prevent pathogen contamination, the NTP has begun irradiating the animal feed because the prepared diet is not formulated to be autoclaved. The nutrient composition of the diet was unaffected by irradiation.

Dr. Medinsky also questioned the appropriateness of analyzing metabolite data normalized to creatinine concentrations rather than total metabolite excreted. Dr. L.T. Burka, NIEHS, explained that creatinine production, which is related to muscle mass, is a good surrogate measure of body weight, providing a convenient method for normalizing data from animals of different size due to age and sex differences. In addition, bulk measures of urine volume are less precise than metabolite concentrations and thus less useful for mathematical modeling.

Dr. Malarkey, the second principal reviewer, agreed with the conclusions. He praised the inclusion of stop-exposure and molecular biology studies and asked for clarification of the specificity of the observed mutations and the sites of β -catenin and *p53* protein accumulation. Dr. Sills replied that the *p53* protein was located primarily in the nucleus and β -catenin predominantly in the cell membrane, although nuclear accumulation of β -catenin was seen in some neoplasm types, such as hepatoblastomas. Dr. Malarkey suggested the lesions in the reproductive tract and endocrine glands might indicate an endocrine-disrupting mechanism of toxicity by the chemical. As a general question for all NTP studies, he asked for details of monitoring for possible *Helicobacter hepaticus* contamination of the animal rooms. Dr. Rao described the monitoring procedures used to detect *Helicobacter* infection following an outbreak in the late 1980s and noted that NTP studies have been free of known viral and bacterial pathogens since 1991.

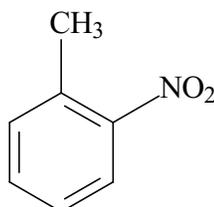
Dr. Klaunig, the third principal reviewer, also agreed with the proposed conclusions. He asked for clarification of the cause of early death of the animals and for more information on the site of origin of the various mesotheliomas. Dr. Dunnick replied that mesotheliomas were a significant cause of mortality and that the majority of the mesotheliomas originated in the tunica vaginalis.

Dr. Chatman questioned the phrasing of the proposed conclusions and suggested that listing certain neoplasms as evidence of carcinogenic activity and others as "also exposure related" or "may have been related" was potentially confusing. Dr. Davis also questioned whether the additional statements implied that the lesions were treatment related. Dr. C.J. Portier, NIEHS,

explained that for each sex/species group, there is one overall conclusion category for the chemical, rather than different categories for different tissue sites. Thus, to convey fully the study results, the NTP mentions not only the neoplasms giving the strongest indication of carcinogenic activity but also other chemically related effects. Dr. Chatman proposed that the lung neoplasms in male rats and liver neoplasms in female rats be included in the *clear evidence* statements. Dr. J.R. Bucher, NIEHS, said the proposed phrasing was

chosen to distinguish that the strength of response for the lung and liver neoplasms was less than that for the other neoplasm types. Dr. Dunnick noted that, were the other neoplasms not present, the lung neoplasms would be classified only as *some evidence*. Dr. Drinkwater agreed that the lung and liver effects were weaker than *clear evidence* and moved that the conclusions be accepted as written. Dr. Klaunig seconded the motion, which was approved by six yes votes to two no votes (Drs. Chatman and Davis).

INTRODUCTION



o-NITROTOLUENE

CAS No. 88-72-2

Chemical Formula: C₇H₇NO₂ Molecular Weight: 137.14

Synonyms: 2-Methylnitrobenzene; *o*-methylnitrobenzene; 2-nitrotoluene; 2-nitrotoluol

CHEMICAL AND PHYSICAL PROPERTIES

The nitrotoluenes are produced by the nitration of toluene with an aqueous acidic mixture of sulfuric acid and nitric acid at a temperature that starts at 25° C and is slowly raised to 37° C. The resulting product contains 55% to 60% *o*-nitrotoluene, 3% to 4% *m*-nitrotoluene, and 35% to 40% *p*-nitrotoluene. The isomers may be separated by a combination of fractional distillation and crystallization (*Kirk-Othmer*, 1981). Isomers of nitrotoluene differ in the position of the nitro group in relation to the methyl group on the benzene ring. While the chemical formula is the same for all isomers, their chemical and physical properties vary (Table 1).

PRODUCTION, USE, AND HUMAN EXPOSURE

o-Nitrotoluene and *p*-nitrotoluene are important commercial chemicals used to synthesize agricultural and rubber chemicals, azo and sulfur dyes, and dyes for cotton, wool, silk, leather, and paper. An estimated 29 million pounds of the *ortho* isomer and 15 million

pounds of the *para* isomer are used annually in the United States. The third isomer, *m*-nitrotoluene, is produced in negligible quantities (*Kirk-Othmer*, 1981; Abshire and Hughes, 1982). *o*-Nitrotoluene is on the U.S. Environmental Protection Agency's (1999) list of high production volume chemicals with an estimated production in 1990 of 45 million to 62 million pounds per year.

Environmental surveys have detected *o*-nitrotoluene in rivers and drinking water (USEPA, 1976); all three isomers of nitrotoluene have been found in wastewater effluent and atmospheric emissions from industrial plants (Forsten, 1973; USEPA, 1976). Microbial systems are capable of biodegrading nitroaromatic compounds (Spain, 1995). The Occupational Safety and Health Administration set an 8-hour, time-weighted average (TWA) permissible exposure limit of 5 ppm (30 mg/m³) for nitrotoluenes (NIOSH, 1997), and the American Conference of Governmental Industrial Hygienists (2000) recommended a threshold limit value of 2 ppm (11 mg/m³) for the 8-hour TWA.

TABLE 1
Chemical and Physical Properties of the Nitrotoluenes^a

	<i>o</i> -Nitrotoluene	<i>m</i> -Nitrotoluene	<i>p</i> -Nitrotoluene
Boiling point	220.4° C	232.6° C	238.3° C
Melting point	-9.3° C	15° C	51.7° C
Density (20° C)	1.163	1.157	1.286
Solubility (H ₂ O, 30° C)	652 mg/L	498 mg/L	442 mg/L
Volatility (20° C)	0.1 mm Hg	0.1 mm Hg	0.1 mm Hg
Volatility (30° C)	0.25 mm Hg	0.25 mm Hg	0.25 mm Hg
Log octanol/water partition coefficient	2.30	2.40	2.37

^a Verschueren, 1983; NTP, 1992

The National Occupational Exposure Survey (1981-1983) (NIOSH, 1990) found exposure to *p*-nitrotoluene among workers in five different occupational groups: biological technicians; painting and paint-spraying machine operators; machine operators; welders and cutters; and operators of separating, filtering, and clarifying machines. The latter group accounted for approximately 60% of potential exposures. Data on potential workplace exposure for the *ortho* and *meta* isomers were not available. An estimated 4,350 people in the United States are potentially exposed to *p*-nitrotoluene in the workplace.

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Experimental Animals

The comparative metabolism of *o*-, *m*-, and *p*-nitrotoluene administered orally was studied in F344 rats (Chism *et al.*, 1984; deBethizy and Rickert, 1984; Chism and Rickert, 1985; Rickert *et al.*, 1987). Following an oral dose of the three radiolabeled compounds as individual chemicals, 73% to 86% of the dose was excreted in the urine within 72 hours. Fecal excretion accounted for 5% to 13% of the dose, and minimal amounts of radiolabel were captured in expired breath (Chism *et al.*, 1984).

Metabolism studies (Appendix L) were designed to compare the *in vivo* metabolism of *o*-nitrotoluene in F344/N rats and B6C3F₁ mice and to determine the effects of dose and of repeated dosing on rates and routes of excretion. The routes of excretion were similar in rats

and mice, with the predominant route being via urine. The slightly higher amount of [¹⁴C]-*o*-nitrotoluene-derived radioactivity in feces of mice can be attributed to contamination of the feces by urine. The rate of excretion was more rapid in rats, with about 90% of the administered radioactivity excreted in urine in the first 24 hours. Less than 70% of the administered radioactivity was excreted in urine by mice in the same time period.

There is good agreement between the current comparative metabolism studies (Appendix L) and those reported by Chism *et al.* (1984) on the major urinary metabolites in male rats. *o*-Aminobenzyl alcohol, a major metabolite identified in the current comparative metabolism studies, was not identified by Chism *et al.* (1984). *o*-Toluidine was also identified as a minor metabolite in some samples in the current studies. The urinary profile after 11 daily doses of 200 mg *o*-nitrotoluene/kg body weight indicated a decreased excretion of *o*-nitrobenzylmercapturic acid, but no other obvious changes (Appendix L).

o-Nitrobenzoic acid and *o*-nitrobenzyl glucuronide are the only metabolites that could be identified in the urine of male mice (Table L3). The major urinary metabolites of the three nitrotoluene isomers that have been identified in rats and mice are shown in Table 2. A detailed metabolic scheme for *o*-nitrotoluene is shown in Figure 1.

All three isomers apparently are converted to the corresponding benzyl alcohol and benzoic acid (Table 2); the *meta* and *para* isomers undergo conjugation with

TABLE 2
Urinary Metabolites of *o*-, *m*-, and *p*-Nitrotoluene in Male Rats and Mice
Administered Gavage Doses of 200 mg/kg^a

	<i>o</i> -Nitrotoluene	<i>m</i> -Nitrotoluene	<i>p</i> -Nitrotoluene
Rats^b	<i>o</i> -Nitrobenzoic acid (29%) <i>o</i> -Nitrobenzyl glucuronide (14%) S-(<i>o</i> -nitrobenzyl)-N-acetylcysteine (12%)	<i>m</i> -Nitrobenzoic acid (21%) <i>m</i> -Nitrohippuric acid (24%) <i>m</i> -Acetamidobenzoic acid (12%)	<i>p</i> -Nitrobenzoic acid (28%) <i>p</i> -Nitrohippuric acid (13%) <i>p</i> -Acetamidobenzoic acid (27%)
Mice^c	<i>o</i> -Nitrobenzoic acid (38%) <i>o</i> -Nitrobenzyl glucuronide (24%)	<i>m</i> -Nitrohippuric acid (52%) <i>m</i> -Nitrobenzoic acid (19%)	<i>p</i> -Nitrohippuric acid (20%) 2-Methyl-5-nitrophenyl glucuronide (13%) 2-Methyl-5-nitrophenyl sulfate (19%)

^a Percentage of administered dose

^b Chism *et al.*, 1984

^c Appendix L; RTI, 1995, 1996a,b

glycine to form the hippuric acid, or nitro reduction and acylation. For *o*-nitrotoluene, formation of *o*-nitrobenzyl alcohol glucuronide is a major metabolic pathway. Conjugation with glucuronic acid is not a major metabolic route for the *meta* and *para* isomers. *o*-Nitrobenzyl glucuronide is excreted via the bile into the intestine, where bacterial enzymes hydrolyze the glucuronic acid and reduce the nitro group to form *o*-aminobenzyl alcohol. *o*-Aminobenzyl alcohol is reabsorbed and further metabolized by hepatic enzymes to a species capable of covalent binding to hepatic DNA. Studies by Chism and Rickert (1985) suggested that *o*-aminobenzyl sulfate is the metabolite of *o*-nitrotoluene responsible for binding covalently to DNA (Figure 2).

An analogous metabolic pathway is followed by 2,6-dinitrotoluene (2,6-DNT), which is oxidized in the liver to 2,6-dinitrobenzyl alcohol and then conjugated with glucuronic acid and excreted in the bile (Kedderis *et al.*, 1984). Intestinal microflora hydrolyze the glucuronide and reduce the nitro group to form 2-amino-6-nitrobenzyl alcohol. A portion of this metabolite is reabsorbed from the intestine and oxidized to a hydroxylamine by hepatic enzymes. The hydroxylamine is then conjugated with sulfate by hepatic sulfotransferase. The unstable N,O-sulfate decomposes to form an electrophilic nitrenium ion that can react with cellular nucleophiles such as DNA. This electrophilic ion is formed in the liver, hence the high carcinogenic activity of

2,6-DNT for rodent liver. 2,6-DNT is more active than 2,4-DNT in an *in vivo/in vitro* hepatocyte unscheduled DNA synthesis assay (Mirsalis and Butterworth, 1980).

The metabolic profiles for 2,6-DNT and *o*-nitrotoluene are similar (Rickert *et al.*, 1987). Both are excreted as glucuronides into the intestine where bacterial enzymes reduce the nitro groups; the aminobenzyl alcohols are reabsorbed and further metabolized in the liver to electrophilic compounds which presumably can interact with DNA. Binding of 2,6-DNT and *o*-nitrotoluene to rat hepatic DNA is decreased by pretreatment with sulfotransferase inhibitors, suggesting that the final step in the activation of each chemical is formation of an unstable N,O-sulfate that decomposes to yield an electrophilic nitrenium ion.

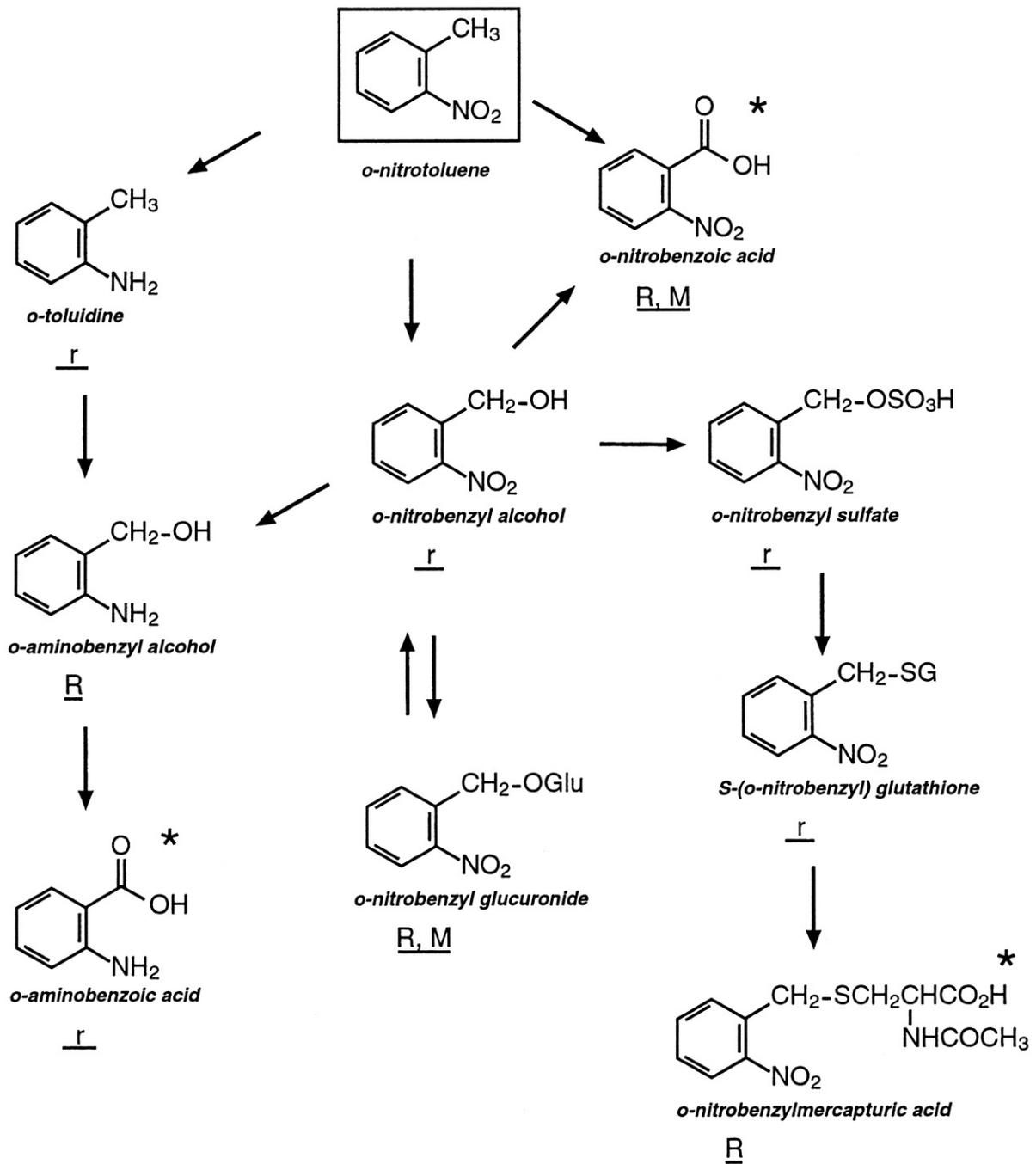
Humans

No information on the absorption, distribution, metabolism, or excretion of *o*-, *m*-, or *p*-nitrotoluene in humans was found in a review of the literature.

TOXICITY

Experimental Animals

Oral LD₅₀ values are 891 mg/kg (rats) and 2,463 mg/kg (mice) for *o*-nitrotoluene, 1,072 mg/kg (rats) and 330 mg/kg (mice) for *m*-nitrotoluene, and 2,144 mg/kg



* Measured in urine in NTP studies

FIGURE 1

Composite Metabolic Scheme for *o*-Nitrotoluene in Rats and Mice (Chism *et al.*, 1984; Appendix L)

Abbreviations: Major (R) or minor (r) urinary metabolite in rats; major (M) metabolite in mice.

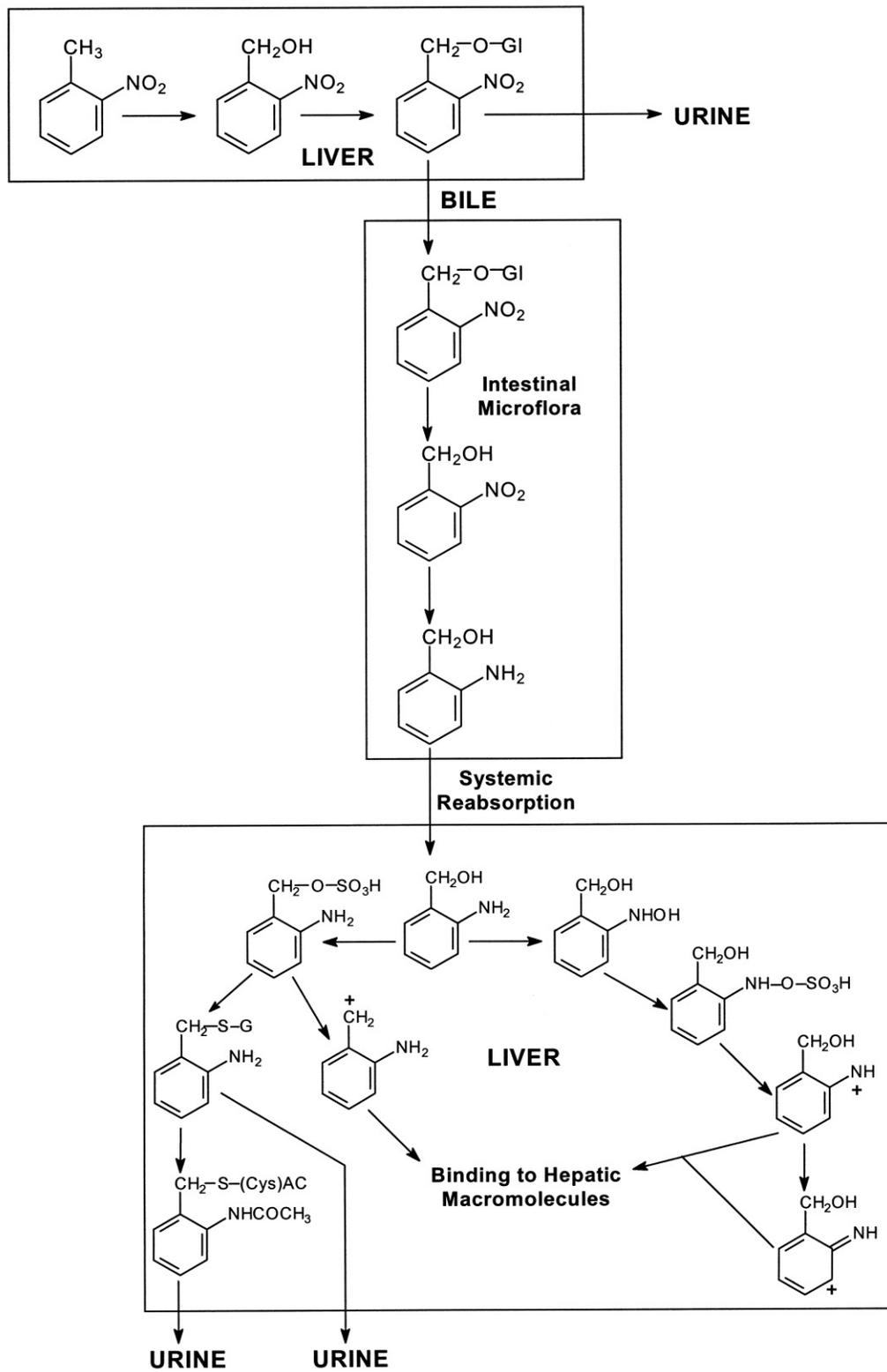


FIGURE 2
Proposed Pathway for Bioactivation of *o*-Nitrotoluene (Chism and Rickert, 1985)

(rats) and 1,231 mg/kg (mice) for *p*-nitrotoluene. These acute toxicity studies did not include histopathologic examination of tissues (Ciss *et al.*, 1980a,b).

In 14-day studies (NTP, 1992), *o*-, *m*-, or *p*-nitrotoluene was administered in feed to male and female F344/N rats and B6C3F₁ mice at concentrations ranging from 388 to 20,000 ppm (equivalent to average daily doses of 55 to 900 mg nitrotoluene/kg body weight). There were no effects on survival or clinical findings of toxicity in these studies, although animals exposed to the higher concentrations showed decreases in body weight gains relative to the controls.

In 13-week studies (NTP, 1992), *o*-, *m*-, or *p*-nitrotoluene was given to male and female F344/N rats and B6C3F₁ mice in feed at concentrations from 625 to 10,000 ppm. The estimated daily doses based on measures of feed consumption were similar for each of the three isomers and ranged from 40 to 725 mg nitrotoluene/kg body weight per day for rats and 45 to 680 mg/kg per day for mice. There were no effects on survival, and clinical findings of toxicity were limited to decreases in feed consumption. Decreased body weight gains occurred in exposed rats and mice at the higher exposure concentrations and were most pronounced in rats receiving *o*-nitrotoluene.

In the 13-week NTP (1992) studies, toxicity to the kidney, spleen, and testis occurred in rats receiving any of the three isomers, and toxicity to the liver and mesothelium occurred in male rats given *o*-nitrotoluene (Table 3). Kidney toxicity in male rats was characterized by the presence of hyaline droplets in tubule epithelial cells, attributed to an increase in the level of α 2u-globulin (as determined by ELISA). No granular casts were seen, and this was considered to be only minimal toxicity to the kidney. In the *p*-nitrotoluene study, pigment, possibly lipofuscin, and karyomegaly were present in the renal tubule epithelium of exposed male and female rats. In the spleen of exposed male and female rats, there were mild increases in the incidences of hematopoiesis, hemosiderin deposition, and/or congestion; this effect was most severe with the *para* isomer, followed by the *ortho* and then the *meta* isomer. Administration of *o*-, *m*-, or *p*-nitrotoluene impaired testicular function in the rat, shown by degeneration of the testis and reduction in sperm concentration, motility, and spermatid number. All three isomers increased the length of the estrous cycle in rats. Hepatic toxicity was characterized by cytoplasmic vacuolization, oval cell

hyperplasia, an increase in the concentration of serum bile acids, and increased sorbitol dehydrogenase and alanine aminotransferase activities in male rats given *o*-nitrotoluene. There was no histopathologic evidence of liver toxicity in male or female rats with the *meta* or *para* isomers or in female rats with the *ortho* isomer, but evidence of liver injury was observed in these groups, as indicated by increased relative liver weights and elevated bile acid concentrations and liver enzyme activities in serum. Mesotheliomas of the tunica vaginalis were observed in three of 10 male rats receiving 5,000 ppm *o*-nitrotoluene, and mesothelial cell hyperplasia was observed in two of 10 male rats receiving 10,000 ppm *o*-nitrotoluene (Table 4).

The only histopathologic evidence of toxicity in mice in the 13-week studies (NTP, 1992) occurred in animals receiving *o*-nitrotoluene, which caused degeneration and metaplasia of the olfactory epithelium. No liver lesions were noted in mice, but the three isomers caused increases in relative liver weights. There was no toxicity to the reproductive system in male or female mice treated with any of the nitrotoluene isomers. No immunotoxicity evaluation was conducted for *o*-nitrotoluene.

Humans

No epidemiological studies or reports of adverse health effects related to exposure to *o*-, *m*-, or *p*-nitrotoluene were found in a review of the literature.

CARCINOGENICITY

Experimental Animals

Interest in the carcinogenicity of mononitrotoluenes stems from the results of long-term rodent studies using technical-grade DNT, 2,4-DNT, or 2,6-DNT. Results of these studies suggest that 2,6-DNT is a potent carcinogen in rat liver (Rickert *et al.*, 1984). The results of Weisburger *et al.* (1978) suggested *ortho*-substituted aromatic compounds are more potent carcinogens than corresponding isomers with *meta* or *para* substitutions; this was seen with *o*-, *m*-, and *p*-toluidine in rats and mice, as well as with other compounds. The toluidine studies are of interest because reduction of the nitro group of the nitrotoluenes yields the corresponding toluidine. The National Cancer Institute (1979) reported that *o*-toluidine hydrochloride was carcinogenic in 2-year studies in male rats (mesotheliomas, splenic sarcomas, subcutaneous fibromas), in female rats

TABLE 3
Summary of Selected Treatment-Related Effects in the 13-Week Nitrotoluene Feed Studies^a

	<i>o</i> -Nitrotoluene		<i>m</i> -Nitrotoluene		<i>p</i> -Nitrotoluene	
	Male	Female	Male	Female	Male	Female
Rats						
Final mean body weight (90% or less of controls)	2,500	2,500	10,000	10,000	5,000	10,000
Changes in hematology parameters	2,500	2,500	5,000	5,000	2,500	2,500
Liver						
Increased relative weight	625	625 ^b	10,000	10,000	5,000	10,000
Increased ALT	5,000	—	—	5,000	—	10,000
Increased SDH	2,500	—	—	—	—	—
Increased bile acids	5,000	10,000	5,000	10,000	10,000	—
Nonneoplastic lesions	2,500	—	—	—	—	—
Kidney						
Increased relative weight	2,500	1,250	10,000	5,000	5,000	10,000
Nonneoplastic lesions	1,250	2,500	625	—	625	625
Spleen						
Nonneoplastic lesions	1,250	2,500	2,500	2,500	625	625
Testis						
Decreased spermatid count	5,000	—	10,000	—	10,000	—
Nonneoplastic lesions	5,000	—	10,000	—	10,000	—
Epididymal mesothelium						
Neoplastic and preneoplastic lesions	5,000	—	—	—	—	—
Increased estrous cycle length	—	10,000	—	5,000	—	10,000
Mice						
Final mean body weight (90% or less of controls)	2,500	2,500	10,000	10,000	10,000	10,000
Nose						
Nonneoplastic lesions	1,250	1,250	—	—	—	—
Liver						
Increased relative weight	2,500	1,250	625	625	625	625

^a NTP, 1992; lowest exposure group (ppm) in which a chemical-related effect was seen; ALT=alanine aminotransferase; SDH=sorbitol dehydrogenase

^b Not observed in any exposure group

TABLE 4
Treatment-Related Lesions in F344/N Rats and B6C3F₁ Mice in the 13-Week Feed Studies of o-Nitrotoluene^a

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Male Rats						
Pancreatic Islets ^b	10	10	10	10	10	10
Cellular Hyperplasia ^c	0	0	0	1 (1.0) ^d	7** (1.0)	10** (1.6)
Liver	10	10	10	10	10	10
Inflammation	5 (1.8)	5 (1.0)	5 (1.6)	10* (1.5)	10* (1.8)	8 (1.8)
Vacuolization	0	0	0	6** (1.3)	9** (1.8)	10** (3.0)
Bile Duct Hyperplasia	0	0	0	2 (1.0)	10** (1.2)	10** (2.2)
Kidney	10	10	10	10	10	10
Nephropathy, Hyaline Droplet	0	0	6** (1.0)	10** (1.6)	10** (2.8)	9** (2.6)
Regeneration	2 (1.0)	6 (1.0)	2 (1.0)	2 (1.0)	5 (1.0)	6 (1.1)
Pigmentation	0	0	0	0	1 (1.0)	10** (1.0)
Spleen	10	10	10	10	10	10
Hematopoiesis	0	0	0	6** (1.3)	10** (2.0)	10** (2.0)
Pigmentation	0	0	0	7** (1.3)	10** (2.0)	10** (2.0)
Capsular Hyperplasia	0	0	1 (1.0)	1 (2.0)	1 (1.0)	9** (1.9)
Salivary Gland	10	10	10	10	10	10
Atrophy	0	0	10** (2.2)	10** (3.1)	10** (3.0)	10** (3.0)
Testis	10	10	10	10	10	10
Seminiferous Tubule Degeneration	0	0	0	0	10** (2.3)	10** (4.0)
Epididymis (Mesothelium)	10	10	10	10	10	10
Hyperplasia ^e	0	0	0	0	0	2
Mesothelioma ^f	0	0	0	0	3	0
Preputial Gland	10	10	10	10	10	10
Atrophy	0	0	4* (1.0)	6** (1.0)	10** (1.9)	10** (1.9)
Female Rats						
Pancreatic Islets	10	10	0	0	10	10
Cellular Hyperplasia	0	0			0	2 (1.0)
Kidney	10	10	10	10	10	10
Pigmentation	0	0	0	3 (1.0)	10** (1.1)	10** (1.8)
Salivary Gland	10	10	10	10	10	10
Atrophy	0	0	10** (1.5)	10** (3.0)	10** (3.0)	10** (2.0)
Spleen	10	10	10	10	10	10
Hematopoiesis	0	0	0	0	1 (1.0)	10** (1.0)
Pigmentation	0	0	0	5* (1.0)	9** (2.0)	10** (2.0)
Capsular Hyperplasia	0	0	0	0	1 (1.0)	2 (1.0)
Clitoral Gland	10	10	10	9	10	10
Atrophy	0	0	0	0	2 (1.0)	10** (1.5)

TABLE 4
Treatment-Related Lesions in F344/N Rats and B6C3F₁ Mice in the 13-Week Feed Studies of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,500 ppm	5,000 ppm	10,000 ppm
Male Mice						
Nose						
Olfactory Epithelium	10	10	10	10	10	10
Degeneration/Metaplasia	0	0	1 (1.0)	2 (1.0)	10** (2.0)	10** (3.0)
Female Mice						
Nose						
Olfactory Epithelium	10	10	10	10	10	10
Degeneration/Metaplasia	0	0	2 (1.5)	9** (1.0)	10** (1.9)	10** (2.9)

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

** $P \leq 0.01$

^a Data presented by Dunnick *et al.* (1994) and PATHCO, Inc. (2000)

^b Number of animals with tissue examined microscopically

^c Number of animals with lesion

^d Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

^e Preneoplastic lesion

^f Neoplasm

(splenic sarcomas, urinary bladder transitional cell tumors, tumors of the mammary gland), in male mice (hemangioma, hemangiosarcoma), and in female mice (liver tumors).

Humans

No epidemiological studies of *o*-, *m*-, or *p*-nitrotoluene were found in a review of the literature.

GENETIC TOXICITY

Testing of the mononitrotoluenes *in vitro* for mutagenicity has generally yielded negative results, although occasional positive responses in various assays have been reported. A recent review of mutagenicity data for the mononitrotoluenes was provided by the International Agency for Research on Cancer (1996). The aromatic nitro group of the nitrotoluenes is considered a structural alert to potential DNA reactivity (Tennant and Ashby, 1991), but such activity would presumably be dependent upon the metabolic capability of the test system. For example, reduction of the nitro group to produce an aromatic amine would likely be necessary for a positive response in the *Salmonella typhimurium* assay.

Although *o*- and *m*-nitrotoluene demonstrated no mutagenic activity in any of several strains of *S. typhimurium*, with or without S9 metabolic activation, isolated positive responses were reported for *p*-nitrotoluene in strain TA100, with and without S9 (Chiu *et al.*, 1978; Miyata *et al.*, 1981; Spangord *et al.*, 1982; Haworth *et al.*, 1983; Suzuki *et al.*, 1983; Shimizu and Yano, 1986; Kawai *et al.*, 1987). *p*-Nitrotoluene also induced cell growth inhibition, a measure of DNA damage, in *Bacillus subtilis* M45/H17 in the absence of S9 (Shimizu and Yano, 1986); *o*-nitrotoluene was weakly positive in this assay, and results with *m*-nitrotoluene were negative.

All three mononitrotoluene isomers induced sister chromatid exchanges in cultured Chinese hamster ovary (CHO) cells; only *m*-nitrotoluene required S9 for a positive response (Galloway *et al.*, 1987). *p*-Nitrotoluene induced chromosomal aberrations in cultured CHO cells in the presence of S9 (Galloway *et al.*, 1987), but no increases in micronuclei or chromosomal aberrations were observed in bone marrow cells of male B6C3F₁ mice administered *p*-nitrotoluene as a single intraperitoneal injection (Furukawa *et al.*, 1989; Ohuchida *et al.*, 1989).

No induction of unscheduled DNA synthesis was observed in male F344 rat hepatocytes or spermatocytes treated with *m*- or *p*-nitrotoluene in the standard *in vitro* assay (Doolittle *et al.*, 1983; Working and Butterworth, 1984) or *in vivo* (Doolittle *et al.*, 1983; Butterworth *et al.*, 1989; Mirsalis *et al.*, 1989). Positive results were obtained, however, in an *in vitro* unscheduled DNA synthesis assay employing serum-free medium (Parton *et al.*, 1995). *o*-Nitrotoluene was also negative in the *in vitro* unscheduled DNA synthesis assay, but in male F344 rats treated *in vivo*, a strongly positive response was observed (Doolittle *et al.*, 1983). No induction of unscheduled DNA synthesis by *o*-nitrotoluene was noted in germ free male rats, indicating that activation of *o*-nitrotoluene or an intermediate metabolic conjugate by intestinal bacteria is necessary to the process. No induction of unscheduled DNA synthesis was observed in hepatocytes of female rats treated with *o*-nitrotoluene *in vivo*; differences in *in vivo* results between males and females may be due to differences in hepatic metabolism or disposition of *o*-nitrotoluene. Different results between males and females have been attributed to males excreting more of the glucuronide conjugates of the nitrotoluenes into the bile and, subsequently, into the intestine where they are metabolized further by bacterial systems. Sex-related differences in metabolism have also been observed with the dinitrotoluenes in rats (Rickert and Long, 1981).

Covalent binding to DNA was measured in hepatocytes of male F344 rats after a single oral dose of *o*-, *m*-, or *p*-nitrotoluene (Rickert *et al.*, 1987). Only *o*-nitrotoluene bound DNA, whereas all three isomers bound protein.

STUDY RATIONALE

The National Institute for Occupational Safety and Health and the NTP nominated the nitrotoluenes for rodent toxicity and carcinogenicity studies based on the considerable human exposure to these chemicals as well as the lack of long-term studies of carcinogenicity in rodents. This Technical Report describes the results of the 2-year studies of *o*-nitrotoluene in F344/N rats and B6C3F₁ mice. The 2-year studies of *p*-nitrotoluene are reported in a companion Technical Report (NTP, 2002).

The exposure concentrations for the core 2-year *o*-nitrotoluene studies were selected based on the findings from the 13-week studies (NTP, 1992). In those studies, body weight effects and/or liver, spleen, and kidney toxicities were seen primarily in rats at exposure concentrations of 2,500 ppm or greater. Male rats receiving 5,000 or 10,000 ppm in those studies had low incidences of mesothelioma or mesothelial hyperplasia after 13 weeks of exposure. The exposure concentrations selected for the 2-year rat studies were 625, 1,250, and 2,000 ppm. A 3-month stop-exposure study in male rats exposed to 2,000 or 5,000 ppm was included to determine the progression/regression of mesothelial lesions and to study the relationship between short-term and 2-year exposures in the development of neoplasms.

In the 13-week study in mice, final mean body weights of 10,000 ppm mice were 22% to 30% less than those of the controls. The exposure concentrations selected for the 2-year study in mice were 1,250, 2,500, and 5,000 ppm, doses at which minimal or no toxicity was seen in the 13-week study.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF *o*-NITROTOLUENE

o-Nitrotoluene was obtained from Aldrich Chemical Company (Milwaukee, WI) in one lot (8056-58-05RTI). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Research Triangle Institute (Research Triangle Park, NC) and the study laboratory (Appendix H). Reports on analyses performed in support of the *o*-nitrotoluene studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a yellow-green liquid, was identified as *o*-nitrotoluene by infrared, ultraviolet/visible, proton nuclear magnetic resonance, and low- and high-resolution mass spectroscopy. The purity of lot 8056-58-05RTI was determined by Karl Fischer water analysis and gas chromatography. Karl Fischer analysis indicated 0.29% water. Gas chromatography by one system indicated one major peak, one impurity with an area of 0.11%, and five minor impurities, each with an area less than 0.1% relative to the major peak; a second system indicated one major peak and six impurities, each with an area less than 0.1% relative to the major peak. A third gas chromatography system indicated no impurities. The overall purity was determined to be greater than 99%.

Stability studies of the bulk chemical were performed by the analytical chemistry laboratory using gas chromatography. These studies indicated that *o*-nitrotoluene was stable as a bulk chemical for at least 2 weeks when stored protected from light at temperatures up to 25° C. To ensure stability, the bulk chemical was stored in amber glass bottles inside metal cans at room temperature. Stability was monitored during the studies using gas chromatography. No degradation of the bulk chemical was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared every 2 weeks by mixing *o*-nitrotoluene with feed (Table H2). Homogeneity and stability studies of the 625 ppm dose formulation and homogeneity studies of the 5,000 ppm dose formulation in nonirradiated NTP-2000 feed were performed by the analytical chemistry laboratory using gas chromatography. Homogeneity studies of the 625, 2,000, and 5,000 ppm dose formulations in irradiated feed were performed by the study laboratory. Homogeneity was confirmed in each study. Stability of the dose formulations was confirmed for 36 days when stored in sealed containers protected from light at temperatures up to 3° C; significant chemical losses due to volatility were seen in the dose formulations under simulated animal room conditions.

Analyses of the dose formulations of *o*-nitrotoluene were conducted at the study laboratory using gas chromatography every 8 to 12 weeks. All dose formulations used were 90% to 115% of the target concentrations. Because of the expected volatility losses during formulation, dose formulations were prepared at up to 115% of the target concentrations. Animal room samples for rats ranged from 75% to 94% and those for mice ranged from 53% to 81% of the target concentrations.

2-YEAR STUDIES

Study Design

In the core study, groups of 60 male and 60 female rats were fed diets containing 625, 1,250, or 2,000 ppm *o*-nitrotoluene for 105 weeks. In a 3-month stop-exposure study, groups of 70 male rats were fed diets containing 2,000 or 5,000 ppm *o*-nitrotoluene for 13 weeks followed by undosed feed for the remainder of the study. A group of 70 male rats receiving undosed feed served as a control group for both male rat studies; 60 female

rats receiving undosed feed were the control group for the female core study. Ten control males and 10 males from each stop-exposure group were sacrificed at 3 months. Groups of 60 male and 60 female mice were fed diets containing 0, 1,250, 2,500, or 5,000 ppm o-nitrotoluene for 105 weeks. An interim evaluation planned for all groups at 15 months was not done due to high mortality in the exposed groups.

Source and Specification of Animals

Male and female F344/N rats and B6C3F₁ mice were obtained from Taconic Laboratory Animals and Services (Germantown, NY) for use in the 2-year studies. Rats and mice were quarantined for 12 to 14 days before the beginning of the studies. Five male and five female rats and mice were randomly selected for parasite evaluation and gross observation of disease. Rats and mice were approximately 6 to 7 weeks old at the beginning of the studies. The health of the animals was monitored during the studies according to the protocols of the NTP Sentinel Animal Program (Appendix K).

Animal Maintenance

Rats were housed up to three (males) or five (females) per cage; mice were housed individually (males) or five (females) per cage. Rats and mice were housed individually during urine collection periods. Feed and water were available *ad libitum*. Feed consumption was measured every 4 weeks. The estimate of dose delivered to the animals (mg/kg) was based on body weight and feed consumption data collected during the course of the 2-year studies and targeted chemical concentration in the feed. Animals were given nonirradiated feed from the beginning of the studies until 3 (rats) or 8 (mice) July 1996 and irradiated feed thereafter. The feed was irradiated to reduce microbial contamination. Cages and racks were rotated every 2 weeks. Dose formulations were replaced in animal room feeders on a 2-day, 2-day, 3-day schedule, due to the formulations' instability under animal room conditions. Further details of animal maintenance are given in Table 5. Information on feed composition and contaminants is provided in Appendix J.

Clinical Examinations and Pathology

All animals were observed twice daily. Clinical findings were recorded every 4 weeks. Animals were weighed initially, during week 4, and every 4 weeks thereafter. Ten male rats in the 0 ppm group and the 2,000 and

5,000 ppm stop-exposure groups were designated for interim evaluation at 3 months.

Complete necropsies and microscopic examinations were performed on all rats and mice. At the 3-month interim evaluation in male rats, the heart, right kidney, liver, lungs, right testis, and thymus were weighed. At necropsy, all organs and tissues were examined for grossly visible lesions, and all major tissues were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 μm , and stained with hematoxylin and eosin for microscopic examination. For all paired organs (e.g., adrenal gland, kidney, ovary), samples from each organ were examined. Tissues examined microscopically are listed in Table 5.

Microscopic evaluations were completed by the study laboratory pathologist, and the pathology data were entered into the Toxicology Data Management System. 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 evaluated by an independent quality assessment laboratory. The individual animal records and tables were compared for accuracy, the slide and tissue counts were verified, and the histotechnique was evaluated. For the 2-year studies, a quality assessment pathologist evaluated slides from all tumors and all potential target organs, which included the bone marrow, kidney, liver, lung, mammary gland, pituitary gland, salivary gland, skin, and spleen of male and female rats; the epididymis, pancreatic islets, peritoneum, preputial gland, prostate gland, and testis of male rats; the clitoral gland and mandibular lymph node of female rats; and the large intestine (cecum), kidney, liver, and stomach (forestomach and glandular) of male and female mice.

The quality assessment report and the reviewed slides were submitted to the NTP Pathology Working Group (PWG) chairperson, who reviewed the selected tissues and addressed any inconsistencies in the diagnoses made by the laboratory and quality assessment pathologists. Representative histopathology slides containing examples of lesions related to chemical administration, examples of disagreements in diagnoses between the laboratory and quality assessment pathologists, or lesions of general interest were presented by the chair person to the PWG for review. The PWG consisted of the quality assessment pathologist and other pathologists

experienced in rodent toxicologic pathology. This group examined the tissues without any knowledge of dose groups or previously rendered diagnoses. When the PWG consensus differed from the opinion of the laboratory pathologist, the diagnosis was changed. Final diagnoses for reviewed lesions represent a consensus between the laboratory pathologist, reviewing pathologist(s), and the PWG. Details of these review procedures have been described, in part, by Maronpot and Boorman (1982) and Boorman *et al.* (1985). For subsequent analyses of the pathology data, the decision of whether to evaluate the diagnosed lesions for each tissue type separately or combined was generally based on the guidelines of McConnell *et al.* (1986).

Urinary Metabolite Analyses

Five male and five female rats and mice from each core study group were randomly selected for urinary metabolite analyses at 2 weeks and 3, 12, and 18 months (Appendix F). Animals were placed in metabolism cages for 24 hours. Urine samples were placed on ice, urine volume and creatinine concentration were measured, and then the samples were frozen pending metabolite analyses.

To establish the correlation between exposure concentration and internal dose and to determine how metabolism of *o*-nitrotoluene may change with chronic exposure and age, three urinary metabolites, *o*-nitrobenzoic acid, *o*-nitrobenzylmercapturic acid, and *o*-aminobenzoic acid were chosen as biomarkers based on the metabolism studies of Chism *et al.* (1984). Metabolite concentrations were determined by high-performance liquid chromatography (HPLC). An internal standard solution of anisic acid in water and sodium hydroxide was added to the rat urine samples, which were then diluted with trifluoroacetic acid in methanol and water. Triethylamine was used to adjust the pH to 2.3, and the resulting mixture was filtered and degassed by sonication. The samples were then analyzed by HPLC on a Zorbax 250 mm × 4.6 mm Rx-C₁₈ column (Varian, Palo Alto, CA) using ultraviolet detection at 254 nm (for *o*-aminobenzoic acid concentration) or 266 nm. Mouse urine data were interpreted with a standard curve generated from the rat urine data. The ratios obtained by dividing the metabolite concentration by the creatinine concentration, rather than the mass of metabolite excreted per 24 hours, were analyzed to normalize the metabolite to body weight and determine the ratio more precisely.

TABLE 5
Experimental Design and Materials and Methods in the 2-Year Feed Studies of o-Nitrotoluene

Study Laboratory

Southern Research Institute (Birmingham, AL)

Strain and Species

F344/N rats

B6C3F₁ mice

Animal Source

Taconic Laboratory Animals and Services (Germantown, NY)

Time Held Before Studies

Rats: 14 days (males) or 12 or 14 days (females)

Mice: 12 days

Average Age When Studies Began

Rats: 6-7 weeks

Mice: 6 weeks

Date of First Exposure

Rats: February 8, 1996

Mice: February 20, 1996

Duration of Exposure

105 weeks, except 2,000 and 5,000 ppm stop-exposure groups which received undosed feed from week 14 until the end of the studies

Date of Last Exposure

Core study rats: February 5-11, 1998

Stop-exposure rats: May 8, 1996

Mice: February 17-23, 1998

Average Age at Necropsy

Rats: 110-112 weeks

Mice: 110-111 weeks

Size of Study Groups

Rats: 60 males and 60 females [0 (females only), 625, 1,250, and 2,000 ppm groups]; 70 males (0 ppm and 2,000 and 5,000 ppm stop-exposure groups)

Mice: 60 males and 60 females

Method of Distribution

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

Animals per Cage

Rats: up to 3 (males) or 5 (females); 1 rat per cage during urine collection periods

Mice: 1 (males) or 5 (females); 1 mouse per cage during urine collection periods

Method of Animal Identification

Tail tattoo

Diet

NTP-2000 open formula meal/pelleted diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*. Animals received nonirradiated feed from the beginning of the studies until July 3 (rats) or 8 (mice), 1996 and irradiated feed thereafter.

TABLE 5
Experimental Design and Materials and Methods in the 2-Year Feed Studies of o-Nitrotoluene

Water

Tap water (Birmingham, AL, municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*

Cages

Polycarbonate (Lab Products, Inc., Maywood, NJ), changed twice weekly

Bedding

Heat-treated hardwood bedding chips (P.J. Murphy Forest Products, Inc., Montville, NJ), changed twice weekly

Cage Filters

Reemay[®] (Andico; Birmingham, AL), changed every 2 weeks

Racks

Stainless steel drawer type (Lab Products, Inc., Maywood, NJ), rotated every 2 weeks

Animal Room Environment

Temperature: 72° ± 3° F

Relative humidity: 50% ± 15%

Room fluorescent light: 12 hours/day

Room air changes: 10/hour

Exposure Concentrations

Core study rats: 0, 625, 1,250, or 2,000 ppm in feed

Stop-exposure rats: 2,000 or 5,000 ppm in feed

Mice: 0, 1,250, 2,500, or 5,000 ppm in feed

Type and Frequency of Observation

Observed twice daily; animals were weighed initially, during week 4, and every 4 weeks thereafter; clinical findings were recorded at 4-week intervals. Feed consumption was measured over a 1-week period every 4 weeks.

Method of Sacrifice

CO₂ asphyxiation

Necropsy

Necropsies were performed on all animals. Organs weighed at the 3-month interim evaluation were the heart, right kidney, liver, lungs, right testis, and thymus.

Urinalysis

Urine was collected during a 24-hour period from five male and five female rats and mice from each core study group at 2 weeks and 3, 12, and 18 months. Parameters evaluated included urine volume and creatinine, o-nitrobenzoic acid, o-nitrobenzylmercapturic acid, and o-aminobenzoic acid concentrations.

Histopathology

Complete histopathology was performed on all rats and mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung, lymph nodes (mandibular and mesenteric), mammary gland (except male mice), nose, ovary, pancreas, pancreatic islets, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, skin, spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, and uterus.

STATISTICAL METHODS

Survival Analyses

The probability of survival was estimated by the product-limit procedure of Kaplan and Meier (1958) and is presented in the form of graphs. Animals found dead of other than natural causes or missing were censored from the survival analyses; animals dying from natural causes were not censored. Statistical analyses for possible dose-related effects on survival used Cox's (1972) method for testing two groups for equality and Tarone's (1975) life table test to identify dose-related trends. All reported P values for the survival analyses are two sided.

Calculation of Incidence

The incidences of neoplasms or nonneoplastic lesions are presented in Tables A1, A5, B1, B5, C1, C5, D1, and D5 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. For calculation of statistical significance, the incidences of most neoplasms (Tables A3a, A3b, B3, C3, and D3) and all nonneoplastic lesions are given as the numbers of animals affected at each site examined microscopically. However, when macroscopic examination was required to detect neoplasms in certain tissues (e.g., hardierian gland, intestine, mammary gland, and skin) before microscopic evaluation, or when neoplasms had multiple potential sites of occurrence (e.g., leukemia or lymphoma), the denominators consist of the number of animals on which a necropsy was performed. Tables A3a, A3b, B3, C3, and D3 also give the survival-adjusted neoplasm rate for each group and each site-specific neoplasm. This survival-adjusted rate (based on the Poly-3 method described below) accounts for differential mortality by assigning a reduced risk of neoplasm, proportional to the third power of the fraction of time on study, to animals that do not reach terminal sacrifice.

Analysis of Neoplasm and Nonneoplastic Lesion Incidences

The Poly-k test (Bailer and Portier, 1988; Portier and Bailer, 1989; Piegorsch and Bailer, 1997) was used to assess neoplasm and nonneoplastic lesion prevalence. This test is a survival-adjusted quantal-response procedure that modifies the Cochran-Armitage linear trend test to take survival differences into account. More specifically, this method modifies the denominator in the quantal estimate of lesion incidence to approximate more closely the total number of animal years at risk.

For analysis of a given site, each animal is assigned a risk weight. This value is one if the animal had a lesion at that site or if it survived until terminal sacrifice; if the animal died prior to terminal sacrifice and did not have a lesion at that site, its risk weight is the fraction of the entire study time that it survived, raised to the kth power.

This method yields a lesion prevalence rate that depends only upon the choice of a shape parameter for a Weibull hazard function describing cumulative lesion incidence over time (Bailer and Portier, 1988). Unless otherwise specified, a value of $k=3$ was used in the analysis of site-specific lesions. This value was recommended by Bailer and Portier (1988) following an evaluation of neoplasm onset time distributions for a variety of site-specific neoplasms in control F344 rats and B6C3F₁ mice (Portier *et al.*, 1986). Bailer and Portier (1988) showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range from 1 to 5. A further advantage of the Poly-3 method is that it does not require lesion lethality assumptions. Variation introduced by the use of risk weights, which reflect differential mortality, was accommodated by adjusting the variance of the Poly-3 statistic as recommended by Bieler and Williams (1993).

Tests of significance included pairwise comparisons of each exposed group with controls and a test for an overall exposure-related trend. Continuity-corrected Poly-3 tests were used in the analysis of lesion incidence, and reported P values are one sided. The significance of lower incidences or decreasing trends in lesions are represented as 1-P with the letter N added (e.g., $P=0.99$ is presented as $P=0.01N$). For neoplasms and nonneoplastic lesions detected at the interim evaluation, the Fisher exact test (Gart *et al.*, 1979), a procedure based on the overall proportion of affected animals, was used.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between exposed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). To make pairwise comparisons, urinary biomarker data were analyzed by Fisher's least significant difference test (following initial ANOVA) (Miller, 1960). Average severity values were analyzed for significance with the Mann-Whitney U test (Hollander and Wolfe, 1973).

Historical Control Data

The concurrent control group represents the most valid comparison to the treated groups and is the only control group analyzed statistically in NTP bioassays. However, historical control data are often helpful in interpreting potential treatment-related effects, particularly for uncommon or rare neoplasm types. For meaningful comparisons, the conditions for studies in the historical database must be generally similar. Until recently, the NTP historical control database consisted of animals fed the NIH-07 diet. In 1995, the NTP changed the diet fed to animals used in toxicity and carcinogenesis studies conducted by the NTP. This new diet (NTP-2000) contains less protein and more fiber and fat than the NIH-07 diet previously used (Rao, 1996, 1997). This dietary change was instituted primarily to increase longevity and decrease the incidence and/or severity of some spontaneous neoplastic and nonneoplastic lesions in the rats and mice used in NTP studies. These studies of *o*-nitrotoluene are among the first in which the animals on study were fed the NTP-2000 diet. Because the incidence of some neoplastic and nonneoplastic lesions may be affected by the dietary change, use of the existing historical control database (NIH-07 diet) may not be appropriate for all neoplasm types.

Currently, the database includes 11 (10 for male rats) studies by various routes in which the NTP-2000 diet was used. Based on the extensive NTP historical database using the NIH-07 diet, incidences of the vast majority of spontaneous neoplasms are not significantly different between control groups regardless of the route of administration. There is no reason to expect this to be different with the NTP-2000 diet. For example, control animals from dosed feed and dosed water studies are treated no differently and no differences in incidence of neoplasms are expected. Exceptions exist for some neoplasms/routes, and if comparisons are necessary for these neoplasm types, only studies with similar routes of administration will be used.

Irradiated Feed

Ionizing energy (irradiation) is known to destroy most, if not all, bacterial and insect contamination without a significant loss of essential nutrients (Rao and Knapka, 1998). The NTP-2000 diet manufactured and used for the NTP studies was irradiated from May 1996 (fed to rats and mice after June 1996) at the FDA-approved level (25 to 50 kilogray) of ionizing radiation. Batches

of diets were evaluated for nutrient concentrations before and after irradiation. The concentrations of nutrients and their byproducts of irradiated diets were not substantially different from the same batches before irradiation and nutritionally adequate rodent diets.

QUALITY ASSURANCE METHODS

The 2-year studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). In addition, as records from the 2-year studies were submitted to the NTP Archives, these studies were audited retrospectively by an independent quality assurance contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Technical Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Technical Report.

GENETIC TOXICOLOGY

The genetic toxicity of *o*-nitrotoluene was assessed by testing the ability of the chemical to induce mutations in various strains of *Salmonella typhimurium*, sister chromatid exchanges and chromosomal aberrations in cultured Chinese hamster ovary cells, micronucleated erythrocytes in rat and mouse bone marrow, and increases in the frequency of micronucleated erythrocytes in mouse peripheral blood. The protocols for these studies and the results are given in Appendix E.

The genetic toxicity studies of *o*-nitrotoluene are part of a larger effort by the NTP to develop a comprehensive database that would permit a critical anticipation of a chemical's carcinogenicity in experimental animals based on numerous considerations, including the molecular structure of the chemical and its observed effects in short-term *in vitro* and *in vivo* genetic toxicity tests (structure-activity relationships). These short-term genetic toxicity tests were originally developed to clarify mechanisms of chemical-induced DNA damage growing out of the earlier electrophilicity/mutagenicity relationship proposed by Miller and Miller (1977) and the somatic mutation theory of cancer (Straus, 1981;

Crawford, 1985). Therefore, the information obtained from these tests applies only to mutagenic carcinogens.

For mutagenic carcinogens, the combination of DNA reactivity and *Salmonella* mutagenicity is highly correlated with the induction of carcinogenicity in multiple species and genders of rodents and at multiple tissue sites (Ashby and Tennant, 1991). Data from NTP studies show that a positive response in *Salmonella* is the most predictive *in vitro* test for rodent carcinogenicity (89% of the *Salmonella* mutagens are rodent carcinogens) and that there is no complementarity among the *in vitro* genetic toxicity tests (Tennant *et al.*, 1987; Zeiger *et al.*, 1990). That is, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. Although other *in vitro* genetic toxicity tests correlate less well with rodent carcinogenicity compared with the *Salmonella* test, these other tests can provide useful information on the types of DNA and chromosomal effects induced by the chemical under investigation.

The predictivity for carcinogenicity of a positive response in the acute *in vivo* bone marrow chromosome aberration test or micronucleus test appears to be less than that in the *Salmonella* test (Shelby *et al.*, 1993; Shelby and Witt, 1995). However, clearly positive results in long-term peripheral blood micronucleus tests are associated with high predictivity for rodent carcinogenicity (Witt *et al.*, 2000); negative results in this assay do not correlate well with either negative or positive results in rodent carcinogenicity studies. Because of the theoretical and observed associations between induced genetic damage and adverse effects in somatic and germ cells, the determination of *in vivo* genetic effects is important to the overall understanding of the risks associated with exposure to a particular chemical. Most organic chemicals that are identified by the International Agency for Research on Cancer as human carcinogens, other than hormones, are genotoxic. The vast majority of these are detected by both the *Salmonella* assay and rodent bone marrow cytogenetics tests (Shelby, 1988; Shelby and Zeiger, 1990).

RESULTS

RATS

Survival

Estimates of 2-year survival probabilities for male and female rats are shown in Table 6 and in the Kaplan-Meier survival curves (Figure 3). All 2,000 ppm core study, all 5,000 ppm stop-exposure, and all but three core study 1,250 ppm male rats died before the end of the study. Survival of 625 ppm core study and 2,000 ppm stop-exposure males and of 2,000 ppm females was significantly less than that of the controls. Due to the poor survival of exposed rats, the planned 15-month interim evaluation was cancelled.

Body Weights, Feed and Compound Consumption, and Clinical Findings

Mean body weights of all exposed groups of males except the 625 ppm group were generally less than those of the controls throughout the study (Table 7 and Figure 4). Mean body weights of 2,000 ppm females were less than those of the controls during year 2 of the study (Table 8 and Figure 4). Feed consumption by exposed males and females was similar to that by the controls throughout the study (Tables I1 and I2). Dietary concentrations of 625, 1,250, or 2,000 ppm resulted in average daily doses of approximately 25, 50, or 90 mg *o*-nitrotoluene/kg body weight to core study males and 30, 60, or 100 mg/kg to females. Dietary concentrations of 2,000 or 5,000 ppm to stop-exposure males resulted in average daily doses of approximately 125 or 315 mg/kg for the first 3 months of the study. Clinical findings included large subcutaneous masses in the torso, head, and appendages of exposed males and females; the numbers of these masses generally increased with exposure concentration. Males in both 2,000 ppm groups and the 5,000 ppm stop-exposure group had small ears and thin tails. Although the cause of the small ears and thin tails is unknown, the presence of this unusual clinical finding in both stop-exposure and core study groups suggests

that the initial insult occurred during the first 3 months of *o*-nitrotoluene exposure.

Biomarkers of Exposure

The results of urinary metabolite determinations in male and female rats are presented in Table F1. With the exception of the 2,000 ppm group at 18 months, the ratios of *o*-nitrobenzoic acid to creatinine excreted in the urine of male rats were significantly larger at 2 weeks than at the later time points. In females, by contrast, the differences between time points were generally not significant. Creatinine concentrations were generally lower at 2 weeks in males and females than at other time points, and the apparent time-related change in the ratios may not reflect a change in *o*-nitrotoluene metabolism. The *o*-nitrobenzoic acid/creatinine ratios were generally larger for females than for males and were linearly related to exposure concentration.

In contrast to the *o*-nitrobenzoic acid/creatinine ratios, larger ratios of *o*-nitrobenzylmercapturic acid to creatinine were seen in urine of all exposed groups at week 2 compared to later time points, with one exception. These ratios were significantly smaller for exposed females than for males. Because the first step in the formation of either *o*-nitrobenzoic acid or *o*-nitrobenzylmercapturic acid is oxidation of the methyl group to a benzyl alcohol, metabolic differences must be either in further oxidation to the carboxylic acid or in formation of conjugates of the alcohol and further reaction with reduced glutathione. The *o*-nitrobenzylmercapturic acid/creatinine ratios were linearly related to exposure concentration in males and females.

The ratios of *o*-aminobenzoic acid to creatinine for control and exposed male and female rats were generally similar. *o*-Aminobenzoic acid is a product of catabolism of tryptophan (White *et al.*, 1978) and is a relatively minor metabolite of *o*-nitrotoluene (Chism *et al.*, 1984). No time- or sex-related analyses of these data were performed.

TABLE 6
Survival of Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Male						
Animals initially in study	70	60	60	60	70	70
3-Month interim evaluation ^a	10	0	0	0	10	10
Accidental deaths ^a	0	0	3	0	0	0
Moribund	18	35	48	53	39	57
Natural deaths	3 ^b	7	6	7	10	3
Animals surviving to study termination	39 ^b	18	3	0	11	0
Percent probability of survival at end of study ^c	65	30	5	0	18	0
Mean survival (days) ^d	698	647	590	508	612	483
Survival analysis ^e	P<0.001	P<0.001	P<0.001	P<0.001		
Survival analysis ^f	P<0.001				P<0.001	P<0.001
Female						
Animals initially in study	60	60	60	60		
Moribund	10	11	19	22		
Natural deaths	3	2	2	5		
Animals surviving to study termination	47 ^g	47 ^b	39	33		
Percent probability of survival at end of study	78	78	65	55		
Mean survival (days)	712	706	696	697		
Survival analysis ^e	P=0.002	P=1.000	P=0.128	P=0.009		

^a Censored from survival analyses

^b Includes one animal that died during the last week of the study

^c Kaplan-Meier determinations

^d Mean of all deaths (uncensored, censored, and terminal sacrifice)

^e The result of the life table trend test (Tarone, 1975) for core study groups is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the exposed group columns.

^f Trend test and pairwise comparisons for stop-exposure groups

^g Includes two animals that died during the last week of the study

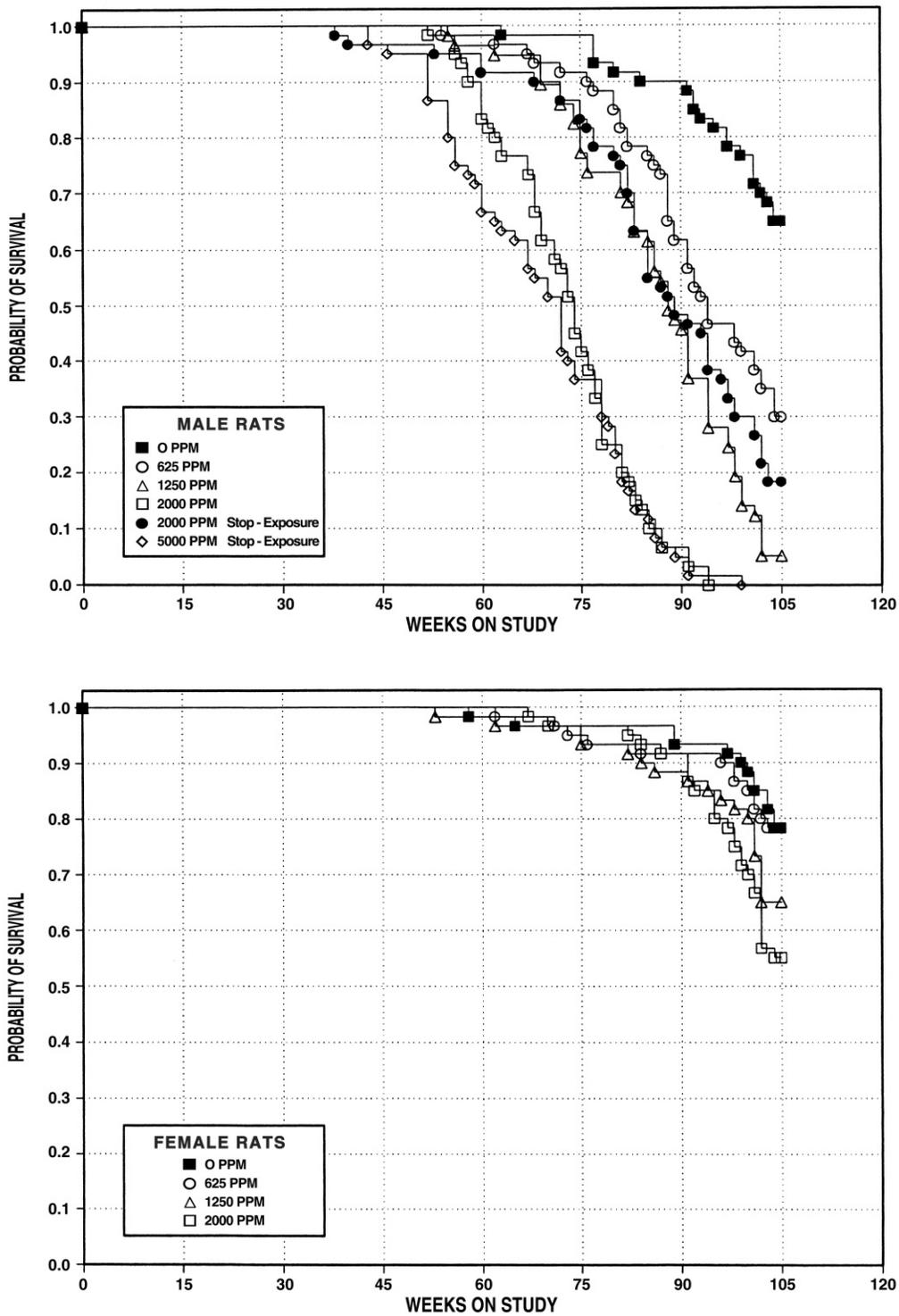


FIGURE 3
Kaplan-Meier Survival Curves for Male and Female Rats
Exposed to *o*-Nitrotoluene in Feed for 2 Years

TABLE 7
Mean Body Weights and Survival of Male Rats in the 2-Year Feed Study of *o*-Nitrotoluene

Weeks on Study	0 ppm		625 ppm			1,250 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	122	70	122	100	60	123	100	60
4	239	70	237	99	60	235	98	60
8	303	70	299	99	60	295	97	60
12	344	70	337	98	60	327	95	60
16 ^a	370	60	363	98	60	350	95	60
20	391	60	378	97	60	366	94	60
24	401	60	385	96	60	372	93	60
28	410	60	399	97	60	384	94	60
32	422	60	406	96	60	392	93	60
36	429	60	413	96	60	399	93	60
40	432	60	414	96	60	400	93	60
44	434	60	416	96	60	400	92	57
48	433	60	417	96	60	402	93	57
52	436	60	419	96	60	404	93	57
56	428	60	415	97	59	398	93	55
60	439	60	423	96	59	403	92	55
64	443	59	427	96	58	409	92	54
68	447	59	432	97	56	417	93	54
72	446	59	433	97	55	418	94	49
76	435	59	424	98	54	409	94	42
80	433	55	419	97	51	405	94	42
84	430	54	423	98	47	407	95	36
88	434	54	420	97	44	411	95	31
92	428	52	425	99	32	408	95	21
96	425	49	424	100	28	395	93	16
100	424	46	421	99	25	408	96	8
104	425	40	415	98	19	395	93	3
Mean for weeks								
1-13	252		249	99		245	97	
14-52	416		401	96		387	93	
53-104	434		423	97		406	94	

TABLE 7
Mean Body Weights and Survival of Male Rats in the 2-Year Feed Study of o-Nitrotoluene

Weeks on Study	2,000 ppm			2,000 ppm (Stop-Exposure)			5,000 ppm (Stop-Exposure)		
	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	122	100	60	123	101	70	121	99	70
4	228	96	60	230	97	70	211	88	70
8	282	93	60	285	94	70	247	81	70
12	315	92	60	316	92	70	263	77	70
16 ^a	329	89	60	341	92	60	279	76	60
20	350	90	60	359	92	60	297	76	60
24	357	89	60	371	92	60	305	76	60
28	363	88	60	381	93	60	312	76	60
32	371	88	60	390	93	60	321	76	60
36	377	88	60	398	93	60	328	76	60
40	378	88	60	400	93	58	329	76	60
44	378	87	60	403	93	58	331	76	58
48	378	87	60	402	93	58	334	77	57
52	383	88	59	408	94	58	336	77	52
56	377	88	57	402	94	57	329	77	45
60	380	86	50	411	94	56	342	78	40
64	388	88	46	417	94	55	346	78	38
68	394	88	40	422	94	54	356	80	33
72	396	89	34	421	94	52	364	82	25
76	393	90	23	415	96	49	367	84	22
80	386	89	15	408	94	46	360	83	14
84	374	87	8	403	94	38	368	86	8
88	368	85	4	413	95	32	349	80	4
92	423	99	2	408	95	28	321	75	1
96				412	97	22	349	82	1
100				412	97	18			
104				404	95	11			
Mean for weeks									
1-13	237	94		239	95		211	84	
14-52	366	88		385	93		317	76	
53-104	388	89		411	95		350	81	

^a Interim evaluations occurred during week 14 (0 ppm group and 2,000 and 5,000 ppm stop-exposure groups).

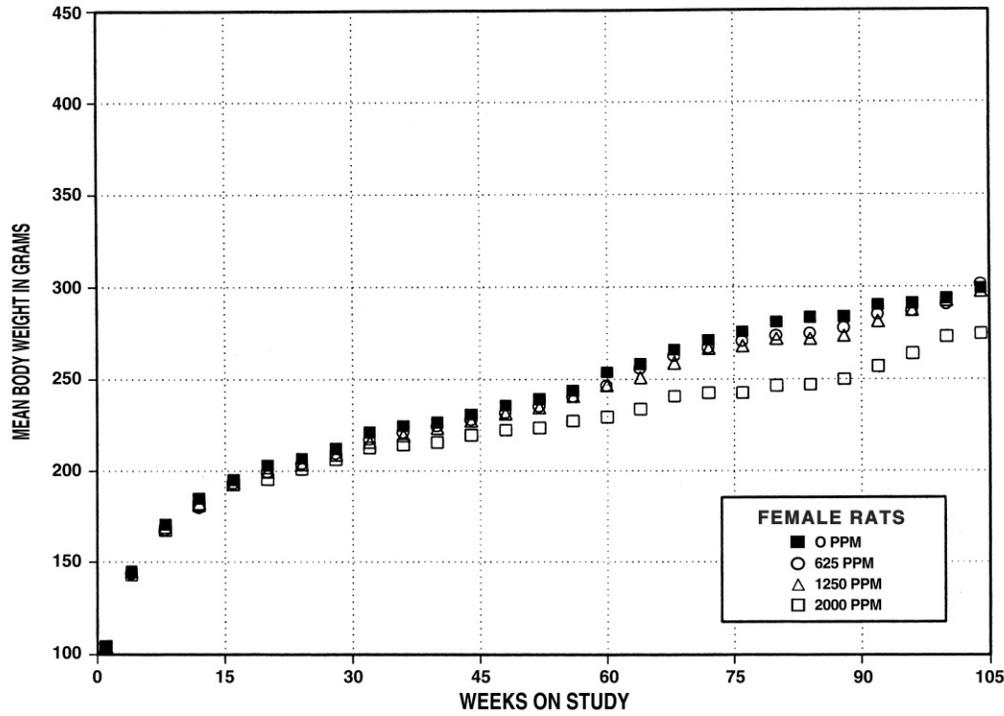
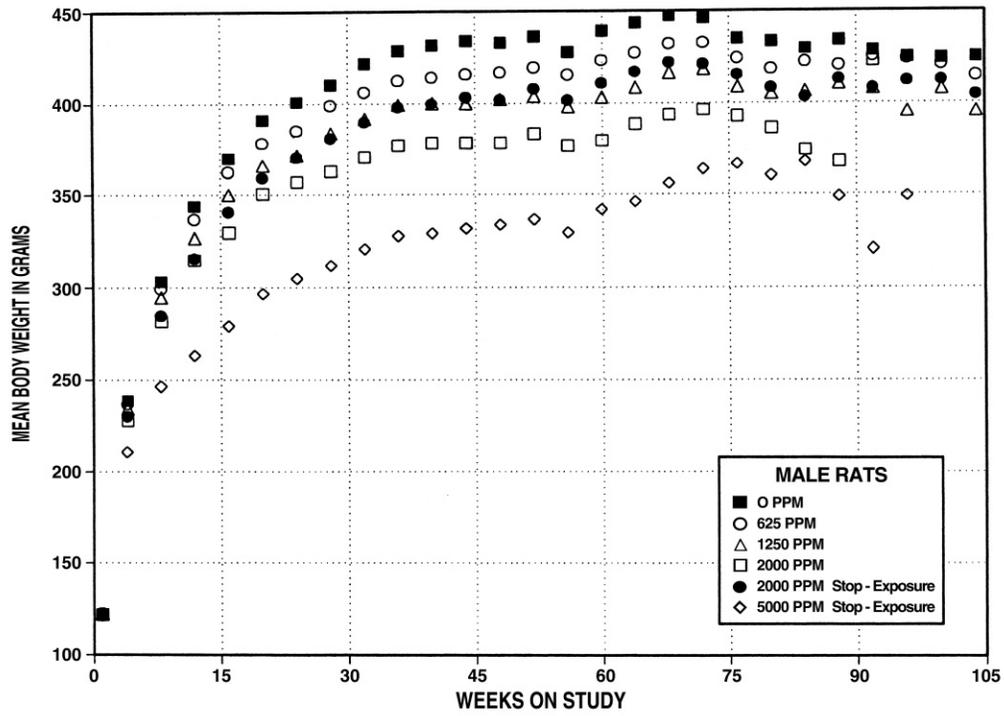


FIGURE 4
Growth Curves for Male and Female Rats
Exposed to *o*-Nitrotoluene in Feed for 2 Years

TABLE 8
Mean Body Weights and Survival of Female Rats in the 2-Year Feed Study of *o*-Nitrotoluene

Weeks on Study	0 ppm		625 ppm			1,250 ppm			2,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	105	60	104	99	60	104	100	60	105	100	60
4	145	60	144	99	60	145	100	60	143	99	60
8	171	60	168	98	60	170	99	60	168	98	60
12	185	60	180	97	60	182	99	60	181	98	60
16	195	60	193	99	60	193	99	60	193	99	60
20	203	60	200	98	60	200	98	60	196	96	60
24	207	60	204	99	60	204	98	60	201	97	60
28	212	60	210	99	60	209	98	60	206	97	60
32	221	60	217	98	60	216	98	60	213	96	60
36	225	60	221	98	60	219	98	60	214	95	60
40	227	60	225	99	60	224	99	60	216	95	60
44	231	60	228	99	60	227	98	60	220	95	60
48	236	60	232	98	60	231	98	60	222	94	60
52	239	60	235	98	60	235	98	60	224	93	60
56	244	60	241	99	60	241	99	59	227	93	60
60	254	59	247	97	60	247	97	59	230	90	60
64	258	59	256	99	59	251	97	58	234	91	60
68	266	58	263	99	59	259	97	58	241	91	59
72	271	58	267	99	58	267	98	58	243	89	58
76	276	58	271	98	57	268	97	56	243	88	58
80	281	58	274	97	56	272	97	56	247	88	58
84	284	58	275	97	56	272	96	55	247	87	57
88	284	58	278	98	55	274	96	53	250	88	55
92	290	56	285	98	55	282	97	52	257	89	52
96	291	56	287	99	55	287	99	51	264	91	48
100	294	54	291	99	52	293	100	49	273	93	43
104	299	48	302	101	47	298	100	39	275	92	33
Mean for weeks											
1-13	152		149	98		150	99		149	98	
14-52	220		217	99		216	98		211	96	
53-104	276		272	99		270	98		249	90	

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of mononuclear cell leukemia and neoplasms and/or non-neoplastic lesions of the mesothelium, skin, mammary gland, liver, lung, circulatory system, testis, and other organs. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix A for male rats and Appendix B for female rats.

Mesothelium: The incidences of malignant mesothelioma occurred with positive trends in males, and the incidences in exposed groups were significantly greater than that in the control group at 2 years (Tables 9, A3a, and A3b). The incidences in the exposed groups exceeded the 2-year historical ranges in control (all

routes) male rats given NTP-2000 diet and in feed study controls given NIH-07 diet (Tables 9 and A4a). The mesotheliomas were associated with the tunica vaginalis of the testis or epididymis (Plate 1). In some males, mesotheliomas were observed on the abdominal wall and on the surface of abdominal organs. The majority of mesotheliomas were large and consisted of papillary or solid areas of pleomorphic mesothelial cells (Plate 2). In some areas, beginning formation of tubules was seen. Extension or implantation of neoplastic cells and invasion of adjacent organs were limited to the serosa or occasionally the capsule of the involved organ. In male F344/N rats, almost all spontaneous mesotheliomas arise from the tunica vaginalis and, consequently, early neoplasms are found adherent to the epididymis or tunica albuginea of the testis, consistent with the predominant location of mesotheliomas in this study. In NTP studies, chemically induced mesotheliomas have been seen almost exclusively in male F344/N rats. Mesotheliomas were not observed in female rats in this study (Table B1).

TABLE 9
Incidences of Malignant Mesothelioma in Male Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Malignant Mesothelioma ^a						
Overall rate ^b	2/60 (3%)	20/60 (33%)	29/60 (48%)	44/60 (73%)	44/60 (73%)	54/60 (90%)
Adjusted rate ^c	3.7%	40.6%	62.4%	87.1%	80.3%	95.1%
Terminal rate ^d	2/39 (5%)	5/18 (28%)	1/3 (33%)	0/0	10/11 (91%)	0/0
First incidence (days)	729 (T)	428	432	359	275	299
Poly-3 test ^e	P<0.001	P<0.001	P<0.001	P<0.001		
Poly-3 test ^f	P<0.001				P<0.001	P<0.001

(T)Terminal sacrifice

^a Historical incidence for 2-year studies with controls given NTP-2000 diet (mean ± standard deviation): 23/609 (3.7% ± 2.9%), range 0%-10%; with feed study controls given NIH-07 diet: 25/1,004 (2.5% ± 2.0%), range 0%-8%

^b Number of animals with neoplasm per number of animals necropsied

^c Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^d Observed incidence at terminal kill

^e Beneath the control incidence is the P value associated with the trend test for the core study groups. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.

^f Trend test and pairwise comparisons for stop-exposure groups

Skin (Subcutaneous): The incidences of fibroma, fibrosarcoma, and fibroma or fibrosarcoma (combined) of the subcutaneous skin in males and of fibroma and fibroma or fibrosarcoma (combined) in females occurred with positive trends, and the incidences in the exposed groups were generally significantly greater than those in the controls at 2 years (Tables 10, A3a, A3b, and B3). Incidences of multiple fibroma and of lipoma were significantly increased in exposed groups of males. The incidences of these neoplasms in the exposed groups generally exceeded the 2-year historical ranges in control (all routes) rats given NTP-2000 diet and in feed study controls given NIH-07 diet (Tables 10, A4b, and B4a).

Fibromas occurred in the subcutis of the skin of the torso, head, and appendages. Fibromas were noninvasive, expansile neoplasms consisting of proliferations of fibrocytes with an abundant collagenous stroma

(Plate 3). Neoplastic cells and collagen formed interlacing bundles or whorling patterns. Mitoses were rare, and cellular atypia was not a feature of fibroma. Fibrosarcomas were generally more cellular and the large, well-formed bundles of mature collagen were less prominent than in fibromas (Plate 4). Fusiform cells were densely packed in interlacing bundles; pleomorphic cells with large nuclei were present in some instances. Mitotic figures were common and often atypical. Lipomas were characterized by focal nodules of variably sized adipocytes often infiltrated with streaks of fibrous tissue; occasionally, a few chronic inflammatory cells were present (Plate 5). Fibromas and fibrosarcomas are the most common neoplasms found in the skin of F344/N rats. Fibromas occur more frequently than fibrosarcomas and are more common in male than female control F344/N rats. Lipomas occur more often in males than in females and are most commonly found in the subcutis (Elwell *et al.*, 1990).

TABLE 10
Incidences of Subcutaneous Skin Neoplasms in Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Male						
Number Necropsied	60	60	60	60	60	60
Lipoma, Multiple ^a	0	0	1	0	0	3
Lipoma (includes multiple) ^b	0	4*	13**	13**	10**	12**
Fibroma, Multiple	1	25**	39**	35**	22**	28**
Fibroma (includes multiple) ^c	5	46**	52**	59**	45**	52**
Fibrosarcoma, Multiple	0	0	4*	5*	0	4*
Fibrosarcoma (includes multiple) ^d	0	7*	17**	20**	8**	12**
Fibroma or Fibrosarcoma^e						
Overall rate	5/60 (8%)	47/60 (78%)	55/60 (92%)	59/60 (98%)	47/60 (78%)	53/60 (88%) ^k
Adjusted rate ^g	9.3%	86.3%	98.7%	99.8%	89.0%	97.8%
Terminal rate ^h	3/39 (8%)	15/18 (83%)	3/3 (100%)	0/0	10/11 (91%)	0/0
First incidence (days)	705	465	384	391	275	299
Poly-3 test ⁱ	P<0.001	P<0.001	P<0.001	P<0.001		
Poly-3 test ^j	P<0.001				P<0.001	P<0.001
Female						
Number Necropsied	60	60	60	60		
Fibroma, Multiple	0	0	0	1		
Fibroma (includes multiple) ^l	3	3	18**	20**		
Fibrosarcoma, Multiple	0	0	0	1		
Fibrosarcoma (includes multiple)	0	0	4	3		
Fibroma or Fibrosarcoma^m						
Overall rate	3/60 (5%)	3/60 (5%)	21/60 (35%)	22/60 (37%)		
Adjusted rate	5.3%	5.4%	37.6%	40.6%		
Terminal rate	3/47 (6%)	3/47 (6%)	16/39 (41%)	16/33		
First incidence (days)	729 (T)	729 (T)	432	663		
Poly-3 test ⁱ	P<0.001	P=0.652	P<0.001	P<0.001		

* Significantly different ($P \leq 0.05$) from the control group by the Poly-3 test

** $P \leq 0.01$

(T) Terminal sacrifice

^a Number of animals with lesion

^b Historical incidence for 2-year studies with controls given NTP-2000 diet (mean \pm standard deviation): 8/609 (1.5% \pm 1.3%), range 0%-4%; with feed study controls given NIH-07 diet: 2/1,004 (0.2% \pm 0.6%), range 0%-2%

^c Historical incidence for NTP-2000 diet: 33/609 (5.1% \pm 4.0%), range 0%-12%; for NIH-07 diet: 56/1,004 (5.6% \pm 3.2%), range 0%-10%

^d Historical incidence for NTP-2000 diet: 8/609 (1.3% \pm 1.4%), range 0%-4%; for NIH-07 diet: 9/1,004 (0.9% \pm 1.4%), range 0%-4%

^e Historical incidence for NTP-2000 diet: 41/609 (6.4% \pm 4.3%), range 2%-14%; for NIH-07 diet: 65/1,004 (6.5% \pm 3.1%), range 2%-10%

^f Number of animals with neoplasm per number of animals necropsied

^g Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^h Observed incidence at terminal kill

ⁱ Beneath the control incidence is the P value associated with the trend test for the core study groups. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.

^j Trend test and pairwise comparisons for stop-exposure groups

^k A single sarcoma occurred in an animal that also had a fibroma.

^l Historical incidence for NTP-2000 diet: 14/659 (2.0% \pm 2.1%), range 0%-6%; for NIH-07 diet: 15/1,001 (1.5% \pm 1.6%), range 0%-4%

^m Historical incidence for NTP-2000 diet: 18/659 (2.6% \pm 2.1%), range 0%-6%; for NIH-07 diet: 19/1,001 (1.9% \pm 2.0%), range 0%-6%

Mammary Gland: In all exposed groups of males and females except 2,000 ppm core study males, the incidences of fibroadenoma of the mammary gland were significantly greater than those in the controls at 2 years (Tables 11, A3a, A3b, and B3). The incidences occurred with positive trends in males and females. The incidences of multiple fibroadenoma were significantly increased in exposed groups of females. The incidences in these groups generally exceeded the 2-year historical ranges in control (all routes) rats given NTP-2000 diet and in feed study controls given NIH-07 diet (Tables 11, A4c, and B4b). Fibroadenomas were characterized by

collections of glandular epithelium arranged in acini and ducts and were surrounded by fibrous connective tissue (Plate 6). The relative amounts of glandular and fibrous elements varied greatly among the neoplasms. Epithelial cells were usually uniform and arranged in a single layer (Plate 7); a few fibroadenomas had small areas of cellular atypia. The incidences of hyperplasia, a precursor of fibroadenoma, were significantly increased in 625 and 1,250 ppm females (Tables 11 and B5). Hyperplasia was characterized by enlarged lobules with an increase in the number of alveoli separated by collagenous stroma.

TABLE 11
Incidences of Mammary Gland Neoplasms in Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Male						
Number Necropsied	60	60	60	60	60	60
Hyperplasia ^a	6 (2.2) ^b	2 (1.5)	2 (2.5)	2 (2.0)	1 (1.0)	4 (1.5)
Fibroadenoma, Multiple	0	0	1	0	1	0
Fibroadenoma (includes multiple) ^c						
Overall rate ^d	0/60 (0%)	7/60 (12%)	10/60 (17%)	2/60 (3%)	13/60 (22%)	20/60 (33%)
Adjusted rate ^e	0.0%	15.6%	26.2%	9.0%	31.2%	61.1%
Terminal rate ^f	0/39 (0%)	4/18 (22%)	1/3 (33%)	0/0	5/11 (46%)	0/0
First incidence (days)	— ^g	621	576	608	523	299
Poly-3 test ^g	P<0.001	P=0.004	P<0.001	P=0.110		
Poly-3 test ^h	P<0.001				P<0.001	P<0.001
Female						
Number Necropsied	60	60	60	60		
Hyperplasia	14 (1.7)	36** (2.3)	30** (2.3)	19 (2.1)		
Fibroadenoma, Multiple	6	35**	39**	42**		
Fibroadenoma (includes multiple) ^j						
Overall rate	23/60 (38%)	47/60 (78%)	52/60 (87%)	56/60 ^k (93%)		
Adjusted rate	40.0%	82.0%	91.7%	96.2%		
Terminal rate	18/47 (38%)	39/47 (83%)	36/39 (92%)	33/33 (100%)		
First incidence (days)	620	505	525	464		
Poly-3 test ^g	P<0.001	P<0.001	P<0.001	P<0.001		
Carcinoma ^l	0	0	1	1		

** Significantly different ($P \leq 0.01$) from the control group by the Poly-3 test

^a Number of animals with lesion

^b Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

^c Historical incidence for 2-year studies with controls given NTP-2000 diet (mean \pm standard deviation): 26/609 (3.8% \pm 2.9%), range 0%-8%; with feed study controls given NIH-07 diet: 42/1,004 (4.2% \pm 3.5%), range 0%-12%

^d Number of animals with neoplasm per number of animals necropsied

^e Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^f Observed incidence at terminal kill

^g Beneath the control incidence is the P value associated with the trend test for the core study groups. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.

^h Trend test and pairwise comparisons for stop-exposure groups

ⁱ Not applicable; no neoplasms in animal group

^j Historical incidence for NTP-2000 diet: 284/659 (41.1% \pm 10.1%), range 28%-56%; for NIH-07 diet: 431/1,001 (43.1% \pm 10.7%), range 24%-60%

^k A single adenoma occurred in an animal that also had a fibroadenoma.

^l Historical incidence for NTP-2000 diet: 15/659 (2.6% \pm 2.2%), range 0%-6%; for NIH-07 diet: 32/1,001 (3.2% \pm 2.6%), range 0%-8%

Liver: At 3 months, liver weights of 5,000 ppm stop-exposure males were significantly greater than those of the controls (Table G1). The incidences of hepatocellular adenoma in 2,000 ppm core study males and females and of hepatocellular adenoma or carcinoma (combined) in 2,000 ppm core study and 5,000 ppm stop-exposure males were significantly greater than those in the controls at 2 years (Tables 12, A3a, A3b, and B3). The incidences in these groups generally exceeded the 2-year historical ranges in control (all routes) rats given NTP-2000 diet and in feed study controls given NIH-07 diet (Tables 12, A4d, and B4c). Hepatocellular adenomas consisted of nodules of hepatocytes that compressed adjacent hepatic parenchyma and lacked the normal lobular and sinusoidal pattern (Plate 8). Hepatocellular carcinomas consisted of solid sheets of hepatocytes or trabeculae three or more cells thick. Neoplastic hepatocytes were anaplastic, with prominent nuclei containing one or more nucleoli and variable amounts of cytoplasm. A single hepatocholangiocarcinoma occurred in a 625 ppm male and in a 2,000 ppm core study male, and cholangiocarcinomas occurred in three 5,000 ppm stop-exposure males. Although the incidences of these neoplasms were low, they were considered exposure related. Hepatocholangiocarcinomas were malignant neoplasms containing cells resembling both hepatocyte and biliary epithelium (Plate 9), while cholangiocarcinomas consisted primarily of neoplastic biliary epithelium. In cholangiocarcinomas, the bile duct epithelium was

atypical, infiltrative, and often surrounded by collagenous connective tissue (Plate 10). No hepatocholangiocarcinomas or cholangiocarcinomas have been observed in male controls given NTP-2000 or NIH-07 diet for 2 years. However, 2 of 20 male F344/N rats given 5,000 ppm *o*-nitrotoluene for 13 weeks followed by a 13-week recovery period and one male F344/N rat given 5,000 ppm *o*-nitrotoluene for 26 weeks had cholangiocarcinomas (NTP, 1996).

At the 3-month interim evaluation, the incidence of hepatocellular cytoplasmic vacuolization was significantly increased in 5,000 ppm stop-exposure males. The vacuolated hepatocytes were observed uniformly throughout the liver lobules. Hepatocellular vacuoles were suggestive of hydropic degeneration and were of moderate severity, and the incidences correlated with the increased liver weights. At 2 years, the incidences of several nonneoplastic lesions of the liver were increased in exposed groups of males and females (Tables 12, A5, and B5). Hepatocellular foci are considered potential preneoplastic lesions and are relatively discrete aggregates of hepatocytes, often enlarged; the lobular pattern of the liver is generally retained. Mixed cell infiltration in males was focal and characterized by minimal chronic active inflammation. Centrilobular necrosis was seen in a small number of exposed rats at all exposure concentrations (except 625 ppm females) and was characterized by focal mild coagulative necrosis of hepatocytes.

TABLE 12
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Rats
in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Male						
3-Month Interim Evaluation						
Number Examined Microscopically	10				10	10
Hepatocellular Cytoplasmic Vacuolization ^a	0				0	10** (2.8) ^b
2-Year Study						
Number Examined Microscopically	60	60	60	60	60	60
Clear Cell Focus	29	29	34	31*	30	34**
Eosinophilic Focus	7	18**	29**	24**	15**	13**
Mixed Cell Focus	5	7	12**	6	12*	8*
Hematopoietic Cell Proliferation	0	6* (1.8)	2 (2.0)	2 (1.5)	11** (1.5)	6** (1.5)
Infiltration Cellular, Mixed Cell	1 (1.0)	5 (1.2)	11** (1.5)	20** (1.7)	15** (1.7)	33** (1.7)
Centrilobular, Necrosis	1 (2.0)	3 (2.3)	8** (1.9)	5* (1.8)	9** (2.6)	3 (2.7)
Hepatocholangiocarcinoma ^c	0	1	0	1	0	0
Cholangiocarcinoma ^c	0	0	0	0	0	3*
Hepatocellular Adenoma, Multiple	0	1	0	0	1	1
Hepatocellular Adenoma (includes multiple) ^d						
Overall rate ^e	2/60 (3%)	3/60 (5%)	3/60 (5%)	7/60 (12%)	3/60 (5%)	4/60 (7%)
Adjusted rate ^f	3.7%	6.8%	8.4%	27.1%	7.6%	18.4%
Terminal rate ^g	1/39 (3%)	1/18 (6%)	0/3 (0%)	0/0	1/11 (9%)	0/0
First incidence (days)	707	621	615	391	579	499
Poly-3 test ^h	P=0.007	P=0.413	P=0.325	P=0.006		
Poly-3 test ⁱ	P=0.062				P=0.362	P=0.079
Hepatocellular Carcinoma, Multiple	0	0	0	1	0	0
Hepatocellular Carcinoma (includes multiple)	1	0	0	1	0	2
Hepatocellular Adenoma or Carcinoma ^j						
Overall rate	3/60 (5%)	3/60 (5%)	3/60 (5%)	8/60 (13%)	3/60 (5%)	6/60 (13%)
Adjusted rate	5.6%	6.8%	8.4%	30.2%	7.6%	25.9%
Terminal rate	2/39 (5%)	1/18 (6%)	0/3 (0%)	0/0	1/11 (9%)	0/0
First incidence (days)	707	621	615	391	579	391
Poly-3 test ^h	P=0.009	P=0.570	P=0.466	P=0.007		
Poly-3 test ⁱ	P=0.030				P=0.511	P=0.029

TABLE 12
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Rats
in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Female				
Number Examined Microscopically	60	59	60	60
Basophilic Focus	51	56*	60**	54
Clear Cell Focus	16	30**	28*	33**
Eosinophilic Focus	5	12	25**	32**
Mixed Cell Focus	6	9	11	28**
Centrilobular, Necrosis	3 (1.7)	0	2 (2.5)	2 (3.0)
Hepatocellular Adenoma, Multiple	0	0	0	1
Hepatocellular Adenoma (includes multiple) ^k				
Overall rate	1/60 (2%)	0/59 (0%)	1/60 (2%)	6/60 (10%)
Adjusted rate	1.8%	0.0%	1.9%	11.2%
Terminal rate	1/47 (2%)	0/47 (0%)	1/39 (3%)	4/33 (12%)
First incidence (days)	729 (T)	—	729 (T)	687
Poly-3 test ^h	P=0.005	P=0.507N	P=0.748	P=0.048

* Significantly different (P≤0.05) from the control group by the Fisher exact test (interim evaluation) or the Poly-3 test (2-year study)

** P≤0.01

(T) Terminal sacrifice

^a Number of animals with lesion

^b Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

^c Historical incidence for 2-year studies with control groups given NTP-2000 diet: 0/609; with feed study controls given NIH-07 diet: 0/1,002

^d Historical incidence for NTP-2000 diet (mean ± standard deviation): 5/609 (0.8% ± 1.2%), range 0%-3%; for NIH-07 diet: 23/1,002 (2.3% ± 3.0%), range 0%-10%

^e Number of animals with neoplasm per number of animals with liver examined microscopically

^f Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^g Observed incidence at terminal kill

^h Beneath the control incidence is the P value associated with the trend test for the core study groups. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice. A lower incidence in an exposure group is indicated by N.

ⁱ Trend test and pairwise comparisons for stop-exposure groups

^j Historical incidence for NTP-2000 diet: 10/609 (1.8% ± 1.9%), range 0%-5%; for NIH-07 diet: 28/1,002 (2.8% ± 3.3%), range 0%-10%

^k Historical incidence for NTP-2000 diet: 4/659 (0.7% ± 1.0%), range 0%-2%; for NIH-07 diet: 4/1,000 (0.4% ± 1.1%), range 0%-4%

^l Not applicable; no neoplasms in animal group

Lung: The incidences of alveolar/bronchiolar adenoma and adenoma or carcinoma (combined) in 5,000 ppm stop-exposure males were significantly greater than those in the controls (Tables 13 and A3b). The incidence of alveolar/bronchiolar adenoma or carcinoma (combined) in the 5,000 ppm stop-exposure group exceeded the 2-year historical ranges in control (all routes) male rats given NTP-2000 diet and in feed study controls given NIH-07 diet (Tables 13 and A4e). The incidences of alveolar epithelial hyperplasia in all exposed groups except 1,250 ppm males and 2,000 ppm

females were increased (Tables 13, A5 and B5). Alveolar/bronchiolar adenomas were usually papillary and distorted the alveolar architecture (Plate 11). Neoplastic cells were cuboidal to columnar and well differentiated (Plate 12). Alveolar/bronchiolar carcinomas were solid or papillary, obliterated the normal pulmonary architecture, and sometimes invaded adjacent structures. Alveolar epithelial hyperplasia was characterized by increased numbers of type II pneumocytes lining the alveoli; however, the alveolar architecture was maintained.

TABLE 13
Incidences of Neoplasms and Nonneoplastic Lesions of the Lung in Rats
in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Male						
Number Examined Microscopically	60	60	60	60	60	60
Alveolar Epithelium, Hyperplasia ^a	2 (3.0) ^b	8* (2.4)	3 (1.7)	7** (2.3)	15** (1.5)	29** (1.6)
Alveolar/bronchiolar Adenoma, Multiple	0	0	0	0	0	1
Alveolar/bronchiolar Adenoma (includes multiple) ^c						
Overall rate ^d	1/60 (2%)	5/60 (8%)	1/60 (2%)	2/60 (3%)	3/60 (5%)	8/60 (13%)
Adjusted rate ^e	1.9%	11.2%	2.9%	8.7%	7.6%	33.0%
Terminal rate ^f	1/39 (3%)	2/18 (11%)	1/3 (33%)	0/0	1/11 (9%)	0/0
First incidence (days)	729 (T)	639	729 (T)	491	615	419
Poly-3 test ^g	P=0.237	P=0.066	P=0.654	P=0.254		
Poly-3 test ^h	P<0.001				P=0.205	P<0.001
Alveolar/bronchiolar Carcinoma (includes multiple)	1	0	0	0	0	3
Alveolar/bronchiolar Adenoma or Carcinoma ⁱ						
Overall rate	2/60 (3%)	5/60 (8%)	1/60 (2%)	2/60 (3%)	3/60 (5%)	11/60 (18%)
Adjusted rate	3.7%	11/2%	2.9%	8.7%	7.6%	42.0%
Terminal rate	2/39 (5%)	2/18 (11%)	1/3 (33%)	0/0	1/11 (9%)	0/0
First incidence (days)	729 (T)	639	729 (T)	419	615	419
Poly-3 test ^g	P=0.390	P=0.149	P=0.644N	P=0.386		
Poly-3 test ^h	P<0.001				P=0.362	P<0.001

TABLE 13
Incidences of Neoplasms and Nonneoplastic Lesions of the Lung in Rats
in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Female				
Number Examined Microscopically	60	60	60	60
Alveolar Epithelium, Hyperplasia	6 (1.7)	14* (2.1)	16* (1.8)	9 (1.8)
Alveolar/bronchiolar Adenoma, Multiple	0	1	0	0
Alveolar/bronchiolar Adenoma (includes multiple)	1	2	0	3
Alveolar/bronchiolar Carcinoma (includes multiple)	0	0	0	1
Alveolar/bronchiolar Adenoma or Carcinoma	1	2	0	4

* Significantly different ($P \leq 0.05$) from the control group by the Poly-3 test

** $P \leq 0.01$

(T) Terminal sacrifice

^a Number of animals with lesion

^b Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

^c Historical incidence for 2-year studies with control groups given NTP-2000 diet: 24/609 (4.2% ± 3.5%), range 0%-12%; with feed study controls given NIH-07 diet: 24/1,002 (2.4% ± 3.2%), range 0%-14%

^d Number of animals with neoplasm per number of animals with liver examined microscopically

^e Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^f Observed incidence at terminal kill

^g Beneath the control incidence is the P value associated with the trend test for the core study groups. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice. A lower incidence in an exposure group is indicated by N.

^h Trend test and pairwise comparisons for stop-exposure groups

ⁱ Historical incidence for NTP-2000 diet: 26/609 (4.5% ± 3.9%), range 0%-14%; for NIH-07 diet: 31/1,002 (3.1% ± 3.5%), range 0%-16%

Circulatory System: The incidences of hemangioma and hemangioma or hemangiosarcoma (combined) were significantly greater in 5,000 ppm stop-exposure males than in the controls (Tables 14, A3a, and A3b). In the 5,000 ppm stop-exposure males, a hemangioma occurred in the spleen of one animal, a second had a hemangioma of the skin and of the skeletal muscle, and a third had a hemangioma in an unspecified tissue (Table A2). These incidences exceeded the 2-year

historical ranges in control (all routes) male rats given NTP-2000 diet and in feed study controls given NIH-07 diet (Tables 14 and A4f). The hemangiosarcoma in the 5,000 ppm stop-exposure male occurred in the skeletal muscle. The presence of benign hemangiomas and a single hemangiosarcoma in a variety of organs in the 5,000 ppm stop-exposure group was not considered to be related to exposure to o-nitrotoluene.

TABLE 14
Incidences of Circulatory System Neoplasms in Male Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Hemangioma, All Organs^a						
Overall rate ^b	0/60 (0%)	3/60 (5%)	0/60 (0%)	2/60 (3%)	0/60 (0%)	3/60 (5%)
Adjusted rate ^c	0.0%	6.7%	0.0%	8.7%	0.0%	14.0%
Terminal rate ^d	0/39 (0%)	1/18 (6%)	0/3 (0%)	0/0	0/11 (9%)	0/0
First incidence (days)	— ^e	632	— ^h	439	—	417
Poly-3 test ^e	P=0.158	P=0.090	— ^h	P=0.112	—	P=0.034
Poly-3 test ^f	P=0.025					
Hemangioma or Hemangiosarcoma, All Organsⁱ						
Overall rate	1/60 (2%)	3/60 (5%)	1/60 (2%)	2/60 (3%)	0/60 (0%)	4/60 (7%)
Adjusted rate	1.9%	6.7%	2.9%	8.7%	0.0%	18.0%
Terminal rate	1/39 (3%)	1/18 (6%)	0/3 (0%)	0/0	0/11 (9%)	0/0
First incidence (days)	729 (T)	632	714	439	—	391
Poly-3 test ^e	P=0.232	P=0.243	P=0.654	P=0.254	P=0.565N	P=0.037
Poly-3 test ^f	P=0.041					

(T) Terminal sacrifice

^a Historical incidence for 2-year studies with control groups given NTP-2000 diet: 3/609 (0.5% ± 1.3%), range 0%-4%; with feed study controls given NIH-07 diet: 3/1,004 (0.3% ± 0.7%), range 0%-2%

^b Number of animals with neoplasm per number of animals necropsied

^c Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^d Observed incidence at terminal kill

^e Beneath the control incidence is the P value associated with the trend test for the core study groups. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice. A lower incidence in an exposure group is indicated by N.

^f Trend test and pairwise comparisons for stop-exposure groups

^g Not applicable; no neoplasms in animal group

^h Value of statistic cannot be computed.

ⁱ Historical incidence for NTP-2000 diet: 6/609 (1.1% ± 1.4%), range 0%-4%; for NIH-07 diet: 10/1,004 (1.0% ± 1.4%), range 0%-4%

Mononuclear Cell Leukemia: The incidences of mononuclear cell leukemia were significantly decreased in all groups of males exposed to 1,250 ppm or greater and in all exposed groups of females, and these incidences were less than the 2-year historical ranges in control (all routes) rats given NTP-2000 diet and in feed study controls given NIH-07 diet (Tables 15, A3a, A3b, A4g, B3, and B4d). Mononuclear cell leukemia is one of the most common neoplasms in the F344/N rat. A

number of factors may contribute to decreases in leukemia incidences in F344/N rats, including splenectomy, X-irradiation, and corn oil gavage. Chemicals that cause toxicity of the spleen may also be associated with decreased incidences of leukemia (Elwell *et al.*, 1990). The decreased incidences of leukemia in both males and females was most likely related to the splenic toxicity previously discussed.

TABLE 15
Incidences of Mononuclear Cell Leukemia in Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Male						
Mononuclear Cell Leukemia ^a						
Overall rate ^b	30/60 (50%)	21/60 (35%)	3/60 (5%)	3/60 (5%)	13/60 (22%)	1/60 (2%)
Adjusted rate ^c	52.2%	42.8%	8.5%	12.7%	31.1%	5.0%
Terminal rate ^d	16/39 (41%)	7/18 (39%)	0/3 (0%)	0/0	4/11 (36%)	0/0
First incidence (days)	533	465	676	419	531	569
Poly-3 test ^e	P<0.001N	P=0.214N	P<0.001N	P=0.003N		
Poly-3 test ^f	P<0.001N				P=0.027N	P<0.001N
Female						
Mononuclear Cell Leukemia ^g						
Overall rate	21/60 (35%)	6/60 (10%)	4/60 (7%)	5/60 (8%)		
Adjusted rate	35.6%	10.6%	7.3%	9.1%		
Terminal rate	11/47 (23%)	3/47 (6%)	1/39 (3%)	1/33 (3%)		
First incidence (days)	453	429	523	488		
Poly-3 test ^e	P=0.001N	P=0.001N	P=0.001N	P=0.001N		

^a Historical incidence for 2-year studies with controls given NTP-2000 diet (mean ± standard deviation): 300/609 (47.3% ± 10.5%), range 32%-68%; with feed study controls given NIH-07 diet: 547/1,004 (54.5% ± 10.7%), range 32%-74%

^b Number of animals with neoplasm per number of animals necropsied

^c Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^d Observed incidence at terminal kill

^e Beneath the control incidence is the P value associated with the trend test for the core study groups. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by N.

^f Trend test and pairwise comparisons for stop-exposure groups

^g Historical incidence for NTP-2000 diet: 185/659 (29.1% ± 8.4%), range 16%-42%; for NIH-07 diet: 293/1,001 (29.3% ± 7.6%), range 16%-42%

Testis: The combined incidence of interstitial cell adenoma (bilateral or unilateral) of the testis was significantly less in the 5,000 ppm stop-exposure group than in the controls (Tables 16 and A3b). The decrease resulted primarily from the lower incidence of bilateral interstitial cell adenoma in the 5,000 ppm stop-exposure group. The incidence of bilateral interstitial adenoma in the 2,000 ppm core study males was also slightly less than that in the controls; however, the combined incidence of unilateral and bilateral interstitial cell adenoma in this group did not differ significantly from that in the controls. The decreased incidence in the 5,000 ppm stop-exposure group could not be explained solely by reduced survival, as the survival rates in that group and the 2,000 ppm core study males were similar. The decreased incidence was likely related to testicular toxicity observed early in the study in the 5,000 ppm stop-exposure group. Interstitial cell adenoma is the most common testicular neoplasm in F344/N rats, occurring with an incidence of 88% in 2-year carcinogenicity studies (Table A4h) and 96% in lifetime studies. Chemicals that cause testicular toxicity characterized by atrophy and degeneration have been associated with decreases in

the incidence of interstitial cell adenoma in 2-year studies (Boorman *et al.*, 1985).

At 3 months, the incidence of testicular atrophy was significantly increased in the 5,000 ppm stop-exposure group (Tables 16 and A5); this lesion was characterized by a reduction in the numbers of normal-appearing spermatozoa and of seminiferous tubules, which varied from a few seminiferous tubules to a reduction of all tubules in some testes. The majority of tubules had undergone degeneration and were lined only by Sertoli cells, with few to no spermatogenic cells remaining. Mineralization was associated with tubular degeneration. In some tubules, cellular debris and multinucleated giant cells were observed, but these were not prominent findings in the atrophic testes. As a result of degeneration and atrophy, the luminal contents of the epididymis were atypical; cellular atypia was characterized by the presence of rounded and enlarged or multinucleated degenerated spermatozoa. In addition to increased incidences of testicular atrophy in most exposed groups at 2 years, the incidence of interstitial cell hyperplasia was increased in the 2,000 ppm core study group.

TABLE 16
Incidences of Neoplasms and Nonneoplastic Lesions of the Testis and Epididymis
in Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
3-Month Interim Evaluation						
Epididymis ^a	10				10	10
Atrophy ^b	0				0	5* (2.4) ^c
Testis	10				10	10
Mineralization	0				0	5* (2.0)
Germinal Epithelium, Atrophy	0				1 (1.0)	9** (2.4)
2-Year Study						
Testis	60	60	60	60	60	60
Germinal Epithelium, Atrophy	13 (2.8)	21* (3.2)	12 (3.2)	19** (3.0)	18* (3.6)	53** (3.9)
Interstitial Cell, Hyperplasia	10 (1.4)	14 (1.6)	13 (1.5)	31** (1.4)	15 (1.5)	4 (1.0)
Interstitial Cell Adenoma, Bilateral	43	34	32	17	31	4**
Interstitial Cell Adenoma, Unilateral	12	19	19	29	19	23
Interstitial Cell Adenoma (bilateral or unilateral) ^d						
Overall rate ^e	55/60 (92%)	53/60 (88%)	51/60 (85%)	46/60 (77%)	50/60 (83%)	27/60 (45%)
Adjusted rate ^f	94.3%	93.7%	96.5%	92.2%	92.6%	74.7%
Terminal rate ^g	38/39 (97%)	18/18 (100%)	3/3 (100%)	0/0	10/11 (91%)	0/0
First incidence (days)	533	465	483	404	370	384
Poly-3 test ^h	P=0.453N	P=0.610N	P=0.457	P=0.475N		
Poly-3 test ⁱ	P<0.001N				P=0.506N	P<0.001N

* Significantly different (P≤0.05) from the control group by the Fisher exact test (interim evaluation) or the Poly-3 test (2-year study)

** P≤0.01

^a Number of animals with tissue examined microscopically

^b Number of animals with lesion

^c Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

^d Historical incidence for 2-year studies with control groups given NTP-2000 diet: 535/609 (86.4% ± 9.1%), range 72%-98%; with feed study controls given NIH-07 diet: 889/1,003 (88.6% ± 6.0%), range 74%-96%

^e Number of animals with neoplasm per number of animals with testis examined microscopically

^f Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^g Observed incidence at terminal kill

^h Beneath the control incidence is the P value associated with the trend test for the core study groups. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by N.

ⁱ Trend test and pairwise comparisons for stop-exposure groups

Other Organs: The incidences of nonneoplastic lesions of the bone marrow, kidney, mediastinal and mandibular lymph nodes, nose, pancreatic islets, pituitary gland, preputial and clitoral glands, salivary gland, and spleen were increased in exposed groups of males and/or females at 3 months and/or 2 years (Tables 17, A5, and B5).

Bone marrow hyperplasia in exposed groups of males and females was characterized by hematopoietic cell proliferation. Hematopoiesis of the spleen in exposed groups of males and females involved primarily the erythropoietic cell series. Although congestion of the spleen occurred in both stop-exposure groups, the pathogenesis of this lesion was not determined. Hematopoietic cell proliferation in the bone marrow and spleen may have been a secondary response because many rats had subcutaneous ulcerated neoplasms with varying degrees of inflammation.

The incidences and severity of lymphoid hyperplasia of the mandibular lymph node were significantly increased in 2,000 ppm females.

At 3 months, hyaline degeneration of the kidney in 5,000 ppm stop-exposure males was characterized by a slight increase in the presence of hyaline droplets within renal tubule epithelial cells. The hyaline droplets were homogeneous, eosinophilic, and rounded (3 to 5 μm in diameter). Larger droplets appeared to be extruded into the tubular lumina. Associated with the hyaline droplet degeneration was minimal nephropathy, which was considered part of the degeneration and, therefore, was not given a separate diagnosis. At 2 years, pigmentation of the kidney of exposed groups of males consisted of fine, dark brown granules within the cytoplasm of cortical renal tubule epithelial cells.

Olfactory epithelial degeneration of the nose occurred in levels II and III of the nasal cavity of stop-exposure males at 3 months and was characterized by disruption, vacuolization, or atrophy of the olfactory epithelium. In some areas, epithelial cells were somewhat flattened, suggesting very early development of squamous cell

metaplasia. The degenerative changes were mostly unilateral and primarily involved the most dorsal aspect of the ethmoid turbinates, nasal septum, and dorsal meatus.

Hyperplasia of the pancreatic islets occurred in 5,000 ppm stop-exposure males at 3 months and 2 years and was characterized by a greater number and size of islets (up to 500 μm in diameter). Some hyperplastic islets were twice the size of those in the controls. Larger islets had slightly enlarged islet cells arranged in a less orderly fashion than the normal glandular pattern seen in the controls. Pancreatic islet pigmentation was observed at 2 years in 5,000 ppm stop-exposure males.

The incidences of atrophy of the preputial gland were significantly increased in most groups of exposed males at 3 months and at 2 years. Atrophy was characterized by glandular epithelial cells with fewer and smaller cytoplasmic granules than normal. The interstitial tissue was more prominent than normal and was likely due to the decreased size of the glandular epithelial tissue. Incidences of atrophy of the clitoral gland, analogous to the preputial gland, were significantly increased in 1,250 and 2,000 ppm females.

Incidences of cytoplasmic alteration of the pituitary gland (pars distalis) were significantly increased in the 2,000 ppm core study and 5,000 ppm stop-exposure male groups at 2 years. The pituitary gland cytoplasmic alteration in exposed male rats consisted of hypertrophy, increased eosinophilia, and cytoplasmic vacuolization of cells in the pars distalis, and may have been related to the atrophy of the testis.

Atrophy of the salivary gland was observed in exposed groups of males and females and was characterized by a reduction in the size and number of eosinophilic cytoplasmic granules in the epithelial cells of secretory ducts in the submandibular salivary gland. In some salivary glands, few or no eosinophilic granules were found and the epithelial cells were reduced in size.

The incidences of pigmentation of the spleen were increased in 2,000 ppm core-study males and females.

TABLE 17
Incidences of Selected Nonneoplastic Lesions in Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Male						
3-Month Interim Evaluation						
Kidney ^a	10				10	10
Renal Tubule, Degeneration, Hyaline ^b	1 (1.0) ^c				0	10** (2.0)
Nose	10				10	10
Olfactory Epithelium, Degeneration	0				4* (1.5)	10** (3.0)
Pancreatic Islets	10				10	10
Hyperplasia	0				0	8** (1.6)
Preputial Gland	10				10	10
Atrophy	0				1 (2.0)	8** (2.0)
Salivary Gland	10				10	10
Atrophy	0				10** (3.0)	10** (3.8)
Spleen	10				10	10
Congestion	0				3 (1.7)	10** (1.9)
Hematopoietic Cell Proliferation	0				2 (1.0)	10** (1.8)
2-Year Study						
Bone Marrow	60	60	60	60	60	60
Hyperplasia	2 (3.5)	25** (3.0)	43** (2.9)	45** (2.9)	37** (3.0)	33** (2.9)
Kidney	60	60	60	60	60	60
Renal Tubule, Pigmentation	5 (2.4)	12* (1.8)	14** (1.6)	9* (1.9)	18** (2.2)	6 (2.7)
Lymph Node	60	60	60	60	60	60
Mediastinal, Pigmentation	6 (2.7)	9 (3.1)	3 (3.0)	8* (3.4)	13* (3.6)	13** (3.2)
Pancreatic Islets	60	60	60	60	60	60
Hyperplasia	2 (1.5)	0	0	2 (1.5)	2 (1.5)	10** (1.9)
Pigmentation	1 (1.0)	0	0	2 (2.0)	0	11** (2.0)
Pituitary Gland	59	60	58	59	57	59
Pars Distalis, Cytoplasmic Alteration	1 (3.0)	0	1 (1.0)	4* (1.3)	1 (1.0)	42** (2.1)
Preputial Gland	60	59	58	56	60	59
Atrophy	7 (2.0)	9 (2.3)	35** (2.3)	41** (2.6)	38** (2.3)	54** (2.9)
Salivary Gland	60	60	59	60	58	59
Atrophy	0	2 (2.0)	18** (2.1)	43** (2.2)	16** (2.1)	49** (2.4)
Spleen	60	60	60	60	60	60
Hematopoietic Cell Proliferation	7 (2.9)	33** (2.6)	38** (2.4)	47** (2.3)	36** (2.6)	35** (2.2)
Pigmentation	9 (2.6)	8 (2.5)	13 (2.6)	16** (2.3)	4 (2.8)	7 (2.7)

TABLE 17
Incidences of Selected Nonneoplastic Lesions in Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Female				
Bone Marrow	60	60	60	60
Hyperplasia	2 (3.0)	7 (2.4)	15** (2.7)	24** (2.7)
Clitoral Gland	59	57	54	53
Atrophy	1 (2.0)	3 (2.0)	6* (2.3)	25** (2.4)
Lymph Node	60	60	59	59
Mandibular, Lymphoid Hyperplasia	3 (2.0)	5 (2.2)	6 (2.2)	15** (2.7)
Salivary Gland	60	60	60	60
Atrophy	2 (3.5)	3 (2.0)	9* (2.1)	48** (2.4)
Spleen	60	59	60	59
Hematopoietic Cell Proliferation	22 (1.6)	38** (1.4)	48** (1.9)	48** (2.1)
Pigmentation	36 (2.5)	44 (2.2)	43 (2.4)	46* (2.3)

* Significantly different ($P \leq 0.05$) from the control group by the Fisher exact test (interim evaluation) or the Poly-3 test (2-year study)

** $P \leq 0.01$

^a Number of animals with tissue examined microscopically

^b Number of animals with lesion

^c Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

MICE

Survival

Estimates of 2-year survival probabilities for male and female mice are shown in Table 18 and in the Kaplan-Meier survival curves (Figure 5). All 2,500 ppm males died by week 101 and all 5,000 ppm males died by week 66. Survival of 1,250 ppm males and 5,000 ppm females was significantly less than that of the controls. Due to the poor survival of exposed mice, the planned 15-month interim evaluation was cancelled.

Body Weights, Feed and Compound Consumption, and Clinical Findings

Mean body weights of exposed males and 5,000 ppm females were generally less than those of the controls throughout the study; mean body weights of 2,500 ppm females were less than those of the controls during the second year of the study (Figure 6; Tables 19 and 20). Feed consumption by 5,000 ppm males was less than

that by the controls; feed consumption by other exposed groups of males and females was similar to that by the controls (Tables 13 and 14). Dietary concentrations of 1,250, 2,500, or 5,000 ppm resulted in average daily doses of approximately 165, 360, or 700 mg *o*-nitrotoluene/kg body weight to males and 150, 320, or 710 mg/kg to females. Clinical findings included large subcutaneous masses in the torso, head, and appendages of males and females; incidences of clinical findings were generally exposure-concentration related.

Biomarkers of Exposure

The results of urinary metabolite determinations in male and female mice are presented in Table F2. At time points with sufficient data to permit determinations, the ratios of *o*-nitrobenzoic acid to creatinine excreted in urine appeared to be linearly related to exposure concentration. The concentrations of *o*-nitrobenzylmercapturic acid and *o*-aminobenzoic acid were generally below the limit of quantitation in male and female mice.

TABLE 18
Survival of Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Male				
Animals initially in study	60	60	60	60
Moribund	3	5	21	31
Natural deaths	5	21	39	29
Animals surviving to study termination	52	34	0	0
Percent probability of survival at end of study ^a	87	57	0	0
Mean survival (days) ^b	712	674	523	386
Survival analysis ^c	P<0.001	P<0.001	P<0.001	P<0.001
Female				
Animals initially in study	60	60	60	60
Moribund	4	6	7	22
Natural deaths	4	8	6	33
Animals surviving to study termination	52	46	47	5
Percent probability of survival at end of study	87	77	78	8
Mean survival (days)	706	692	716	626
Survival analysis	P<0.001	P=0.236	P=0.355	P<0.001

^a Kaplan-Meier determinations

^b Mean of all deaths (uncensored, censored, and terminal sacrifice)

^c The result of the life table trend test (Tarone, 1975) is in the control column, and the results of the life table pairwise comparisons (Cox, 1972) with the controls are in the exposed group columns.

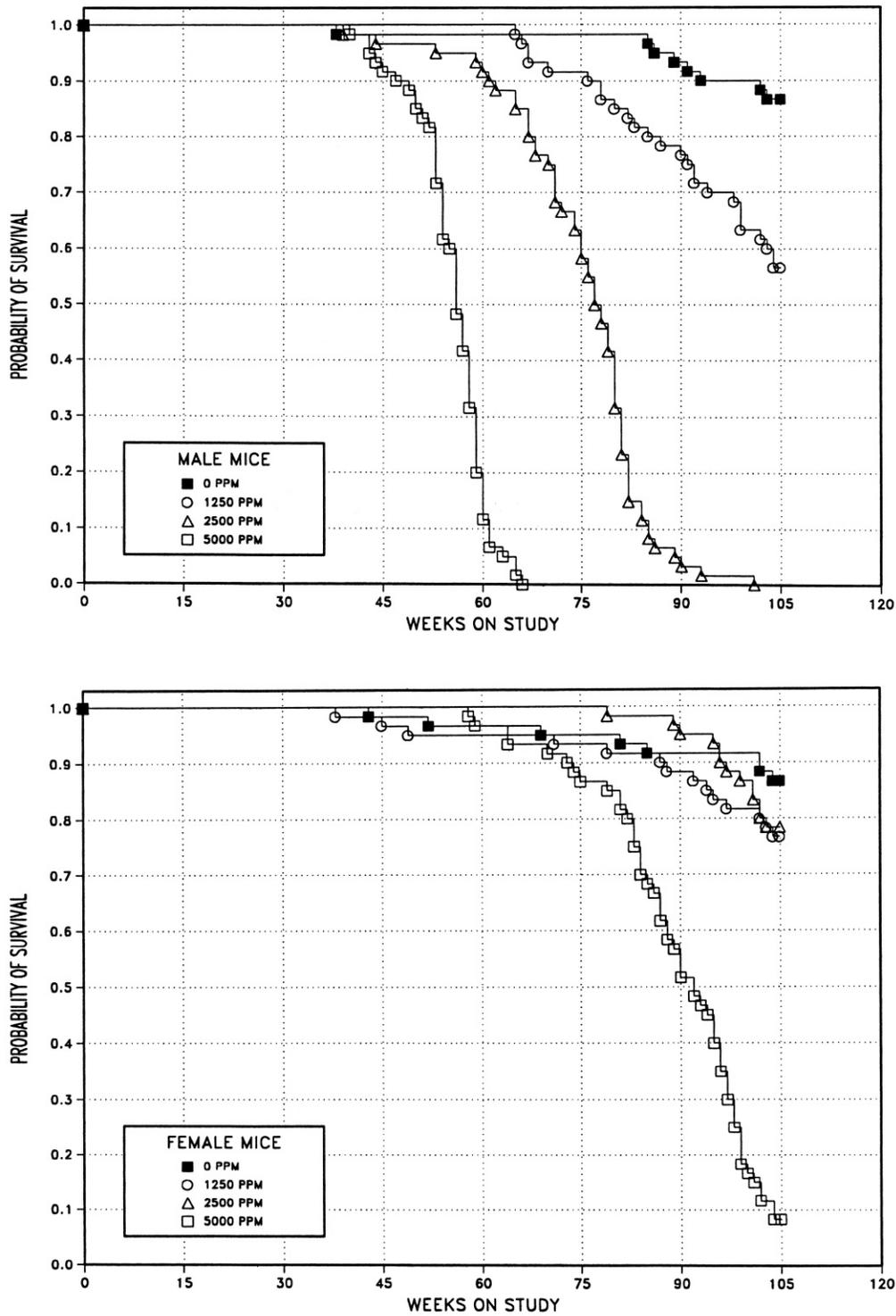


FIGURE 5
Kaplan-Meier Survival Curves for Male and Female Mice
Exposed to *o*-Nitrotoluene in Feed for 2 Years

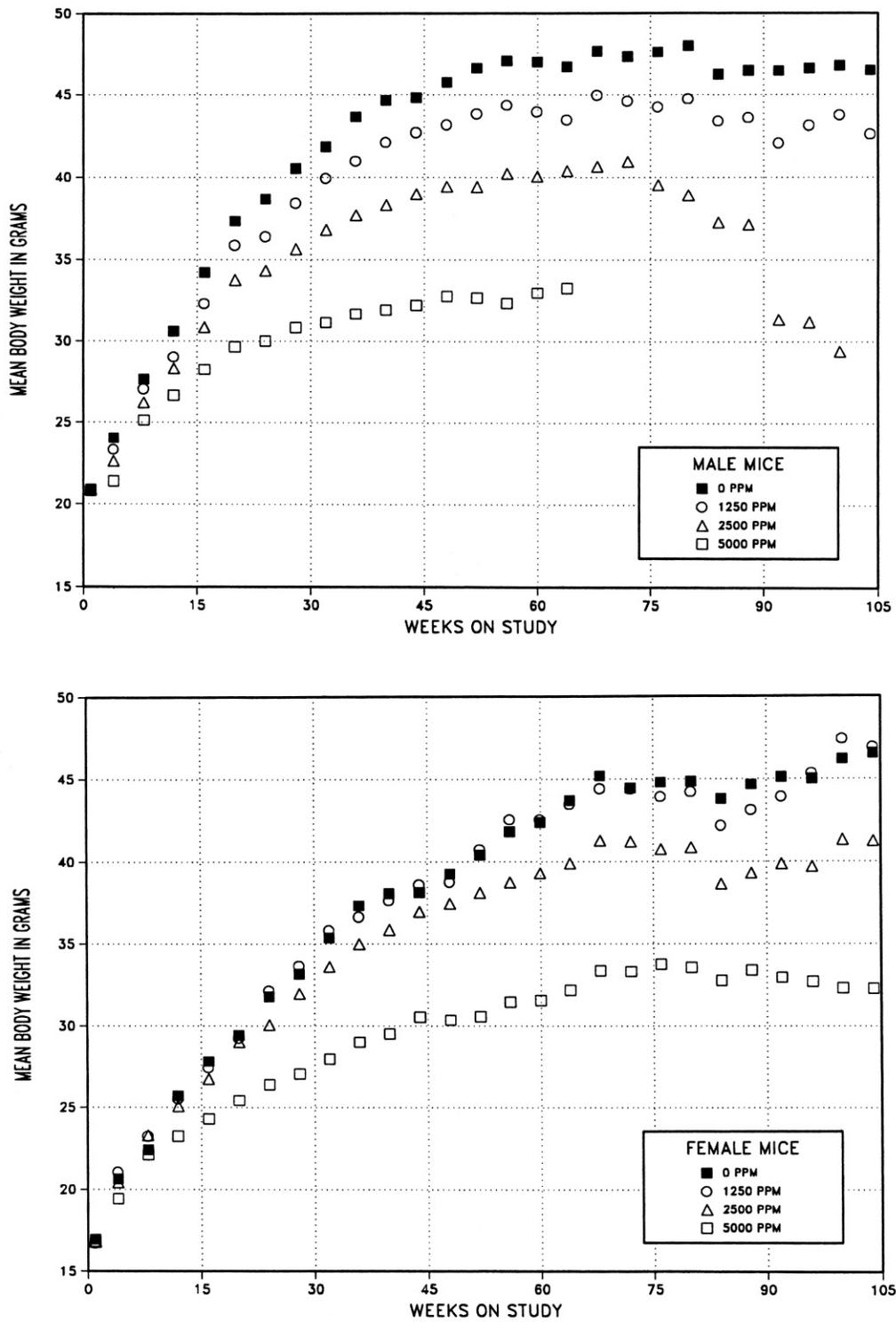


FIGURE 6
Growth Curves for Male and Female Mice
Exposed to *o*-Nitrotoluene in Feed for 2 Years

TABLE 19
Mean Body Weights and Survival of Male Mice in the 2-Year Feed Study of o-Nitrotoluene

Weeks on Study	0 ppm		1,250 ppm			2,500 ppm			5,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	20.9	60	20.8	100	60	20.9	100	60	20.8	100	60
4	24.0	60	23.4	98	60	22.6	94	60	21.4	89	60
8	27.7	60	27.1	98	60	26.2	95	60	25.1	91	60
12	30.6	60	29.0	95	60	28.3	93	60	26.7	87	60
16	34.2	60	32.3	94	60	30.9	90	60	28.3	83	60
20	37.3	60	35.9	96	60	33.8	91	60	29.7	80	60
24	38.7	60	36.4	94	60	34.3	89	60	30.0	78	60
28	40.5	60	38.4	95	60	35.6	88	60	30.8	76	60
32	41.9	60	39.9	95	60	36.8	88	60	31.1	74	60
36	43.6	60	41.0	94	60	37.7	87	60	31.7	73	60
40	44.7	59	42.1	94	60	38.3	86	59	31.9	71	60
44	44.8	59	42.7	95	60	39.0	87	59	32.2	72	57
48	45.8	59	43.2	94	60	39.4	86	58	32.8	72	54
52	46.6	59	43.8	94	60	39.4	85	58	32.7	70	49
56	47.1	59	44.4	94	60	40.2	85	57	32.4	69	35
60	47.0	59	44.0	94	60	40.1	85	56	33.0	70	8
64	46.7	59	43.4	93	60	40.4	87	53	33.3	71	3
68	47.7	59	45.0	94	56	40.7	85	48			
72	47.3	59	44.6	94	55	41.0	87	41			
76	47.6	59	44.2	93	54	39.5	83	34			
80	48.0	59	44.8	93	52	38.9	81	24			
84	46.3	59	43.4	94	49	37.3	81	9			
88	46.5	57	43.6	94	47	37.1	80	4			
92	46.5	55	42.1	91	45	31.4	68	2			
96	46.6	54	43.1	93	42	31.2	67	1			
100	46.8	54	43.7	93	38	29.4	63	1			
104	46.5	52	42.6	92	36						
Mean for weeks											
1-13	25.8		25.1	97		24.5	95		23.5	91	
14-52	41.8		39.6	95		36.5	87		31.1	74	
53-104	47.0		43.8	93		37.3	79		32.9	70	

TABLE 20
Mean Body Weights and Survival of Female Mice in the 2-Year Feed Study of *o*-Nitrotoluene

Weeks on Study	0 ppm		1,250 ppm			2,500 ppm			5,000 ppm		
	Av. Wt. (g)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors	Av. Wt. (g)	Wt. (% of controls)	No. of Survivors
1	16.9	60	16.7	99	60	16.8	99	60	16.7	99	60
4	20.6	60	21.1	102	60	20.4	99	60	19.4	94	60
8	22.4	60	23.2	104	60	23.3	104	60	22.1	99	60
12	25.7	60	25.5	99	60	25.1	98	60	23.2	90	60
16	27.8	60	27.4	99	60	26.8	96	60	24.3	87	60
20	29.4	60	29.3	100	60	29.0	99	60	25.4	86	60
24	31.8	60	32.2	101	60	30.1	95	60	26.4	83	60
28	33.2	60	33.6	101	60	32.0	96	60	27.1	82	60
32	35.4	60	35.8	101	60	33.6	95	60	28.0	79	60
36	37.3	60	36.7	98	60	35.0	94	60	29.0	78	60
40	38.1	60	37.7	99	59	35.9	94	60	29.5	77	60
44	38.1	59	38.6	101	59	37.0	97	60	30.5	80	60
48	39.3	59	38.8	99	58	37.5	95	60	30.4	77	60
52	40.4	59	40.7	101	57	38.1	94	60	30.6	76	60
56	41.8	58	42.6	102	57	38.8	93	60	31.5	75	60
60	42.4	58	42.5	100	57	39.3	93	60	31.6	75	58
64	43.7	58	43.5	100	57	39.9	91	60	32.2	74	57
68	45.2	58	44.4	98	57	41.3	91	60	33.4	74	56
72	44.5	57	44.4	100	56	41.2	93	60	33.3	75	55
76	44.8	57	43.9	98	56	40.8	91	60	33.8	75	52
80	44.8	57	44.2	99	55	40.9	91	59	33.6	75	51
84	43.8	56	42.2	96	55	38.7	88	59	32.8	75	43
88	44.7	55	43.1	96	54	39.3	88	59	33.4	75	35
92	45.1	55	43.9	97	53	39.9	89	57	33.0	73	30
96	45.0	55	45.3	101	50	39.7	88	56	32.7	73	23
100	46.2	55	47.4	103	49	41.3	89	52	32.3	70	10
104	46.6	53	46.9	101	46	41.2	88	47	32.3	69	6
Mean for weeks											
1-13	21.4		21.6	101		21.4	100		20.4	95	
14-52	35.1		35.1	100		33.5	95		28.1	80	
53-104	44.5		44.2	99		40.2	90		32.8	74	

Pathology and Statistical Analyses

This section describes the statistically significant or biologically noteworthy changes in the incidences of neoplasms and/or nonneoplastic lesions of the circulatory system, large intestine (cecum), liver, kidney, nose, spleen, skin, and prostate gland. Summaries of the incidences of neoplasms and nonneoplastic lesions, individual animal tumor diagnoses, statistical analyses of primary neoplasms that occurred with an incidence of at least 5% in at least one animal group, and historical incidences for the neoplasms mentioned in this section are presented in Appendix C for male mice and Appendix D for female mice.

Circulatory System: The incidences of hemangiosarcoma (all organs) in all exposed groups of males and in 5,000 ppm females were significantly greater than those in the controls (Tables 21, C3, and D3). The incidences exceeded the 2-year historical ranges in control (all routes) mice given NTP-2000 diet and in feed study controls given NIH-07 diet (Tables 21, C4a, and D4a).

Hemangiosarcomas in exposed males and females occurred primarily in the mesentery (Plates 13 and 14), skeletal muscle (Plates 15 and 16), and subcutis of the skin (Tables 21, C3, and D3). In some cases, mice with multiple and/or large hemangiosarcomas had small hemangiosarcomas in other tissues, primarily the lung (Tables C1 and D1). Many of these neoplasms were considered to be metastases. Hemangiosarcomas ranged from small neoplasms, approximately 1 to 2 mm in diameter, to large masses with diameters greater than 1 cm. Microscopically, hemangiosarcomas consisted of numerous, irregular, variably sized, blood-filled vascular channels lined by large, pleomorphic endothelial cells and separated by variable amounts of fibrous stroma. The central portions of most hemangiosarcomas had undergone necrosis, leaving a large, blood-filled cavity surrounded by a rim of neoplastic vascular tissue. Generally, in the B6C3F₁ mouse, hemangiosarcomas can be multicentric, and often arise primarily in the spleen and liver (Ward *et al.*, 1999).

TABLE 21
Incidences of Hemangiosarcoma in Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Male				
Number Necropsied	60	60	60	60
Hemangiosarcoma, Mesentery, Multiple ^a	0	0	8**	14**
Hemangiosarcoma, Mesentery (includes multiple)	0	8**	38**	38**
Hemangiosarcoma, Skeletal Muscle, Multiple	0	0	8**	26**
Hemangiosarcoma, Skeletal Muscle (includes multiple)	0	6*	33**	45**
Hemangiosarcoma, Subcutaneous Skin, Multiple	0	0	0	8**
Hemangiosarcoma, Subcutaneous Skin, (includes multiple)	0	4*	8**	20**
Hemangiosarcoma (includes multiple), All Organs ^b				
Overall rate ^c	4/60 (7%)	17/60 (28%)	55/60 (92%)	60/60 (100%)
Adjusted rate ^d	7.0%	32.7%	97.9%	100.0%
Terminal rate ^e	3/52 (6%)	9/34 (27%)	0/0	0/0
First incidence (days)	710	542	365	277
Poly-3 test ^f	P<0.001	P<0.001	P<0.001	P<0.001
Female				
Number Necropsied	60	60	60	60
Hemangiosarcoma, Mesentery, Multiple	0	0	0	7*
Hemangiosarcoma, Mesentery (includes multiple)	0	0	0	32**
Hemangiosarcoma, Skeletal Muscle, Multiple	0	0	0	2
Hemangiosarcoma, Skeletal Muscle (includes multiple)	0	0	0	16**
Hemangiosarcoma, Subcutaneous Skin, Multiple	0	0	0	3
Hemangiosarcoma, Subcutaneous Skin (includes multiple)	0	0	2	19**
Hemangiosarcoma (includes multiple), All Organs ^g				
Overall rate	0/60 (0%)	2/60 (3%)	3/60 (5%)	50/60 (83%)
Adjusted rate	0.0%	3.6%	5.2%	90.2%
Terminal rate	0/52 (0%)	1/46 (2%)	2/47 (4%)	4/5 (80%)
First incidence (days)	— ^h	343	624	409
Poly-3 test	P<0.001	P=0.232	P=0.124	P<0.001

* Significantly different (P≤0.05) from the control group by the Poly-3 test

** P≤0.01

^a Number of animals with lesion

^b Historical incidence for 2-year studies with controls given NTP-2000 diet (mean ± standard deviation): 37/659 (5.8% ± 3.2%), range 2%-14%; with feed study controls given NIH-07 diet: 53/952 (5.6% ± 3.5%), range 2%-14%

^c Number of animals with neoplasm per number of animals necropsied

^d Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^e Observed incidence at terminal kill

^f Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.

^g Historical incidence for NTP-2000 diet: 15/659 (2.6% ± 2.7%), range 0%-8%; for NIH-07 diet: 31/953 (3.3% ± 2.4%), range 0%-8%

^h Not applicable; no neoplasms in animal group

Large Intestine (Cecum): The incidences of carcinoma of the cecum in 1,250 and 2,500 ppm males were significantly increased and exceeded the 2-year historical range in control (all routes) male mice given NTP-2000 diet; this neoplasm has not been observed in feed study controls given NIH-07 diet (Tables 22, C3, and C4b). Although the increased incidences of carcinoma in exposed groups of females were not significant, they were considered to be exposure related because this neoplasm is very rare and has not been observed in female controls given NTP-2000 diet (0/659; all routes) or NIH-07 diet (0/953; feed studies) for 2 years. In the current study, no cecal carcinomas were observed in either control group or in 5,000 ppm males. All 5,000 ppm males died by week 66; hemangiosarcomas may have caused death before cecal carcinomas developed. Microscopically, the carcinomas were characterized by proliferation of glandular structures composed of

moderately pleomorphic mucosal epithelial cells that invaded the cecal wall. In some smaller carcinomas, minimal invasion was observed, while in large carcinomas, extensive invasion was observed and was characterized by neoplastic cells extending through the cecal wall into the attached mesentery (Plates 17 and 18) or invaded vascular structure (Plate 19). In some carcinomas, the submucosa was diffusely thickened by solid masses of large cells resembling “signet ring cells” with abundant clear cytoplasm and a peripherally located nucleus and mixed with smaller numbers of epithelial cells with large nuclei and small amounts of cytoplasm. Invasion of the cecal wall by these neoplasms was not apparent, although similar cells were seen in other carcinomas with extensive invasion. All carcinomas of the cecum were confirmed to be of epithelial cell origin (cytokeratin-20 positive) and were negative for lysozyme (macrophage marker).

TABLE 22
Incidences of Carcinoma of the Large Intestine (Cecum) in Mice
in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Male				
Carcinoma ^a				
Overall rate ^b	0/60 (0%)	12/60 (20%)	9/60 (15%)	0/60 (0%)
Adjusted rate ^c	0.0%	22.7%	31.6%	0.0%
Terminal rate ^d	0/52 (0%)	4/34 (12%)	0/0	0/0
First incidence (days)	— ^e	485	270	—
Poly-3 test ^f	P<0.001	P<0.001	P<0.001	— ^g
Female				
Carcinoma ^h				
Overall rate	0/60 (0%)	1/60 (2%)	4/60 (7%)	3/60 (5%)
Adjusted rate	0.0%	1.9%	7.0%	7.4%
Terminal rate	0/52 (0%)	1/46 (2%)	3/47 (6%)	1/5 (20%)
First incidence (days)	—	729 (T)	715	619
Poly-3 test	P=0.024	P=0.492	P=0.064	P=0.072

(T) Terminal sacrifice

^a Historical incidence for 2-year studies with controls given NTP-2000 diet (mean ± standard deviation): 1/659 (0.2% ± 0.6%), range 0%-2%; with feed study controls given NIH-07 diet: 0/952

^b Number of animals with neoplasm per number of animals necropsied

^c Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^d Observed incidence at terminal kill

^e Not applicable; no neoplasms in animal group

^f Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.

^g Value of statistic cannot be computed.

^h Historical incidence for NTP-2000 diet: 0/659; for NIH-07 diet: 0/953

Liver: The incidences of hepatocellular adenoma in 2,500 and 5,000 ppm females, hepatocellular carcinoma in 5,000 ppm females, and hepatocellular adenoma or carcinoma (combined) in 2,500 and 5,000 ppm females were significantly greater than those in the controls and exceeded the 2-year historical ranges for these neoplasms in control (all routes) female mice given NTP-2000 diet (Tables 23, D3, and D4b). In addition, the incidences of hepatocellular carcinoma and hepatocellular adenoma or carcinoma (combined) in 5,000 ppm females exceeded the historical ranges for feed study controls given NIH-07 diet. Due to low survival, these neoplasms occurred less frequently in 2,500 and 5,000 ppm males than in the concurrent or historical controls (Tables 18, 23, C3, and C4c). The incidences of eosinophilic foci in 1,250 ppm males and 5,000 ppm females and of basophilic foci in 1,250 and 2,500 ppm males and 1,250 and 5,000 ppm females were significantly increased (Tables 23, C5, and D5). Microscopically, foci, hepatocellular adenoma, and hepatocellular carcinoma represent a continuum. These lesions had an appearance typical of that seen in B6C3F₁ mice. Eosinophilic and basophilic foci were small to moderately large lesions composed of hepatocytes with eosinophilic and basophilic cytoplasm; generally, these hepatocytes were somewhat enlarged. The hepatocytes were arranged in normal hepatic cords that merged with the surrounding normal hepatocytes. Most foci had little or no compression of the surrounding normal hepatocytes, although some degree of compression was present in some larger foci. Adenomas were discrete masses with distinct borders that caused compression of the surrounding normal hepatic parenchyma. Adenomas usually were composed of hepatocytes that appeared similar to those seen in eosinophilic foci, except that in adenomas the normal lobular architecture was not apparent, and plates

of neoplastic hepatocytes intersected the surrounding normal hepatocytes at sharp angles rather than merging with them as in foci. Carcinomas were discrete masses that generally had irregular borders due to localized areas of growth of neoplastic hepatocytes into the surrounding normal parenchyma. The neoplastic hepatocytes often were somewhat atypical, but the major distinguishing feature of carcinomas was the presence of abnormal patterns of growth. The most common abnormal growth pattern was formation of trabeculae of neoplastic hepatocytes that were three or more cell layers thick (Plate 20), while less commonly the neoplastic cells formed glandular structures or solid masses.

Incidences of focal syncytial alteration of hepatocytes were significantly increased in all groups of exposed males (Tables 23 and C5). Microscopically, syncytial alteration was generally a minimal change consisting of one to several scattered hepatocytes containing multiple (usually four to six) small nuclei. The significance of this lesion is uncertain; the detection of this minimal change was most likely due to the lower number of hepatocellular neoplasms in males that died early, making it possible to detect more syncytial cells microscopically. The incidences of necrosis in all exposed male groups and the incidences of necrosis, focal hepatocyte necrosis, and focal cytoplasmic vacuolization in 5,000 ppm females were significantly increased (Tables 23, C5, and D5). Necrosis was characterized by one or more scattered discrete foci of coagulative necrosis consisting of hepatocytes with uniformly eosinophilic cytoplasm and pyknotic or karyorrhectic nuclei. Focal hepatocyte necrosis was characterized by small numbers of necrotic hepatocytes that were associated with mixed inflammatory cells.

TABLE 23
Incidences of Neoplasms and Nonneoplastic Lesions of the Liver in Mice
in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Male				
Number Examined Microscopically	60	59	57	60
Basophilic Focus ^a	0	6*	4*	0
Eosinophilic Focus	3	14**	1	1
Necrosis	1 (1.0) ^b	15** (2.0)	27** (1.9)	30** (1.5)
Hepatocyte, Syncytial Alteration, Focal	16 (1.0)	26* (1.1)	43** (1.2)	39** (1.4)
Hepatocellular Adenoma, Multiple	3	6	2	0
Hepatocellular Adenoma (includes multiple)	18	18	3	0
Hepatocellular Carcinoma, Multiple	3	7	1	0
Hepatocellular Carcinoma (includes multiple)	12	16	5	2
Hepatocellular Adenoma or Carcinoma ^c	27	28	7	2
Female				
Number Examined Microscopically	60	59	59	60
Basophilic Focus	1	6*	2	6*
Eosinophilic Focus	2	3	6	28**
Necrosis	3 (1.7)	0	2 (1.5)	13** (2.2)
Hepatocyte, Necrosis, Focal	0	0	0	6** (1.5)
Hepatocyte, Cytoplasmic Vacuolization, Focal	1 (2.0)	2 (2.5)	2 (1.5)	9** (1.4)
Hepatocellular Adenoma, Multiple	0	0	3	20**
Hepatocellular Adenoma (includes multiple) ^d	7	5	19**	29**
Hepatocellular Carcinoma, Multiple	0	1	2	7
Hepatocellular Carcinoma (includes multiple) ^e	2	4	6	16**
Hepatocellular Adenoma or Carcinoma ^f				
Overall rate ^g	9/60 (15%)	9/59 (15%)	24/59 (41%)	39/60 (65%)
Adjusted rate ^h	15.7%	16.9%	42.1%	79.1%
Terminal rate ⁱ	8/52 (15%)	9/46 (20%)	20/47 (43%)	5/5 (100%)
First incidence (days)	362	729 (T)	661	567
Poly-3 test ^j	P<0.001	P=0.538	P<0.001	P<0.001

* Significantly different ($P \leq 0.05$) from the control group by the Poly-3 test

** $P \leq 0.01$

(T) Terminal sacrifice

^a Number of animals with lesion

^b Average severity grade of lesions in affected animals: 1=minimal, 2=mild; 3=moderate, 4=marked

^c Historical incidence for 2-year studies with control groups given NTP-2000 diet (mean \pm standard deviation): 304/659 (47.8% \pm 12.9%), range 28%-72%; with feed study controls given NIH-07 diet: 493/950 (51.9% \pm 7.9%), range 40%-68%

^d Historical incidence for NTP-2000 diet: 101/655 (16.0% \pm 6.3%), range 8%-28%; for NIH-07 diet: 218/951 (22.9% \pm 9.9%), range 12%-50%

^e Historical incidence for NTP-2000 diet: 49/655 (8.0% \pm 4.7%), range 3%-16%; for NIH-07 diet: 104/951 (10.9% \pm 4.6%), range 4%-20%

^f Historical incidence for NTP-2000 diet: 143/655 (22.8% \pm 9.6%), range 12%-40%; for NIH-07 diet: 292/951 (30.7% \pm 10.2%), range 12%-56%

^g Number of animals with neoplasm per number of animals with liver examined microscopically

^h Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

ⁱ Observed incidence at terminal kill

^j Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for differential mortality in animals that do not reach terminal sacrifice.

Kidney: The incidences of renal tubule pigmentation in all exposed groups of males and 5,000 ppm females were significantly greater than those in the controls (Tables 24, C5, and D5). Microscopically, pigmentation was a minimal to moderate change consisting of fine, dark brown granules within the cytoplasm of cortical

renal tubular epithelial cells. The incidence of hyaline droplet accumulation in renal tubule cells was significantly increased in 2,500 ppm males and in 5,000 ppm females. Microscopically, hyaline droplet accumulation consisted of small, homogeneous, brightly eosinophilic droplets within the epithelium of renal cortical tubules.

TABLE 24
Incidences of Selected Nonneoplastic Lesions of the Kidney in Mice
in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Male				
Number Examined Microscopically	58	59	58	60
Renal Tubule Pigmentation ^a	1 (1.0) ^b	6* (2.8)	32** (2.3)	35** (2.2)
Renal Tubule, Hyaline Droplet Accumulation	1 (4.0)	2 (3.0)	5* (2.0)	3 (1.3)
Female				
Number Examined Microscopically	59	56	58	59
Renal Tubule Pigmentation	0	1 (2.0)	3 (2.0)	35** (2.9)
Renal Tubule, Hyaline Droplet Accumulation	1 (3.0)	3 (3.0)	2 (2.0)	10** (2.3)

* Significantly different ($P \leq 0.05$) from the control group by the Poly-3 test

** $P \leq 0.01$

^a Number of animals with lesion

^b Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

Nose: Olfactory epithelial degeneration occurred in all mice that received 2,500 or 5,000 ppm (males: 0/60, 36/60, 60/60, 60/60; females: 0/60, 28/60, 59/59, 57/57; Tables C5 and D5). Severities of this lesion increased with increasing exposure concentration (males: 1.1, 1.8, 3.0; females: 1.0, 2.0, 3.0). Olfactory degeneration was a complex lesion and consisted of necrosis, atrophy, regeneration, hyperplasia, hypertrophy, and metaplasia. The most prominent changes were atrophy of the olfactory epithelium, Bowman's glands, and olfactory nerve bundles accompanied by replacement of the olfactory epithelium with a respiratory-type epithelium composed of ciliated columnar cells that covered the dorsal meatus surface and extended into the mucosa to form dilated pseudoglands. Olfactory degeneration was similar to that seen in mice exposed to *o*-nitrotoluene for 13 weeks (NTP, 1992).

Spleen: The incidences of hematopoietic cell proliferation were significantly increased in all exposed groups of males (13/60, 24/60, 49/58, 60/60; Table C5) and the 2,500 and 5,000 ppm females (11/59, 19/57, 21/58, 54/57; Table D5). These increases were considered secondary to the increased incidences of hemangiosarcoma in these groups.

Skin: The incidences of edema of the subcutaneous tissue in 2,500 and 5,000 ppm males (0/60, 3/60, 14/60, 22/60; Table C5) and in the 5,000 ppm females (0/60, 1/60, 2/60, 4/60; Table D5) were significantly greater than those in the control groups. Microscopically, the thickened subcutaneous tissue was characterized by separation of the connective tissue by abundant clear spaces caused by the accumulation of fluid. The cause of the edema was unclear. However, the presence of dilated

lymphatics in the areas of edema suggested that the edema may have resulted from lymphatic obstruction caused by the presence of subcutaneous hemangiosarcomas.

Prostate Gland: The incidences of chronic inflammation in 2,500 and 5,000 ppm males were significantly increased (0 ppm, 0/60; 1,250 ppm, 1/59; 2,500 ppm, 7/57; 5,000 ppm, 4/58; Table C5). These increases were minimal and were not considered to be exposure related.

GENETIC TOXICOLOGY

o-Nitrotoluene (3.0 to 1,000 µg/plate) was not mutagenic in *Salmonella typhimurium* strain TA98, TA100, TA1535, or TA1537, with or without Aroclor-induced rat or hamster liver S9 (Table E1; Haworth *et al.*, 1983). Significantly increased sister chromatid exchange frequencies were observed in cultured Chinese hamster ovary cells treated with *o*-nitrotoluene with S9; an equivocal response was seen without S9 (Table E2; Galloway *et al.*, 1987). Due to *o*-nitrotoluene-induced cell cycle delay in the trial without S9, an extended culture time was employed to permit accumulation of sufficient second-division metaphase cells for analysis. *o*-Nitrotoluene did not induce chromosomal aberrations in cultured Chinese hamster ovary cells with or without S9 (Table E3; Galloway *et al.*, 1987). No increases in

the frequencies of micronucleated polychromatic erythrocytes were observed *in vivo* in the bone marrow of male rats or male mice treated with *o*-nitrotoluene. In male F344/N rats, an acute micronucleus test was performed using two protocols (Table E4). The initial test used a single intraperitoneal injection of *o*-nitrotoluene followed by bone marrow analysis 24 hours later; in the second test, bone marrow was harvested for analysis 48 hours after a single intraperitoneal injection of *o*-nitrotoluene. In neither test was a positive response observed. In male mice, three intraperitoneal injections also yielded negative results, although a small increase in the frequency of micronucleated polychromatic erythrocytes was observed at all doses tested (Table E5). *o*-Nitrotoluene, administered in feed for 13 weeks, did not increase the frequency of micronucleated normochromatic (mature) erythrocytes in peripheral blood of female mice. However, a small increase in the frequency of micronucleated normochromatic erythrocytes was noted in male mice at the highest exposure concentration tested, 10,000 ppm (Table E6). The increase in male mice was sufficient to generate a significant trend test ($P=0.003$), but because none of the frequencies in individual exposed groups were significantly increased over the corresponding control group and because the increase in the frequency of micronuclei in the 10,000 ppm group was small, the test in male mice was judged to be equivocal.

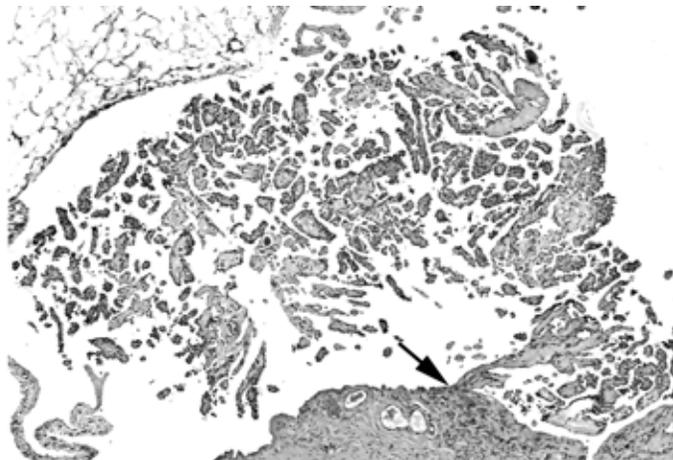


PLATE 1

Malignant mesothelioma in a male F344/N rat exposed to 5,000 ppm (stop-exposure) *o*-nitrotoluene in the 2-year feed study. Note the mesothelioma extending from the tunica vaginalis (arrow). H&E; 16X

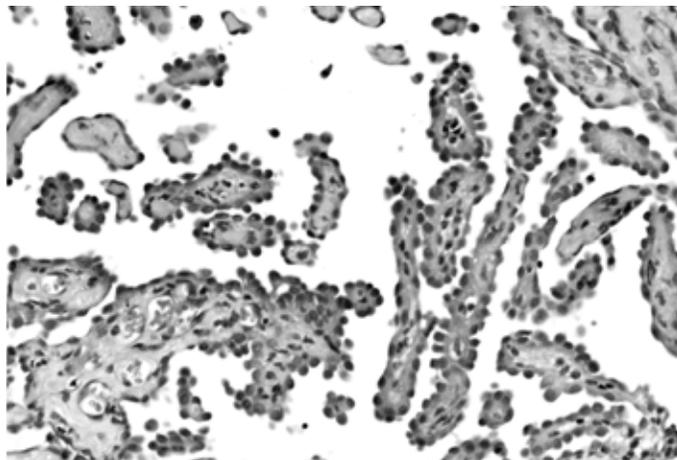


PLATE 2

Higher magnification of papillary portion of the mesothelioma in Plate 1. Note the malignant mesothelial cells covering the stroma. H&E; 80X

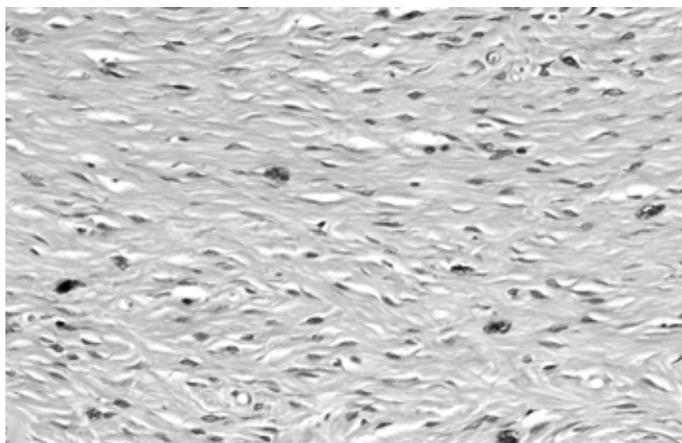


PLATE 3

Subcutaneous skin fibroma in a male F344/N rat exposed to 2,000 ppm *o*-nitrotoluene in feed for 2 years. Note the proliferation of fibrocytes, lack of cellular atypia, and the abundant collagenous stroma. H&E; 80X

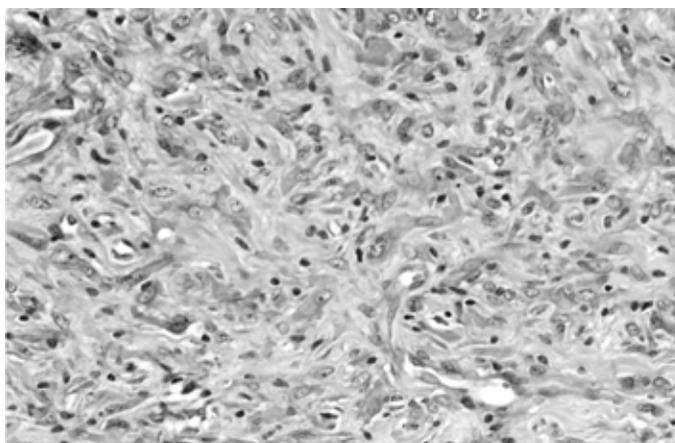


PLATE 4

Subcutaneous skin fibrosarcoma in a male F344/N rat exposed to 1,250 ppm *o*-nitrotoluene in feed for 2 years. Compared to the fibroma in Plate 3, the fibrosarcoma is more cellular, neoplastic cells are more pleomorphic, and the collagenous stroma is less prominent. H&E; 80X

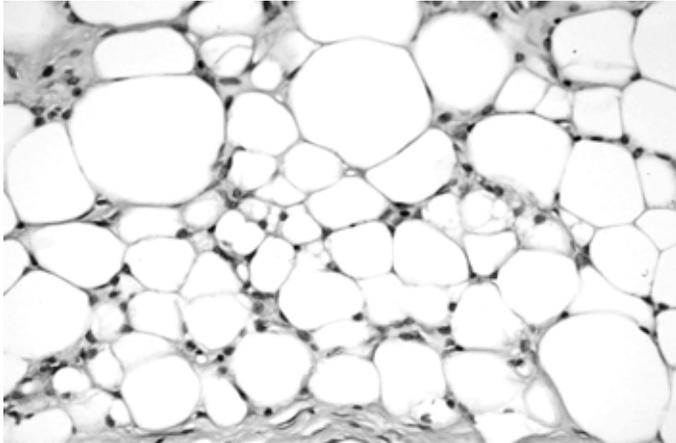


PLATE 5

Subcutaneous lipoma in a male F344/N rat exposed to 2,000 ppm *o*-nitrotoluene in feed for 2 years. Note the sheet of variably sized adipocytes within the subcutis. H&E; 80x

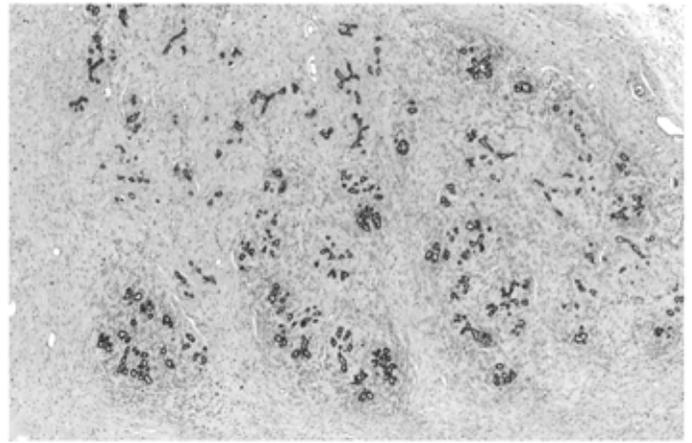


PLATE 6

Mammary gland fibroadenoma in a male F344/N rat exposed to 1,250 ppm *o*-nitrotoluene in feed for 2 years. Note the collection of glandular epithelium surrounded by fibrous connective tissue. H&E; 10x

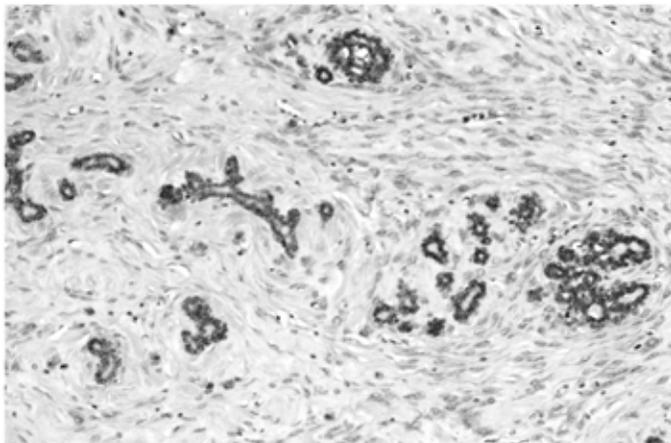


PLATE 7

Higher magnification of the fibroadenoma in Plate 6. Uniform epithelial cells are arranged in a single layer forming glands and ducts. H&E; 40x

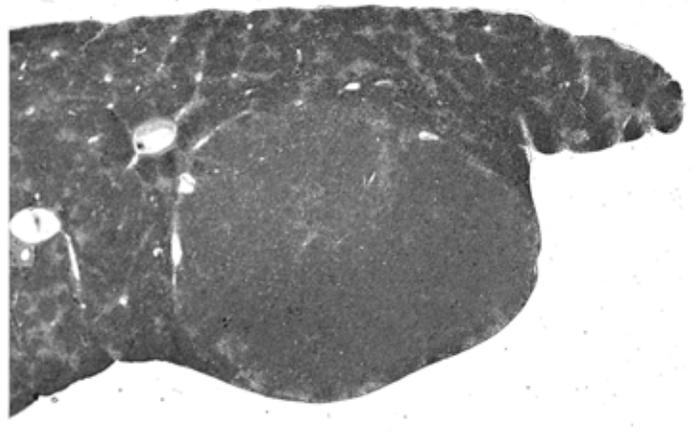


PLATE 8

Hepatocellular adenoma in a male F344/N rat exposed to 2,000 ppm *o*-nitrotoluene in feed for 2 years. Note the compression of adjacent hepatocytes and loss of normal lobular parenchyma. H&E; 5x

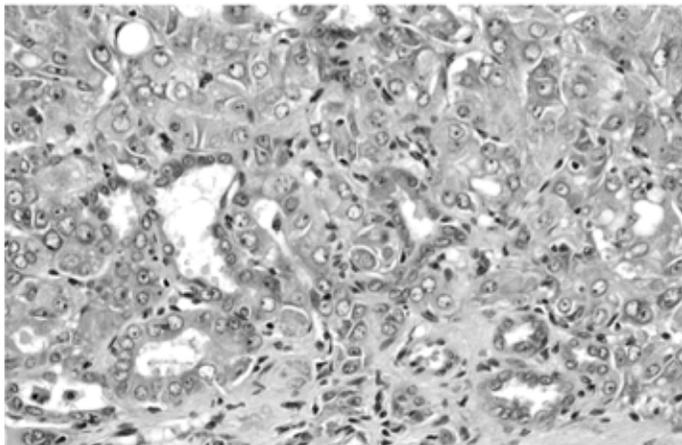


PLATE 9
Hepatocholangiocarcinoma in a male F344/N rat exposed to 2,000 ppm *o*-nitrotoluene in feed for 2 years. Note the presence of cells resembling hepatocytes and bile ductules. H&E; 80x

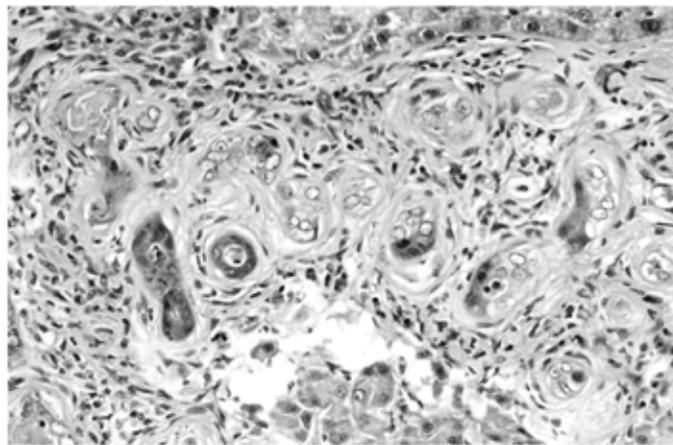


PLATE 10
Cholangiocarcinoma in the liver of a male F344/N rat exposed to 5,000 ppm (stop-exposure) *o*-nitrotoluene in the 2-year feed study. Note the atypical bile duct cells surrounded by scirrhous-type response. H&E; 80x

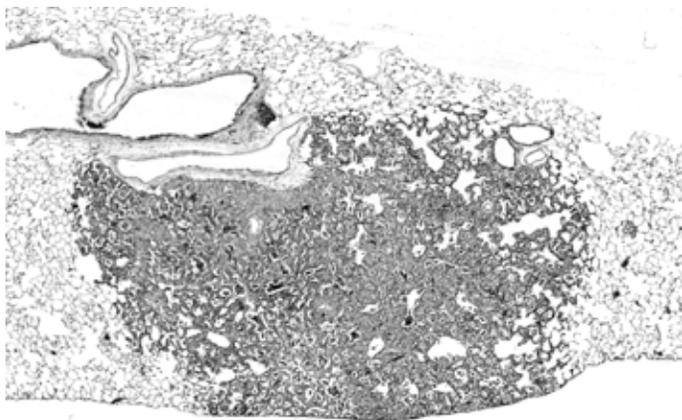


PLATE 11
Alveolar/bronchiolar adenoma in a male F344/N rat exposed to 5,000 ppm (stop-exposure) *o*-nitrotoluene in the 2-year feed study. Note that the normal alveolar architecture is distorted. H&E; 8x

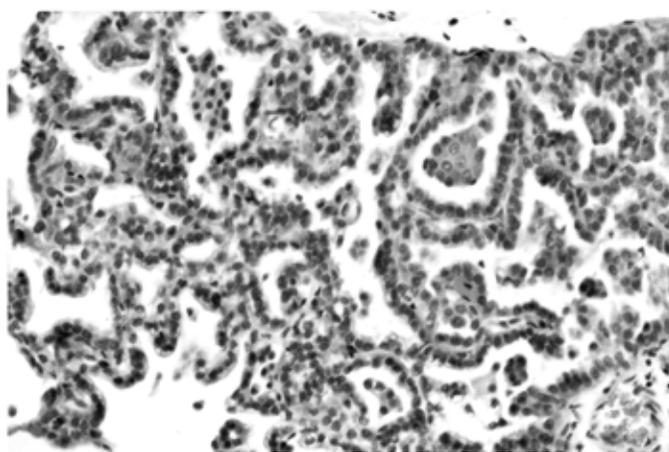


PLATE 12
Higher magnification of the alveolar/bronchiolar adenoma in Plate 11. Note the papillary arrangement of well-differentiated neoplastic cells. H&E; 80x

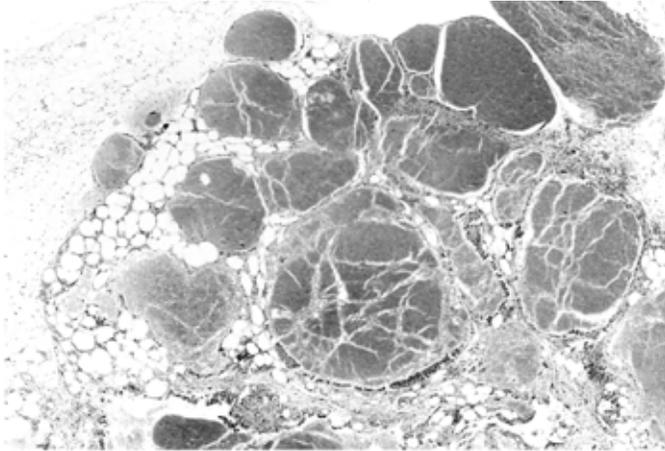


PLATE 13

Hemangiosarcoma in the mesentery from a male B6C3F₁ mouse exposed to 5,000 ppm *o*-nitrotoluene in feed for 2 years. Note the variably sized and large blood-filled cavernous spaces lined by malignant endothelial cells. H&E; 10X□

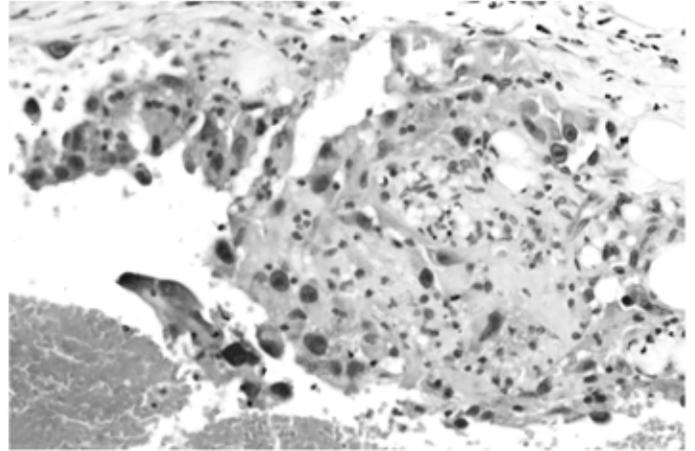


PLATE 14

Higher magnification of Plate 13. Malignant endothelial cells line the cavernous spaces. Note the nuclear pleomorphism and hyperchromatic nuclei. H&E; 80X□

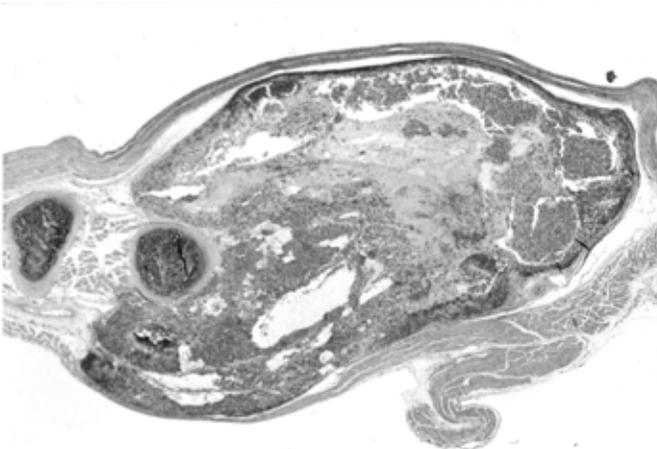


PLATE 15

Hemangiosarcoma in the skeletal muscle (thorax) of a male B6C3F₁ mouse exposed to 5,000 ppm *o*-nitrotoluene in feed for 2 years. Note that the central portion of the hemangiosarcoma has undergone necrosis, leaving a large blood-filled cavity surrounded by a rim of neoplastic endothelial cells. H&E; 8X□

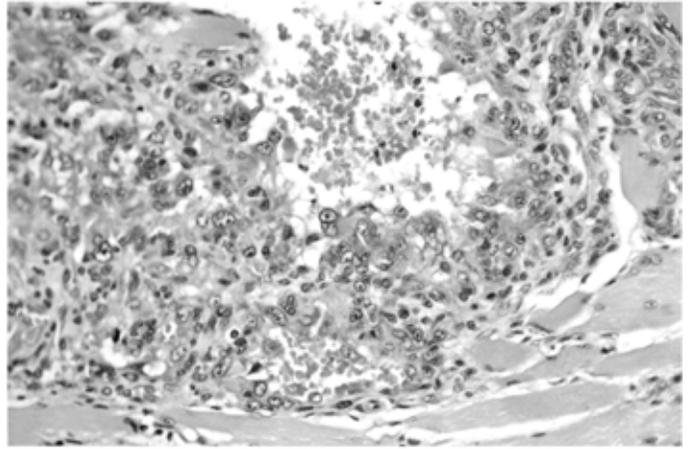


PLATE 16

Higher magnification of Plate 15. A sheet of malignant endothelial cells is associated with the skeletal muscle (right bottom part of figure). H&E; 80X□

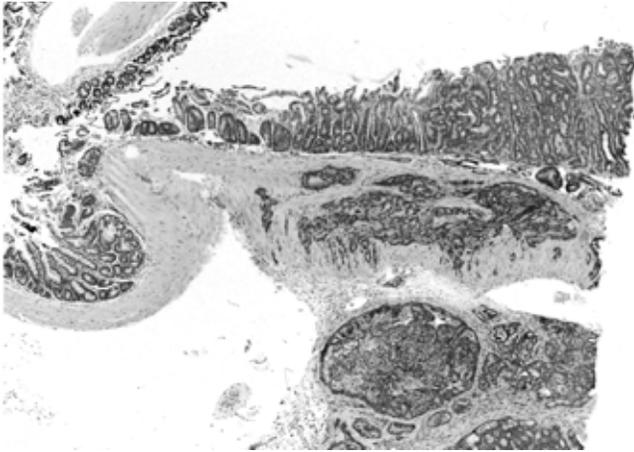


PLATE 17

Carcinoma in the cecum of a male B6C3F₁ mouse exposed to 2,500 ppm *o*-nitrotoluene in feed for 2 years. Note the neoplastic epithelial cells extending through muscle layers of the cecum and within the mesentery. H&E; 10x□

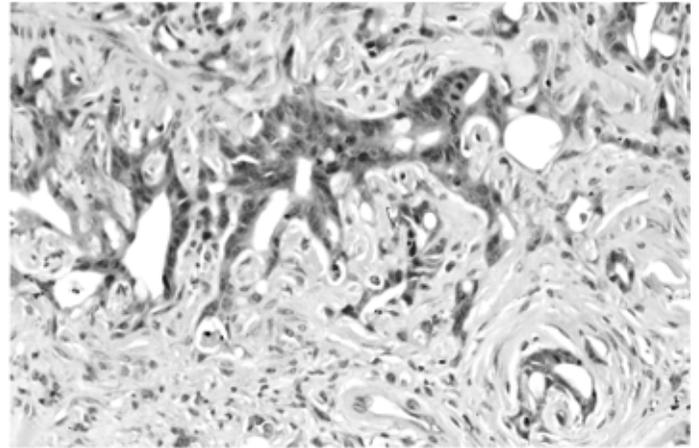


PLATE 18

Higher magnification of Plate 17. Note the infiltrative nature of the anaplastic epithelial cells within the cecal wall. H&E; 16x□

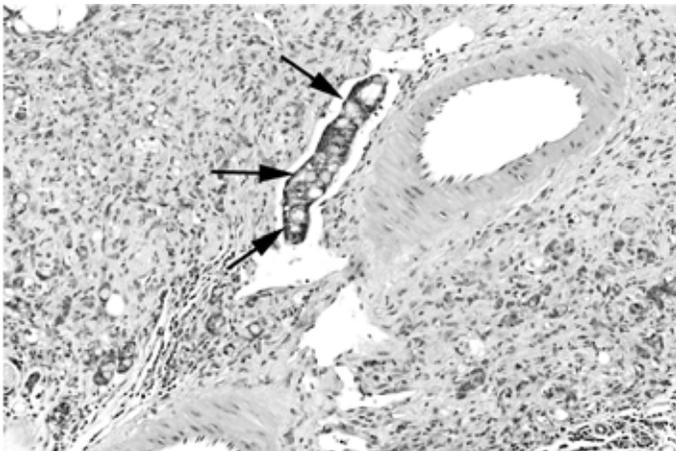


PLATE 19

Carcinoma in the cecum of a female B6C3F₁ mouse exposed to 2,500 ppm *o*-nitrotoluene in feed for 2 years. Note the neoplastic epithelial cells within the lymphatic vasculature (arrows). H&E; 40x□

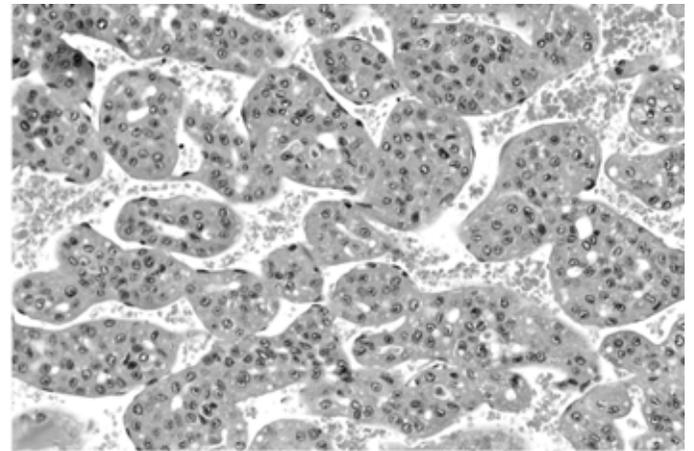


PLATE 20

Hepatocellular carcinoma in a female B6C3F₁ mouse exposed to 5,000 ppm *o*-nitrotoluene in feed for 2 years. Note the trabeculae of neoplastic hepatocytes that are three or more cell layers thick. H&E; 66x□

DISCUSSION AND CONCLUSIONS

This NTP Technical Report continues the reporting of the comparative biologic effects of the nitrotoluene isomers. The results for the 2-year carcinogenicity studies of *o*-nitrotoluene in F344/N rats and B6C3F₁ mice are described here. The results of the 2-year studies of *p*-nitrotoluene in rats and mice are reported in NTP Technical Report 498 (NTP, 2002).

Comparative 13-week toxicity studies of *o*-, *m*-, and *p*-nitrotoluene (NTP, 1992) showed that all three isomers caused mild toxicity in the kidney, spleen, liver, and/or reproductive system, particularly at the highest concentration of 10,000 ppm. In general, toxicity was most severe with the *ortho* isomer. *o*-Nitrotoluene was carcinogenic in male rats, as indicated by the occurrence of mesotheliomas in the 5,000 ppm group and mesothelial cell hyperplasia in the 10,000 ppm group. The 2-year studies were conducted to further evaluate the carcinogenic potential of *o*-nitrotoluene.

In the 2-year studies, *o*-nitrotoluene caused multiple carcinogenic effects in rats and mice at all exposure concentrations. The average daily dose for core study male rats receiving 625, 1,250, or 2,000 ppm was approximately 25, 50, or 90 mg *o*-nitrotoluene/kg body weight, respectively; male rats in the 2,000 or 5,000 ppm stop-exposure groups received approximately 125 or 315 mg/kg for the first 3 months of the study. Female rats exposed to 625, 1,250, or 2,000 ppm received an average daily dose of approximately 30, 60, or 100 mg/kg. The average daily doses were approximately 165, 360, or 700 mg/kg for male mice and 150, 320, or 710 mg/kg for female mice receiving 1,250, 2,500, or 5,000 ppm.

Survival was reduced in all exposed groups of male rats and male mice because of the development of neoplasms. The development of neoplasms also reduced survival of 2,000 ppm female rats and 5,000 ppm female mice. Reduced survival of exposed rats and mice led to cancellation of the planned 15-month interim evaluations.

Increased incidences of neoplasms in stop-exposure males, including mesothelioma, skin neoplasms, and mammary gland fibroadenoma, indicated that 3 months of *o*-nitrotoluene exposure were sufficient to produce a carcinogenic effect.

Mean body weights of exposed male rats except the 625 ppm group were generally less than those of the controls throughout the study, and those of 2,000 ppm females were less during the second year of the study. Mean body weights of exposed groups of male mice and 5,000 ppm females were less than those of controls throughout most of the study; those of 2,500 ppm females were less during the second year of the study.

Malignant mesothelioma is a neoplasm arising from the surface serosal cells of the pleural, peritoneal, and pericardial cavities and from the tunica vaginalis (Attanoos and Gibbs, 1997). Mesotheliomas or mesothelial hyperplasia occurred in male rats after 13 or 26 weeks of exposure to 5,000 or 10,000 ppm *o*-nitrotoluene (NTP, 1992, 1996). In these studies, mesotheliomas did not occur in control rats and occurred in less than 4% of historical control male rats in 2-year studies. At the 3-month interim sacrifice in the current 2-year study, no mesotheliomas or mesothelial hyperplasia were observed in exposed male rats. However, as time progressed, chemical-induced mesotheliomas occurred in all exposed male rat groups, including the stop-exposure groups, and the incidences were significantly increased by both the pairwise and trend statistics. The incidences in the exposed male rats exceeded the historical ranges for control male rats given NTP-2000 or NIH-07 diet. The occurrence of mesothelioma in male rats was considered to be clear evidence of carcinogenic activity.

Malignant mesotheliomas occurred with incidences of 33%, 48%, and 73% in the 625, 1,250, and 2,000 ppm core study male rat groups, respectively. The incidences of malignant mesothelioma were 73% and 90% in the 2,000 and 5,000 ppm stop-exposure male rat groups. The incidence of mesothelioma was higher in the

2,000 ppm stop-exposure group than in the 625 ppm group even though the latter group received approximately 50% more total exposure to *o*-nitrotoluene. The incidences of mesothelioma were similar in the 2,000 ppm core study and stop-exposure groups of male rats. Thus, critical events leading to mesothelioma occurred early in the study, and this damage was irreversible.

The mesotheliomas varied from small lesions localized to the serosa of the epididymis or testis to massive mesothelial cell proliferation overlying the serosa of multiple organs. These malignant and invasive forms of mesothelioma are typically found in the surface of the epididymis and testis, although they may occur less frequently in the abdominal cavity and metastasize to the lymph nodes in the thorax. Malignant mesotheliomas of the tunica vaginalis also occur in humans (Jones *et al.*, 1995).

In this study, as well as in other NTP studies, chemical induction of mesothelioma is found primarily in male rats. Mesotheliomas have been reported in male rats after exposure to 10 other chemicals in 2-year NTP studies: acronycine (NCI, 1978a); cytembena (NTP, 1981); 1,2-dibromoethane (NTP, 1982); 3,3'-dimethoxybenzidine dihydrochloride (NTP, 1990a), 2,3-dibromo-1-propanol (NTP, 1993); 3,3'-dimethylbenzidine dihydrochloride (NTP, 1991); glycidol (NTP, 1990b); pentachlorophenol (NTP, 1999); phenoxybenzamine hydrochloride (NCI, 1978b); and *o*-toluidine hydrochloride (NCI, 1979).

Studies of other aromatic amines show that the position of the substitution on the aromatic ring is important for predicting the carcinogenic effect. *o*-Toluidine hydrochloride induced a high incidence of mesothelioma in male rats, while no mesotheliomas were observed after treatment with *m*- or *p*-toluidine (Weisburger *et al.*, 1978). In studies of *o*- and *p*-anisidine hydrochloride, only the *ortho* isomer induced a carcinogenic response (urinary bladder neoplasms in rats and mice; kidney and thyroid gland neoplasms in male rats) (NCI, 1978c,d). The structural similarity of *o*-toluidine hydrochloride, *o*-anisidine hydrochloride, and *o*-nitrotoluene (all contain a benzene ring with a methyl or methoxy and nitrogen-containing group on adjacent carbons) suggests a similar intermediate may be responsible for the carcinogenic effects. Chemicals with an *ortho* substitution on the aromatic ring are more likely to be carcinogenic than

chemicals with *meta* or *para* substitution on the aromatic ring (Weisburger *et al.*, 1978).

The molecular pathogenesis of mesotheliomas is not well understood. Active-oxygen free radicals are considered to be important mediators in asbestos-induced mesotheliomas (Attanoos and Gibbs, 1997). Human mesotheliomas have cyclooxygenase-2 and nitric oxide synthase gene alterations (Marrogi *et al.*, 2000) and *p16* gene deletions (Xiao *et al.*, 1995). Studies of human mesotheliomas have also found deletions on chromosomes 1p, 3p, and 22q, which may be the loci of yet-unidentified tumor suppressor genes important in the neoplastic transformation process (Attanoos and Gibbs, 1997). Trisomy of chromosome 7 is another frequent change in mesothelioma (Walker *et al.*, 1992). Mutations in the *ras* gene are not commonly seen in mesothelioma cells in humans (Ramael *et al.*, 1993; Cristaudo *et al.*, 1995) or in rat mesotheliomas induced by ferric nitrilotriacetate (Nishiyama *et al.*, 1995). Mutations of the *p53* gene also were not seen in a sample of rat mesothelioma (Nishiyama *et al.*, 1995) or in human malignant mesotheliomas (Ramael *et al.*, 1992; Mor *et al.*, 1997).

At 2 years, significant increases were seen in the incidences of subcutaneous skin fibroma and fibrosarcoma in all exposed groups of male rats, fibroma in 1,250 and 2,000 ppm female rats, and mammary gland fibroadenoma in most exposed groups of male and female rats. The subcutaneous skin and mammary gland neoplasms were also increased in multiplicity. The incidences exceeded the historical control ranges for these neoplasms in control rats given NTP-2000 or NIH-07 diet. Subcutaneous skin fibrosarcomas and mammary gland fibroadenomas in male and female rats were clear evidence of a carcinogenic response because the effects were seen in both sexes at 1,250 and 2,000 ppm and incidences for these neoplasms exceeded historical rates.

There were increases in the incidences of hepatocellular and/or bile duct neoplasms in 2,000 ppm core study and 5,000 ppm stop-exposure male rats. The incidence of hepatocellular adenoma or carcinoma (combined) in the 2,000 ppm core study group exceeded the historical control ranges in control rats given NTP-2000 or NIH-07 diet. In the 5,000 ppm stop-exposure group, three animals had cholangiocarcinoma, and six had hepatocellular neoplasms.

In a previous study in which male rats received 5,000 ppm *o*-nitrotoluene in feed for 13 weeks and undosed feed for 13 weeks, cholangiocarcinomas occurred in two of 20 male rats (NTP, 1996). Cholangiocarcinomas have also been seen in a 2-year study of dinitrotoluene (Rickert, 1984a). Liver and bile duct neoplasms in male rats were considered to be clear evidence for a carcinogenic effect because the increased incidences were significant, even though many of the male rats died early due to other neoplasms, such as mesotheliomas. The incidence of hepatocellular adenoma was also increased in 2,000 ppm female rats and was considered related to exposure to *o*-nitrotoluene. No cholangiocarcinomas were seen in female rats.

The incidence of alveolar/bronchiolar adenoma or carcinoma (combined) was increased in 5,000 ppm stop-exposure male rats, and this incidence exceeded the historical ranges in control rats given either NTP-2000 or NIH-07 diet. In addition, the incidences of alveolar epithelial hyperplasia were increased in the 625 and 2,000 ppm core study male rats and in the 2,000 and 5,000 ppm stop-exposure groups. The increased incidences of the lung neoplasms were considered related to chemical exposure. While there were increases in the incidences of alveolar epithelial hyperplasia in 625 and 1,250 ppm female rats, there was no evidence for an increased incidence of alveolar/bronchiolar neoplasms in female rats.

The incidences of hemangioma and hemangioma or hemangiosarcoma (combined) were significantly increased in 5,000 ppm stop-exposure male rats. The presence of benign hemangiomas and a single hemangiosarcoma in a variety of organs in this group was not considered to be related to exposure to *o*-nitrotoluene.

Nitroaromatic chemicals that cause hematopoiesis and hemosiderin pigment accumulation in the spleen also may cause a decreased incidence of mononuclear cell leukemia (Elwell *et al.*, 1996). The spleen plays a critical role in the pathogenesis of mononuclear cell leukemia. Although the stem cell for mononuclear cell leukemia in Fischer rats is considered to be a lymphocyte of bone marrow origin, the initial histologic evidence for proliferation and expansion of these neoplastic cells occurs in the spleen, where the leukemia cells fill the sinusoids. Alteration of the spleen microenvironment can effect the development of mononuclear cell leukemia (Elwell *et al.*, 1996). The findings of increased

spleen toxicity and decreased incidences of mononuclear cell leukemia are consistent with similar response patterns seen with other nitroaromatic compounds, including *p*-nitrotoluene. Decreases in the incidence of interstitial cell adenoma of the testis were also seen in exposed male rats.

At 3 months, testicular and epididymal atrophy and salivary gland atrophy probably were related to the lower body weights of male rats exposed to 2,000 or 5,000 ppm. The pituitary gland cytoplasmic alteration in exposed male rats consisted of hypertrophy, increased eosinophilia, and cytoplasmic vacuolization of cells in the pars distalis and may have been related to the atrophy of the testis. The clitoral gland atrophy observed in exposed female rats may also have been related to reduced body weights. The salivary, preputial, and clitoral gland atrophy and pituitary gland alterations in male and female rats may have been related to the compromised state of the animals with the development of neoplasms.

There were exposure-related increased incidences of hemangiosarcoma in male and female mice. Hemangiosarcomas are malignant vascular neoplasms of the blood vessels (Booth and Sundberg, 1995) and may occur in many tissues. In this study, the hemangiosarcomas occurred primarily in the skeletal muscle, skin (subcutaneous tissue), and/or mesentery. This was considered to be clear evidence of a carcinogenic effect because the neoplasms were malignant, the incidences were significant by both the pairwise and trend statistics and exceeded historical control ranges, and the effect was seen in males and females.

The NTP has identified other chemicals that caused hemangiosarcomas in B6C3F₁ mice: 1,3-butadiene (NTP, 1984), pentachlorophenol Dowicide EC-7 (NTP, 1989), 1,2-propylene oxide (NTP, 1985), chloroprene (NTP, 1998), and tetrafluoroethylene (NTP, 1997). The neoplasm site varied with the study. For example, in the 1,3-butadiene study, hemangiosarcomas occurred in the heart; in the chloroprene studies hemangiosarcomas were observed most frequently in the liver, mesentery, and skin; and in the tetrafluoroethylene study, hemangiosarcomas were observed primarily in the liver.

The carcinogenic effects of 1,3-butadiene and chloroprene have been attributed to a mutagenic epoxide intermediate (Melnick and Kohn, 1995). Vinyl chloride,

another epoxide-forming chemical, also causes hemangiosarcomas in mice (Guengerich, 1992; Singer, 1996). Recent studies demonstrated that both *ras* and *p53* mutations may play a role in the pathogenesis of hemangiosarcomas following exposure to 1,3-butadiene or vinyl chloride in rodents (Hollstein *et al.*, 1994; Hong *et al.*, 2000), and to vinyl chloride in humans (Barbin *et al.*, 1997; Marion *et al.*, 1991).

In a study to determine if the same mechanism might be applicable to *o*-nitrotoluene, 15 hemangiosarcomas from *o*-nitrotoluene-exposed mice and 13 hemangiosarcomas from control mice from other NTP studies were examined for genetic alterations (Appendix M). The purpose of these genetic toxicity studies was to compare the spectrum of genetic changes in spontaneous hemangiosarcomas with that in *o*-nitrotoluene-induced hemangiosarcomas. Alterations in *p53* and β -catenin protein distribution were screened using immunohistochemical analysis of formalin-fixed sections.

No indication of *p53* or β -catenin protein accumulation was observed in the hemangiosarcomas from the control mice, but *p53* protein had accumulated in all 15 hemangiosarcomas from *o*-nitrotoluene-exposed mice. β -Catenin had accumulated in seven of the *o*-nitrotoluene-induced hemangiosarcomas.

Wild-type *p53* protein has a short half-life, and protein accumulation in the cytoplasm or nucleus does not occur in a normal cell. The *p53* protein accumulation in the *o*-nitrotoluene-induced hemangiosarcomas suggests that a mutation has occurred in the *p53* gene, leading to the formation of an altered protein that accumulates in the nucleus. Similarly, β -catenin is usually rapidly degraded in normal cells, and the accumulation of this protein in the *o*-nitrotoluene-induced hemangiosarcomas suggests that a mutation may have occurred. Further analysis of the DNA from these hemangiosarcomas indicated specific mutations in the *p53* and β -catenin genes. The occurrence of *p53* and β -catenin mutations in the *o*-nitrotoluene-induced hemangiosarcomas, but not in the spontaneous hemangiosarcomas, suggests that the pathways leading to *o*-nitrotoluene-induced cancer differ from the pathways in spontaneous hemangiosarcomas.

Exposure to *o*-nitrotoluene caused carcinomas of the cecum in male and female mice. None of the approximately 500 other chemicals evaluated by the NTP have caused carcinomas of the cecum in mice. The occurrence of cecum carcinomas in the current study was

considered clear evidence of a carcinogenic effect because these are malignant neoplasms and the incidences were significantly increased in 1,250 and 2,500 ppm male mice. Carcinomas of the cecum were not observed in 5,000 ppm male mice, probably due to the early hemangiosarcoma-related deaths of those animals; mean survival was 712 days in control male mice and 386 days in 5,000 ppm males.

In the 2-year study, the incidences of hepatocellular adenoma, carcinoma, and/or the combined incidence of hepatocellular neoplasms were increased in 2,500 and 5,000 ppm female mice. The incidence of hepatocellular carcinoma in 5,000 ppm female mice exceeded the historical range for control mice given NTP-2000 or NIH-07 diet. The increases in the incidences of benign and malignant liver neoplasms in female mice were considered to be clear evidence for a carcinogenic effect. Exposed male mice died early because of the development of hemangiosarcomas, which may explain why the later-developing hepatocellular neoplasms were not seen in male mice.

The predominant metabolic pathway for *o*-nitrotoluene is oxidation of the methyl group, first to an alcohol and subsequently to a carboxylic acid (Figure 1). *o*-Nitrobenzylmercapturic acid is likely formed from glutathione transferase-catalyzed conjugation of glutathione with *o*-nitrobenzyl sulfate and subsequent catabolism via the mercapturic acid pathway. Conjugation of *p*-nitrobenzyl sulfate with glutathione has been demonstrated *in vitro* (deBethizy and Rickert, 1983). Nitro-group reduction appears to be a relatively minor pathway, at least as determined from the urinary metabolite profile. *o*-Aminobenzyl alcohol and *o*-aminobenzoic acid are representatives of this pathway. In the current studies, *o*-nitrobenzoic acid, *o*-nitrobenzylmercapturic acid, and *o*-aminobenzoic acid were measured in the urine of rats and mice at 2 weeks and at 3, 12, and 18 months (Appendix F). The contribution of *o*-nitrotoluene metabolism to the *o*-aminobenzoic acid in the urine was overshadowed by endogenous *o*-aminobenzoic acid produced via catabolism of tryptophan. For the other two metabolites in rats, and in mice where there were sufficient data, the metabolite to creatinine ratio generally increased with increasing exposure concentration. In rats, the metabolite/creatinine ratio was generally larger at the 2-week time point than at later times. At this age, the animals are at about half their final weight, but food consumption is high. Therefore, exposure is highest on a

weight basis for the younger animals. This fact is not as obvious from the daily metabolite data. There appear to be differences in metabolism between male and female rats, with males excreting more *o*-nitrobenzylmercapturic acid and females excreting more *o*-nitrobenzoic acid.

o-Nitrotoluene generally is not genotoxic in *Salmonella* or other *in vitro* short-term tests. Metabolic activation of the chemical requires reduction of the nitro group to an aromatic amine before activation to the ultimate carcinogen. The reduction of this nitro group is thought to be catalyzed by bacterial nitroreductases in the intestine (Doolittle *et al.*, 1983).

Measurement of chemical-induced DNA repair as unscheduled DNA synthesis is an indicator of genotoxic and carcinogenic potential (Mirsalis and Butterworth, 1980; Mirsalis *et al.*, 1982a,b). *o*-Nitrotoluene's induction of unscheduled DNA synthesis in rat hepatocytes after an oral dose of 200 mg/kg to male rats, but not in cultured hepatocytes exposed *in vitro* or in germ free rats, suggests intestinal bacteria are essential for the metabolic activation of this chemical to a genotoxicant (Doolittle *et al.*, 1983).

In the 2-year studies of *o*- and *p*-nitrotoluene, the *ortho* isomer caused a spectrum of carcinogenic responses in rats and mice that differed from that seen with the *para* isomer (Table 25). Earlier studies showed that covalent binding of radioactivity from [¹⁴C]-*o*-nitrotoluene to total rat hepatic macromolecules is 3.5 times greater than that of *m*- or *p*-nitrotoluene. *o*-Nitrotoluene also binds to male F344 rat hepatic DNA, but this binding could not be detected for *m*- or *p*-nitrotoluene (Rickert *et al.*, 1984b). *o*-Nitrotoluene, but not *m*- or *p*-nitrotoluene, induced DNA repair in the *in vivo/in vitro* hepatocyte unscheduled DNA synthesis assay in male, but not female, F344 rats (Doolittle *et al.*, 1983).

Quantitative differences in metabolism of the mononitrotoluenes are a result of differences in the hepatic conjugation or further oxidation of the first metabolic intermediates, the mononitrobenzyl alcohols. The *o*-nitrotoluene metabolite *o*-nitrobenzyl alcohol is a better substrate for microsomal glucuronyl transferase

than are the *m*- or *p*-nitrobenzyl alcohols (Rickert *et al.*, 1985). Once formed, part of the *o*-nitrobenzyl glucuronide is excreted via the bile into the intestine, where intestinal microflora hydrolyze the glucuronide and reduce the nitro group, resulting in the formation of *o*-aminobenzyl alcohol, which may be reabsorbed (Chism and Rickert, 1985).

o-Aminobenzyl alcohol has received considerable attention as a key intermediate in the bioactivation of *o*-nitrotoluene. It has been suggested that sulfation of *o*-aminobenzyl alcohol may lead to covalent binding to DNA (Chism and Rickert, 1989). The reactivity of *o*-aminobenzyl sulfate could be related to the ease of formation of a reactive benzyl cation due to the electron-donating ability of the amino group. Alternatively, sulfation could lead to formation of a quinone-imine via elimination of sulfate. *o*-Aminobenzyl alcohol also provides a link between *o*-toluidine and *o*-nitrotoluene, as it is likely a metabolite of both chemicals. Although *o*-aminobenzyl alcohol has not been identified as a metabolite of *o*-toluidine, the presence of N-acetyl-*o*-aminobenzyl alcohol as a urinary metabolite in rats is evidence that it may be present *in vivo* (Son *et al.*, 1980). Chism and Rickert (1989) also provided evidence that oxidation of the amino group to a hydroxylamine followed by sulfation, a metabolic activation process for some aryl amines, was not responsible for DNA covalent binding by *o*-aminobenzyl alcohol.

The difference in genotoxic responses of *ortho*- versus *meta*- or *para*-substituted arylamines has been studied by Marques *et al.* (1997). Their data indicated that the conformation of the DNA adduct is a determinant factor in the genotoxic response. Adducts of *ortho*-analogues tend to adopt a *syn* conformation while *meta*- and *para*-analogues tend to adopt an *anti* conformation. While the work of Marques *et al.* (1997) focused on reaction at the nitrogen of the aryl amine, the same principle may apply for reaction at the benzylic carbon of an *o*-nitrotoluene metabolite.

No *o*-nitrotoluene epidemiology studies have been reported in the literature. However, excess cancers have been found in workers exposed to a related chemical, *o*-toluidine (Ward *et al.*, 1991).

TABLE 25
Comparison of Selected Neoplasms in the 2-Year Feed Studies of p-Nitrotoluene and o-Nitrotoluene

	p-Nitrotoluene ^a				o-Nitrotoluene				
	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm	0 ppm	625 ppm	1,250 ppm	2,000 ppm	5,000 ppm
Male Rats									
Average Daily Dose (mg/kg)	0	55	110	240	0	25	50	90	315
Survival	31/50	38/50	38/50	40/50	39/60	18/60	3/60	0/60	0/60
Mesothelium									
Malignant Mesothelioma ^b	5/50	2/50	1/50	4/50	2/60	20/60	29/60	44/60	54/60
Skin (Subcutaneous)									
Lipoma	0/50	0/50	0/50	0/50	0/60	4/60	13/60	13/60	12/60
Fibroma	1/50	2/50	7/50	1/50	5/60	46/60	52/60	59/60	52/60
Fibrosarcoma	0/50	2/50	7/50	1/50	0/60	7/60	17/60	20/60	12/60
Fibroma or Fibrosarcoma	1/50	2/50	9/50	1/50	5/60	47/60	55/60	59/60	53/60
Mammary Gland									
Fibroadenoma	0/50	0/50	0/50	0/50	0/60	7/60	10/60	2/60	20/60
Liver									
Hepatocellular Adenoma	0/50	0/50	0/50	0/50	2/60	3/60	3/60	7/60	4/60
Hepatocellular Adenoma or Carcinoma	1/50	0/50	0/50	0/50	3/60	3/60	3/60	8/60	6/60
Cholangiocarcinoma	0/50	0/50	0/50	0/50	0/60	0/60	0/60	0/60	3/60
Hepatocholangiocarcinoma	0/50	0/50	0/50	0/50	0/60	1/60	0/60	1/60	0/60
Lung									
Alveolar/bronchiolar Adenoma	1/50	0/50	2/50	2/50	1/60	5/60	1/60	2/60	8/60
Alveolar/bronchiolar Adenoma or Carcinoma	1/50	1/50	2/50	3/50	2/60	5/60	1/60	2/60	11/60

^a NTP, 2002

^b Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for liver and lung; for other tissues, denominator is number of animals necropsied.

TABLE 25
Comparison of Selected Neoplasms in the 2-Year Feed Studies of *p*-Nitrotoluene and *o*-Nitrotoluene

	<i>p</i> -Nitrotoluene				<i>o</i> -Nitrotoluene			
	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Female Rats								
Average Daily Dose (mg/kg)	0	60	125	265	0	30	60	100
Survival	39/50	37/50	39/50	41/50	47/60	47/60	39/60	33/60
Skin (Subcutaneous) Fibroma	0/50	0/50	0/50	1/49	3/59	3/60	18/60	19/60
Fibroma or Fibrosarcoma	1/50	1/50	0/50	2/50	3/60	3/60	21/60	22/60
Mammary Gland Fibroadenoma	14/50	17/50	20/50	5/50	23/60	47/60	52/60	56/60
Liver Hepatocellular Adenoma	0/50	0/50	1/50	0/50	1/60	0/59	1/60	6/60
Clitoral Gland Adenoma or Carcinoma	8/50	12/50	20/50	8/49	14/59	13/57	6/54	3/53
Male Mice								
Average Daily Dose (mg/kg)	0	170	345	690	0	165	360	700
Survival	46/50	46/50	45/50	42/50	52/60	46/60	47/60	5/60
Circulatory System Hemangiosarcoma	1/50	1/50	3/50	2/50	4/60	17/60	55/60	60/60
Large Intestine (Cecum) Carcinoma	1/48	0/48	0/48	0/49	0/56	12/49	9/36	0/44
Lung Alveolar/bronchiolar Adenoma or Carcinoma	8/50	14/50	12/50	19/50	14/60	7/60	6/60	0/60

TABLE 25
Comparison of Selected Neoplasms in the 2-Year Feed Studies of *p*-Nitrotoluene and *o*-Nitrotoluene

	<i>p</i> -Nitrotoluene			<i>o</i> -Nitrotoluene		
	0 ppm	1,250 ppm	5,000 ppm	0 ppm	1,250 ppm	5,000 ppm
Female Mice						
Average Daily Dose (mg/kg)	0	155	660	0	150	710
Survival	46/50	47/50	49/50	52/60	46/60	5/60
Circulatory System						
Hemangiosarcoma	1/50	1/50	1/50	0/60	2/60	50/60
Large Intestine (Cecum)						
Carcinoma	0/49	0/48	0/50	0/60	1/60	3/60
Liver						
Hepatocellular Adenoma	6/49	3/50	5/50	7/60	5/59	29/60
Hepatocellular Carcinoma	3/49	4/50	1/50	2/60	4/59	16/60
Hepatocellular Adenoma or Carcinoma	8/49	6/50	6/50	9/60	9/59	39/60

CONCLUSIONS

Under the conditions of these studies, there was *clear evidence of carcinogenic activity** of *o*-nitrotoluene in male rats based on increased incidences of malignant mesothelioma, subcutaneous skin neoplasms, mammary gland fibroadenoma, and liver neoplasms. The increased incidences of lung neoplasms in male rats were also considered to be exposure related. There was *clear evidence of carcinogenic activity* of *o*-nitrotoluene in female rats based on increased incidences of subcutaneous skin neoplasms and mammary gland fibroadenoma. The increased incidence of hepatocellular adenoma in female rats was also considered to be exposure related. There was *clear evidence of carcinogenic activity* of

o-nitrotoluene in male and female mice based on increased incidences of hemangiosarcoma, carcinoma of the large intestine (cecum), and hepatocellular neoplasms (females only).

Exposure to *o*-nitrotoluene caused increased incidences of nonneoplastic lesions of the mammary gland (females only), liver, bone marrow, spleen, lung, and mandibular lymph node (females only) in male and female rats and of the liver, kidney, and nose in male and female mice.

Decreased incidences of mononuclear cell leukemia occurred in exposed groups of rats; the incidence of testicular interstitial cell adenoma was decreased in exposed male rats.

* Explanation of Levels of Evidence of Carcinogenic Activity is on page 13. A summary of the Technical Reports Review Subcommittee comments and public discussion on this Technical Report appears on page 15.

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APPENDIX A
SUMMARY OF LESIONS IN MALE RATS
IN THE 2-YEAR FEED STUDY
OF *o*-NITROTOLUENE

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TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of o-Nitrotoluene^a

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Disposition Summary						
Animals initially in study	70	60	60	60	70	70
3-Month interim evaluation	10				10	10
Early deaths						
Accidental deaths			3			
Moribund	18	35	48	53	39	57
Natural deaths	3	7	6	7	10	3
Survivors						
Died last week of study	1					
Terminal sacrifice	38	18	3		11	
Animals examined microscopically	70	60	60	60	70	70
Systems Examined at 3 Months with No Neoplasms Observed						
Alimentary System						
Cardiovascular System						
Endocrine System						
General Body System						
Genital System						
Hematopoietic System						
Integumentary System						
Musculoskeletal System						
Nervous System						
Respiratory System						
Special Senses System						
Urinary System						
2-Year Study						
Alimentary System						
Intestine large, colon	(60)	(59)	(60)	(60)	(59)	(60)
Polyp adenomatous	1 (2%)			1 (2%)		2 (3%)
Intestine large, rectum	(59)	(60)	(60)	(59)	(60)	(58)
Leiomyosarcoma					1 (2%)	
Intestine large, cecum	(60)	(60)	(60)	(58)	(59)	(60)
Leiomyoma					1 (2%)	1 (2%)
Leiomyosarcoma			1 (2%)			
Intestine small, jejunum	(59)	(60)	(58)	(53)	(57)	(60)
Carcinoma					1 (2%)	
Intestine small, ileum	(58)	(58)	(58)	(58)	(56)	(58)
Liver	(60)	(60)	(60)	(60)	(60)	(60)
Cholangiocarcinoma						3 (5%)
Hemangiosarcoma		1 (2%)				
Hepatocellular carcinoma	1 (2%)					2 (3%)
Hepatocellular carcinoma, multiple				1 (2%)		
Hepatocellular adenoma	2 (3%)	2 (3%)	3 (5%)	7 (12%)	2 (3%)	3 (5%)
Hepatocellular adenoma, multiple		1 (2%)			1 (2%)	1 (2%)
Hepatocholangiocarcinoma		1 (2%)		1 (2%)		
Histiocytic sarcoma	1 (2%)				1 (2%)	

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
2-Year Study (continued)						
Alimentary System (continued)						
Mesentery	(16)	(6)	(5)	(4)	(1)	(3)
Fibroma			1 (20%)			
Fibrosarcoma		1 (17%)				2 (67%)
Lipoma		1 (17%)				
Oral mucosa			(1)	(1)		
Hemangioma				1 (100%)		
Pancreas	(60)	(60)	(60)	(60)	(60)	(60)
Histiocytic sarcoma					1 (2%)	
Mixed tumor benign					2 (3%)	
Acinus, adenoma			1 (2%)			3 (5%)
Islets, pancreatic, adenoma	1 (2%)					
Salivary glands	(60)	(60)	(59)	(60)	(58)	(59)
Fibrosarcoma					1 (2%)	
Fibrosarcoma, metastatic, skin					1 (2%)	
Rhabdomyosarcoma, metastatic, skeletal muscle		1 (2%)				
Sarcoma			1 (2%)			
Schwannoma malignant, metastatic, skin		1 (2%)				
Stomach, forestomach	(59)	(60)	(60)	(60)	(60)	(60)
Squamous cell papilloma					1 (2%)	
Stomach, glandular	(59)	(60)	(60)	(60)	(60)	(60)
Tongue	(1)		(1)			
Squamous cell papilloma			1 (100%)			
Tooth				(1)		(1)
Fibroma						1 (100%)
Cardiovascular System						
Heart	(60)	(60)	(60)	(60)	(60)	(60)
Schwannoma malignant						1 (2%)
Endocrine System						
Adrenal cortex	(60)	(60)	(60)	(60)	(60)	(60)
Adenoma				1 (2%)		
Adrenal medulla	(60)	(60)	(60)	(59)	(60)	(60)
Neuroblastoma				1 (2%)		
Pheochromocytoma malignant	2 (3%)				1 (2%)	
Pheochromocytoma benign	1 (2%)	4 (7%)	6 (10%)	2 (3%)	4 (7%)	2 (3%)
Bilateral, pheochromocytoma benign	1 (2%)					
Islets, pancreatic	(60)	(60)	(60)	(60)	(60)	(60)
Adenoma	2 (3%)					1 (2%)
Carcinoma		2 (3%)				
Parathyroid gland	(58)	(55)	(59)	(58)	(58)	(57)
Adenoma		1 (2%)			1 (2%)	
Pituitary gland	(59)	(60)	(58)	(59)	(57)	(59)
Craniopharyngioma					1 (2%)	
Pars distalis, adenoma	11 (19%)	9 (15%)	3 (5%)	9 (15%)	10 (18%)	5 (8%)
Pars intermedia, adenoma		1 (2%)				

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
2-Year Study (continued)						
Endocrine System (continued)						
Thyroid gland	(59)	(60)	(60)	(60)	(60)	(60)
Rhabdomyosarcoma, metastatic, skeletal muscle		1 (2%)				
Sarcoma, metastatic, salivary glands			1 (2%)			
Bilateral, C-cell, adenoma	1 (2%)					
C-cell, adenoma	9 (15%)	6 (10%)		1 (2%)	3 (5%)	4 (7%)
C-cell, carcinoma		4 (7%)		1 (2%)	1 (2%)	
Follicular cell, adenoma			1 (2%)			
Follicular cell, carcinoma	1 (2%)	1 (2%)		1 (2%)		
General Body System						
Peritoneum	(3)	(21)	(33)	(46)	(47)	(54)
Tissue NOS		(1)				(1)
Hemangioma						1 (100%)
Genital System						
Preputial gland	(60)	(59)	(58)	(56)	(60)	(59)
Adenoma	2 (3%)	3 (5%)	2 (3%)	1 (2%)	1 (2%)	1 (2%)
Carcinoma	2 (3%)	2 (3%)		1 (2%)	1 (2%)	2 (3%)
Prostate	(60)	(59)	(60)	(60)	(60)	(60)
Ventral, adenoma	2 (3%)				1 (2%)	
Seminal vesicle	(60)	(59)	(60)	(60)	(60)	(59)
Testes	(60)	(60)	(60)	(60)	(60)	(60)
Bilateral, interstitial cell, adenoma	43 (72%)	34 (57%)	32 (53%)	17 (28%)	31 (52%)	4 (7%)
Interstitial cell, adenoma	12 (20%)	19 (32%)	19 (32%)	29 (48%)	19 (32%)	23 (38%)
Hematopoietic System						
Bone marrow	(60)	(60)	(60)	(60)	(60)	(60)
Histiocytic sarcoma	1 (2%)					
Lymph node	(16)	(14)	(4)	(9)	(18)	(16)
Squamous cell carcinoma, metastatic, skin					1 (6%)	
Mediastinal, histiocytic sarcoma					1 (6%)	
Lymph node, mandibular	(58)	(59)	(58)	(59)	(58)	(59)
Histiocytic sarcoma	1 (2%)					
Rhabdomyosarcoma, metastatic, skeletal muscle		1 (2%)				
Lymph node, mesenteric	(60)	(60)	(59)	(58)	(60)	(59)
Spleen	(60)	(60)	(60)	(60)	(60)	(60)
Fibroma					1 (2%)	
Fibrosarcoma			1 (2%)		1 (2%)	
Hemangioma						1 (2%)
Hemangiosarcoma			1 (2%)			
Histiocytic sarcoma		1 (2%)			1 (2%)	
Thymus	(56)	(54)	(55)	(55)	(56)	(56)
Alveolar/bronchiolar carcinoma, metastatic, lung						1 (2%)
Fibrosarcoma, metastatic, spleen					1 (2%)	
Thymoma benign		1 (2%)				

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
2-Year Study (continued)						
Integumentary System						
Mammary gland	(57)	(47)	(46)	(43)	(51)	(50)
Fibroadenoma		7 (15%)	9 (20%)	2 (5%)	12 (24%)	20 (40%)
Fibroadenoma, multiple			1 (2%)		1 (2%)	
Hemangioma		1 (2%)				
Skin	(60)	(60)	(60)	(60)	(60)	(60)
Basal cell adenoma	2 (3%)			1 (2%)		
Hemangioma						1 (2%)
Histiocytic sarcoma		1 (2%)			1 (2%)	
Keratoacanthoma	5 (8%)	3 (5%)	1 (2%)		1 (2%)	
Schwannoma malignant						1 (2%)
Squamous cell carcinoma					1 (2%)	
Squamous cell papilloma						2 (3%)
Subcutaneous tissue, fibroma	4 (7%)	21 (35%)	13 (22%)	24 (40%)	45 (75%)	24 (40%)
Subcutaneous tissue, fibroma, multiple	1 (2%)	25 (42%)	39 (65%)	35 (58%)	22 (37%)	28 (47%)
Subcutaneous tissue, fibrosarcoma		7 (12%)	13 (22%)	15 (25%)	8 (13%)	8 (13%)
Subcutaneous tissue, fibrosarcoma, multiple			4 (7%)	5 (8%)		4 (7%)
Subcutaneous tissue, hemangioma		2 (3%)				
Subcutaneous tissue, hemangiopericytoma				1 (2%)		
Subcutaneous tissue, hemangiosarcoma	1 (2%)					
Subcutaneous tissue, lipoma		4 (7%)	12 (20%)	13 (22%)	10 (17%)	9 (15%)
Subcutaneous tissue, lipoma, multiple			1 (2%)			3 (5%)
Subcutaneous tissue, osteosarcoma					1 (2%)	
Subcutaneous tissue, sarcoma						1 (2%)
Subcutaneous tissue, schwannoma malignant		1 (2%)			1 (2%)	1 (2%)
Musculoskeletal System						
Bone	(60)	(60)	(60)	(60)	(60)	(60)
Osteosarcoma	1 (2%)	1 (2%)		1 (2%)	2 (3%)	3 (5%)
Skeletal muscle	(1)	(5)	(3)		(6)	(5)
Alveolar/bronchiolar carcinoma, metastatic, lung						1 (20%)
Hemangioma						1 (20%)
Hemangiosarcoma						1 (20%)
Rhabdomyosarcoma		1 (20%)			1 (17%)	1 (20%)
Nervous System						
Brain	(60)	(60)	(60)	(60)	(60)	(60)
Carcinoma, metastatic, pituitary gland				1 (2%)		
Granular cell tumor benign		1 (2%)				
Histiocytic sarcoma	1 (2%)					
Oligodendroglioma malignant						1 (2%)
Rhabdomyosarcoma, metastatic, skeletal muscle		1 (2%)				
Cranial nerve, schwannoma malignant			1 (2%)			

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
2-Year Study (continued)						
Respiratory System						
Lung	(60)	(60)	(60)	(60)	(60)	(60)
Alveolar/bronchiolar adenoma	1 (2%)	5 (8%)	1 (2%)	2 (3%)	3 (5%)	7 (12%)
Alveolar/bronchiolar adenoma, multiple						1 (2%)
Alveolar/bronchiolar carcinoma	1 (2%)					1 (2%)
Alveolar/bronchiolar carcinoma, multiple						2 (3%)
Carcinoma, metastatic, Zymbal's gland		1 (2%)				
Histiocytic sarcoma	1 (2%)				1 (2%)	
Squamous cell carcinoma					1 (2%)	
Nose	(60)	(60)	(60)	(60)	(60)	(60)
Special Senses System						
Eye	(2)	(1)	(2)	(1)		
Schwannoma malignant, metastatic, brain			1 (50%)			
Harderian gland		(1)	(1)			
Schwannoma malignant, metastatic, brain			1 (100%)			
Zymbal's gland		(2)		(2)	(2)	(1)
Carcinoma		2 (100%)		2 (100%)	2 (100%)	1 (100%)
Urinary System						
Kidney	(60)	(60)	(60)	(60)	(60)	(60)
Urinary bladder	(60)	(60)	(60)	(59)	(60)	(59)
Hemangioma				1 (2%)		
Leiomyoma					1 (2%)	
Papilloma	1 (2%)		1 (2%)			
Squamous cell papilloma		1 (2%)				
Transitional epithelium, papilloma		2 (3%)				
Systemic Lesions						
Multiple organs ^b	(60)	(60)	(60)	(60)	(60)	(60)
Histiocytic sarcoma	1 (2%)	2 (3%)			2 (3%)	
Leukemia mononuclear	30 (50%)	21 (35%)	3 (5%)	3 (5%)	13 (22%)	1 (2%)
Lymphoma malignant			1 (2%)			
Mesothelioma malignant	2 (3%)	20 (33%)	29 (48%)	44 (73%)	44 (73%)	54 (90%)

TABLE A1
Summary of the Incidence of Neoplasms in Male Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Neoplasm Summary						
Total animals with primary neoplasms ^c						
2-Year study	60	60	57	60	60	60
Total primary neoplasms						
2-Year study	144	221	202	224	235	239
Total animals with benign neoplasms						
2-Year study	60	58	55	59	57	56
Total benign neoplasms						
2-Year study	102	154	147	148	152	149
Total animals with malignant neoplasms						
2-Year study	37	47	42	51	54	58
Total malignant neoplasms						
2-Year study	42	67	55	76	83	90
Total animals with metastatic neoplasms						
2-Year study		4	3	1	6	5
Total metastatic neoplasms						
2-Year study		7	4	1	8	11

^a Number of animals examined microscopically at the site and the number of animals with neoplasm

^b Number of animals with any tissue examined microscopically

^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE A3a
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Adrenal Medulla: Benign Pheochromocytoma				
Overall rate ^a	2/60 (3%)	4/60 (7%)	6/60 (10%)	2/59 (3%)
Adjusted rate ^b	3.7%	8.9%	16.3%	8.9%
Terminal rate ^c	2/39 (5%)	1/18 (6%)	0/3 (0%)	0/0
First incidence (days)	729 (T)	567	531	471
Poly-3 test ^d	P=0.064	P=0.261	P=0.048	P=0.380
Adrenal Medulla: Benign or Malignant Pheochromocytoma				
Overall rate	4/60 (7%)	4/60 (7%)	6/60 (10%)	2/59 (3%)
Adjusted rate	7.4%	8.9%	16.3%	8.9%
Terminal rate	4/39 (10%)	1/18 (6%)	0/3 (0%)	0/0
First incidence (days)	729 (T)	567	531	471
Poly-3 test	P=0.220	P=0.543	P=0.170	P=0.584
Liver: Hepatocellular Adenoma				
Overall rate	2/60 (3%)	3/60 (5%)	3/60 (5%)	7/60 (12%)
Adjusted rate	3.7%	6.8%	8.4%	27.1%
Terminal rate	1/39 (3%)	1/18 (6%)	0/3 (0%)	0/0
First incidence (days)	707	621	615	391
Poly-3 test	P=0.007	P=0.413	P=0.325	P=0.006
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	3/60 (5%)	3/60 (5%)	3/60 (5%)	8/60 (13%)
Adjusted rate	5.6%	6.8%	8.4%	30.2%
Terminal rate	2/39 (5%)	1/18 (6%)	0/3 (0%)	0/0
First incidence (days)	707	621	615	391
Poly-3 test	P=0.009	P=0.570	P=0.466	P=0.007
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	1/60 (2%)	5/60 (8%)	1/60 (2%)	2/60 (3%)
Adjusted rate	1.9%	11.2%	2.9%	8.7%
Terminal rate	1/39 (3%)	2/18 (11%)	1/3 (33%)	0/0
First incidence (days)	729 (T)	639	729 (T)	419
Poly-3 test	P=0.237	P=0.066	P=0.654	P=0.254
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	2/60 (3%)	5/60 (8%)	1/60 (2%)	2/60 (3%)
Adjusted rate	3.7%	11.2%	2.9%	8.7%
Terminal rate	2/39 (5%)	2/18 (11%)	1/3 (33%)	0/0
First incidence (days)	729 (T)	639	729 (T)	419
Poly-3 test	P=0.390	P=0.149	P=0.644N	P=0.386
Mammary Gland: Fibroadenoma				
Overall rate	0/60 (0%)	7/60 (12%)	10/60 (17%)	2/60 (3%)
Adjusted rate	0.0%	15.6%	26.2%	9.0%
Terminal rate	0/39 (0%)	4/18 (22%)	1/3 (33%)	0/0
First incidence (days)	— ^e	621	576	608
Poly-3 test	P<0.001	P=0.004	P<0.001	P=0.110
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	11/59 (19%)	9/60 (15%)	3/58 (5%)	9/59 (15%)
Adjusted rate	19.9%	19.6%	8.9%	34.2%
Terminal rate	6/39 (15%)	3/18 (17%)	1/3 (33%)	0/0
First incidence (days)	441	569	621	391
Poly-3 test	P=0.390	P=0.581N	P=0.149N	P=0.155

TABLE A3a
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Preputial Gland: Adenoma				
Overall rate	2/60 (3%)	3/59 (5%)	2/58 (3%)	1/56 (2%)
Adjusted rate	3.7%	6.8%	5.9%	5.0%
Terminal rate	2/39 (5%)	1/17 (6%)	0/3 (0%)	0/0
First incidence (days)	729 (T)	465	519	471
Poly-3 test	P=0.420	P=0.413	P=0.519	P=0.634
Preputial Gland: Adenoma or Carcinoma				
Overall rate	4/60 (7%)	5/59 (8%)	2/58 (3%)	2/56 (4%)
Adjusted rate	7.4%	11.2%	5.9%	9.7%
Terminal rate	2/39 (8%)	2/17 (12%)	0/3 (0%)	0/0
First incidence (days)	720	465	519	471
Poly-3 test	P=0.542	P=0.387	P=0.559N	P=0.551
Skin: Keratoacanthoma				
Overall rate	5/60 (8%)	3/60 (5%)	1/60 (2%)	0/60 (0%)
Adjusted rate	9.2%	6.7%	2.8%	0.0%
Terminal rate	3/39 (8%)	1/18 (6%)	0/3 (0%)	0/0
First incidence (days)	537	616	564	—
Poly-3 test	P=0.079N	P=0.470N	P=0.238N	P=0.218N
Skin: Keratoacanthoma or Basal Cell Adenoma				
Overall rate	7/60 (12%)	3/60 (5%)	1/60 (2%)	1/60 (2%)
Adjusted rate	12.9%	6.7%	2.8%	4.6%
Terminal rate	5/39 (13%)	1/18 (6%)	0/3 (0%)	0/0
First incidence (days)	537	616	564	608
Poly-3 test	P=0.053N	P=0.252N	P=0.114N	P=0.291N
Skin (Subcutaneous Tissue): Lipoma				
Overall rate	0/60 (0%)	4/60 (7%)	13/60 (22%)	13/60 (22%)
Adjusted rate	0.0%	8.9%	33.0%	44.8%
Terminal rate	0/39 (0%)	1/18 (6%)	0/3 (0%)	0/0
First incidence (days)	—	569	502	414
Poly-3 test	P<0.001	P=0.041	P<0.001	P<0.001
Skin (Subcutaneous Tissue): Fibroma				
Overall rate	5/60 (8%)	46/60 (77%)	52/60 (87%)	59/60 (98%)
Adjusted rate	9.3%	85.0%	96.2%	99.8%
Terminal rate	3/39 (8%)	15/18 (83%)	3/3 (100%)	0/0
First incidence (days)	705	465	432	391
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
Skin (Subcutaneous Tissue): Fibrosarcoma				
Overall rate	0/60 (0%)	7/60 (12%)	17/60 (28%)	20/60 (33%)
Adjusted rate	0.0%	15.3%	42.3%	59.4%
Terminal rate	0/39 (0%)	1/18 (6%)	1/3 (33%)	0/0
First incidence (days)	—	569	384	439
Poly-3 test	P<0.001	P=0.004	P<0.001	P<0.001
Skin (Subcutaneous Tissue): Fibroma or Fibrosarcoma				
Overall rate	5/60 (8%)	47/60 (78%)	55/60 (92%)	59/60 (98%)
Adjusted rate	9.3%	86.3%	98.7%	99.8%
Terminal rate	3/39 (8%)	15/18 (83%)	3/3 (100%)	0/0
First incidence (days)	705	465	384	391
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001

TABLE A3a
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Testes: Adenoma				
Overall rate	55/60 (92%)	53/60 (88%)	51/60 (85%)	46/60 (77%)
Adjusted rate	94.3%	93.7%	96.5%	92.2%
Terminal rate	38/39 (97%)	18/18 (100%)	3/3 (100%)	0/0
First incidence (days)	533	465	483	404
Poly-3 test	P=0.453N	P=0.610N	P=0.457	P=0.475N
Thyroid Gland (C-Cell): Adenoma				
Overall rate	10/59 (17%)	6/60 (10%)	0/60 (0%)	1/60 (2%)
Adjusted rate	18.6%	13.6%	0.0%	4.5%
Terminal rate	6/38 (16%)	6/18 (33%)	0/3 (0%)	0/0
First incidence (days)	533	729 (T)	—	404
Poly-3 test	P=0.006N	P=0.352N	P=0.010N	P=0.147N
Thyroid Gland (C-Cell): Carcinoma				
Overall rate	0/59 (0%)	4/60 (7%)	0/60 (0%)	1/60 (2%)
Adjusted rate	0.0%	9.1%	0.0%	4.6%
Terminal rate	0/38 (0%)	3/18 (17%)	0/3 (0%)	0/0
First incidence (days)	—	691	— ^f	632
Poly-3 test	P=0.323	P=0.042	—	P=0.350
Thyroid Gland (C-Cell): Adenoma or Carcinoma				
Overall rate	10/59 (17%)	10/60 (17%)	0/60 (0%)	2/60 (3%)
Adjusted rate	18.6%	22.6%	0.0%	8.8%
Terminal rate	6/38 (16%)	9/18 (50%)	0/3 (0%)	0/0
First incidence (days)	533	691	—	404
Poly-3 test	P=0.029N	P=0.403	P=0.010N	P=0.269N
All Organs: Hemangioma				
Overall rate	0/60 (0%)	3/60 (5%)	0/60 (0%)	2/60 (3%)
Adjusted rate	0.0%	6.7%	0.0%	8.7%
Terminal rate	0/39 (0%)	1/18 (6%)	0/3 (0%)	0/0
First incidence (days)	—	632	—	439
Poly-3 test	P=0.158	P=0.090	—	P=0.112
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	1/60 (2%)	3/60 (5%)	1/60 (2%)	2/60 (3%)
Adjusted rate	1.9%	6.7%	2.9%	8.7%
Terminal rate	1/39 (3%)	1/18 (6%)	0/3 (0%)	0/0
First incidence (days)	729 (T)	632	714	439
Poly-3 test	P=0.232	P=0.243	P=0.654	P=0.254
All Organs: Mononuclear Cell Leukemia				
Overall rate	30/60 (50%)	21/60 (35%)	3/60 (5%)	3/60 (5%)
Adjusted rate	52.2%	42.8%	8.5%	12.7%
Terminal rate	16/39 (41%)	7/18 (39%)	0/3 (0%)	0/0
First incidence (days)	533	465	676	419
Poly-3 test	P<0.001N	P=0.214N	P<0.001N	P=0.003N
All Organs: Malignant Mesothelioma				
Overall rate	2/60 (3%)	20/60 (33%)	29/60 (48%)	44/60 (73%)
Adjusted rate	3.7%	40.6%	62.4%	87.1%
Terminal rate	2/39 (5%)	5/18 (28%)	1/3 (33%)	0/0
First incidence (days)	729 (T)	428	432	359
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001

TABLE A3a
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
All Organs: Benign Neoplasms				
Overall rate	60/60 (100%)	58/60 (97%)	55/60 (92%)	59/60 (98%)
Adjusted rate	100.0%	99.4%	99.1%	99.8%
Terminal rate	39/39 (100%)	18/18 (100%)	3/3 (100%)	0/0
First incidence (days)	441	465	432	391
Poly-3 test	P=0.799N	P=0.996N	P=0.974N	P=1.000N
All Organs: Malignant Neoplasms				
Overall rate	37/60 (62%)	47/60 (78%)	42/60 (70%)	51/60 (85%)
Adjusted rate	63.7%	83.6%	81.8%	94.0%
Terminal rate	21/39 (54%)	14/18 (78%)	2/3 (67%)	0/0
First incidence (days)	533	378	384	359
Poly-3 test	P<0.001	P=0.010	P=0.023	P<0.001
All Organs: Benign or Malignant Neoplasms				
Overall rate	60/60 (100%)	60/60 (100%)	57/60 (95%)	60/60 (100%)
Adjusted rate	100.0%	100.0%	99.6%	100.0%
Terminal rate	39/39 (100%)	18/18 (100%)	3/3 (100%)	0/0
First incidence (days)	441	378	384	359
Poly-3 test	P=1.000N	—	P=1.000N	—

(T) Terminal sacrifice

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, liver, lung, pituitary gland, preputial gland, testes, and thyroid gland; for other tissues, denominator is number of animals necropsied.

^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by N.

^e Not applicable; no neoplasms in animal group

^f Value of statistic cannot be computed.

TABLE A3b
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Stop-Exposure Feed Study of o-Nitrotoluene

	0 ppm	2,000 ppm	5,000 ppm
Adrenal Medulla: Benign Pheochromocytoma			
Overall rate ^a	2/60 (3%)	4/60 (7%)	2/60 (3%)
Adjusted rate ^b	3.7%	10.1%	9.9%
Terminal rate ^c	2/39 (5%)	2/11 (18%)	0/0
First incidence (days)	729 (T)	569	567
Poly-3 test ^d	P=0.187	P=0.210	P=0.352
Adrenal Medulla: Benign or Malignant Pheochromocytoma			
Overall rate	4/60 (7%)	5/60 (8%)	2/60 (3%)
Adjusted rate	7.4%	12.7%	9.9%
Terminal rate	4/39 (10%)	3/11 (27%)	0/0
First incidence (days)	729 (T)	569	567
Poly-3 test	P=0.359	P=0.315	P=0.544
Bone: Osteosarcoma			
Overall rate	1/60 (2%)	2/60 (3%)	3/60 (5%)
Adjusted rate	1.9%	5.0%	14.0%
Terminal rate	0/39 (0%)	0/11 (0%)	0/0
First incidence (days)	727	470	359
Poly-3 test	P=0.074	P=0.399	P=0.097
Liver: Cholangiocarcinoma			
Overall rate	0/60 (0%)	0/60 (0%)	3/60 (5%)
Adjusted rate	0.0%	0.0%	13.9%
Terminal rate	0/39 (0%)	0/11 (0%)	0/0
First incidence (days)	— ^e	— ^f	384
Poly-3 test	P=0.025	— ^f	P=0.034
Liver: Hepatocellular Adenoma			
Overall rate	2/60 (3%)	3/60 (5%)	4/60 (7%)
Adjusted rate	3.7%	7.6%	18.4%
Terminal rate	1/39 (3%)	1/11 (9%)	0/0
First incidence (days)	707	579	499
Poly-3 test	P=0.062	P=0.362	P=0.079
Liver: Hepatocellular Adenoma or Carcinoma			
Overall rate	3/60 (5%)	3/60 (5%)	6/60 (10%)
Adjusted rate	5.6%	7.6%	25.9%
Terminal rate	2/39 (5%)	1/11 (9%)	0/0
First incidence (days)	707	579	391
Poly-3 test	P=0.030	P=0.511	P=0.029
Lung: Alveolar/bronchiolar Adenoma			
Overall rate	1/60 (2%)	3/60 (5%)	8/60 (13%)
Adjusted rate	1.9%	7.6%	33.0%
Terminal rate	1/39 (3%)	1/11 (9%)	0/0
First incidence (days)	729 (T)	615	419
Poly-3 test	P<0.001	P=0.205	P<0.001
Lung: Alveolar/bronchiolar Carcinoma			
Overall rate	1/60 (2%)	0/60 (0%)	3/60 (5%)
Adjusted rate	1.9%	0.0%	14.1%
Terminal rate	1/39 (3%)	0/11 (0%)	0/0
First incidence (days)	729 (T)	—	499
Poly-3 test	P=0.115	P=0.565N	P=0.096

TABLE A3b
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Stop-Exposure Feed Study of *o*-Nitrotoluene

	0 ppm	2,000 ppm	5,000 ppm
Lung: Alveolar/bronchiolar Adenoma or Carcinoma			
Overall rate	2/60 (3%)	3/60 (5%)	11/60 (18%)
Adjusted rate	3.7%	7.6%	42.0%
Terminal rate	2/39 (5%)	1/11 (9%)	0/0
First incidence (days)	729 (T)	615	419
Poly-3 test	P<0.001	P=0.362	P<0.001
Mammary Gland: Fibroadenoma			
Overall rate	0/60 (3%)	13/60 (22%)	20/60 (33%)
Adjusted rate	0.0%	31.2%	61.1%
Terminal rate	0/39 (0%)	5/11 (46%)	0/0
First incidence (days)	—	523	299
Poly-3 test	P<0.001	P<0.001	P<0.001
Pancreas: Adenoma			
Overall rate	1/60 (2%)	0/60 (0%)	3/60 (5%)
Adjusted rate	1.9%	0.0%	14.3%
Terminal rate	0/39 (0%)	0/11 (0%)	0/0
First incidence (days)	705	—	471
Poly-3 test	P=0.117	P=0.565N	P=0.094
Pituitary Gland (Pars Distalis): Adenoma			
Overall rate	11/59 (19%)	10/57 (18%)	5/59 (8%)
Adjusted rate	19.9%	25.5%	23.6%
Terminal rate	6/39 (15%)	4/11 (36%)	0/0
First incidence (days)	441	579	567
Poly-3 test	P=0.360	P=0.351	P=0.486
Preputial Gland: Adenoma or Carcinoma			
Overall rate	4/60 (7%)	2/60 (3%)	3/59 (5%)
Adjusted rate	7.4%	5.1%	14.1%
Terminal rate	3/39 (8%)	0/11 (0%)	0/0
First incidence (days)	720	649	384
Poly-3 test	P=0.411	P=0.491N	P=0.352
Skin: Keratoacanthoma			
Overall rate	5/60 (8%)	1/60 (2%)	0/60 (0%)
Adjusted rate	9.2%	2.6%	0.0%
Terminal rate	3/39 (8%)	0/11 (0%)	0/0
First incidence (days)	537	579	—
Poly-3 test	P=0.087N	P=0.200N	P=0.248N
Skin: Squamous Cell Papilloma or Keratoacanthoma			
Overall rate	5/60 (8%)	1/60 (2%)	2/60 (3%)
Adjusted rate	9.2%	2.6%	9.8%
Terminal rate	3/39 (8%)	0/11 (0%)	0/0
First incidence (days)	537	579	540
Poly-3 test	P=0.406N	P=0.200N	P=0.615
Skin: Squamous Cell Papilloma, Keratoacanthoma, or Squamous Cell Carcinoma			
Overall rate	5/60 (8%)	2/60 (3%)	2/60 (3%)
Adjusted rate	9.2%	5.0%	9.8%
Terminal rate	3/39 (8%)	0/11 (0%)	0/0
First incidence (days)	537	569	540
Poly-3 test	P=0.492N	P=0.364N	P=0.615

TABLE A3b
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Stop-Exposure Feed Study of o-Nitrotoluene

	0 ppm	2,000 ppm	5,000 ppm
Skin: Squamous Cell Papilloma, Keratoacanthoma, Basal Cell Adenoma, or Squamous Cell Carcinoma			
Overall rate	7/60 (12%)	2/60 (3%)	2/60 (3%)
Adjusted rate	12.9%	5.0%	9.8%
Terminal rate	5/39 (13%)	0/11 (0%)	0/0
First incidence (days)	537	569	540
Poly-3 test	P=0.268N	P=0.184N	P=0.513N
Skin (Subcutaneous Tissue): Lipoma			
Overall rate	0/60 (0%)	10/60 (17%)	12/60 (20%)
Adjusted rate	0.0%	24.6%	44.7%
Terminal rate	0/39 (0%)	3/11 (27%)	0/0
First incidence (days)	—	523	362
Poly-3 test	P<0.001	P<0.001	P<0.001
Skin (Subcutaneous Tissue): Fibroma			
Overall rate	5/60 (8%)	45/60 (75%)	52/60 (87%)
Adjusted rate	9.3%	87.6%	97.2%
Terminal rate	3/39 (8%)	10/11 (91%)	0/0
First incidence (days)	705	370	299
Poly-3 test	P<0.001	P<0.001	P<0.001
Skin (Subcutaneous Tissue): Fibrosarcoma			
Overall rate	0/60 (0%)	8/60 (13%)	12/60 (20%)
Adjusted rate	0.0%	19.6%	44.7%
Terminal rate	0/39 (0%)	2/11 (18%)	0/0
First incidence (days)	—	275	359
Poly-3 test	P<0.001	P<0.001	P<0.001
Skin (Subcutaneous Tissue): Fibrosarcoma or Sarcoma			
Overall rate	0/60 (0%)	8/60 (13%)	13/60 (22%)
Adjusted rate	0.0%	19.6%	47.5%
Terminal rate	0/39 (0%)	2/11 (18%)	0/0
First incidence (days)	—	275	359
Poly-3 test	P<0.001	P<0.001	P<0.001
Skin (Subcutaneous Tissue): Fibroma, Fibrosarcoma, or Sarcoma			
Overall rate	5/60 (8%)	47/60 (78%)	53/60 (88%)
Adjusted rate	9.3%	89.0%	97.8%
Terminal rate	3/39 (8%)	10/11 (91%)	0/0
First incidence (days)	705	275	299
Poly-3 test	P<0.001	P<0.001	P<0.001
Testes: Adenoma			
Overall rate	55/60 (92%)	50/60 (83%)	27/60 (45%)
Adjusted rate	94.3%	92.6%	74.7%
Terminal rate	38/39 (97%)	10/11 (91%)	0/0
First incidence (days)	533	370	384
Poly-3 test	P<0.001N	P=0.506N	P<0.001N
Thyroid Gland (C-Cell): Adenoma			
Overall rate	10/59 (17%)	3/60 (5%)	4/60 (7%)
Adjusted rate	18.6%	7.7%	18.4%
Terminal rate	6/38 (16%)	2/11 (18%)	0/0
First incidence (days)	533	531	417
Poly-3 test	P=0.326N	P=0.119N	P=0.603N

TABLE A3b
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Stop-Exposure Feed Study of o-Nitrotoluene

	0 ppm	2,000 ppm	5,000 ppm
Thyroid Gland (C-Cell): Adenoma or Carcinoma			
Overall rate	10/59 (17%)	4/60 (7%)	4/60 (7%)
Adjusted rate	18.6%	10.2%	18.4%
Terminal rate	6/38 (16%)	2/11 (18%)	0/0
First incidence (days)	533	531	417
Poly-3 test	P=0.384N	P=0.209N	P=0.603N
All Organs: Hemangioma			
Overall rate	0/60 (0%)	0/60 (0%)	3/60 (5%)
Adjusted rate	0.0%	0.0%	14.0%
Terminal rate	0/39 (0%)	0/11 (0%)	0/0
First incidence (days)	—	—	417
Poly-3 test	P=0.025	—	P=0.034
All Organs: Hemangioma or Hemangiosarcoma			
Overall rate	1/60 (2%)	0/60 (0%)	4/60 (7%)
Adjusted rate	1.9%	0.0%	18.0%
Terminal rate	1/39 (3%)	0/11 (0%)	0/0
First incidence (days)	729 (T)	—	391
Poly-3 test	P=0.041	P=0.565N	P=0.037
All Organs: Osteosarcoma			
Overall rate	1/60 (2%)	3/60 (5%)	3/60 (5%)
Adjusted rate	1.9%	7.3%	14.0%
Terminal rate	0/39 (0%)	0/11 (0%)	0/0
First incidence (days)	727	260	359
Poly-3 test	P=0.060	P=0.217	P=0.097
All Organs: Mononuclear Cell Leukemia			
Overall rate	30/60 (50%)	13/60 (22%)	1/60 (2%)
Adjusted rate	52.2%	31.1%	5.0%
Terminal rate	16/39 (41%)	4/11 (36%)	0/0
First incidence (days)	533	531	569
Poly-3 test	P<0.001N	P=0.027N	P<0.001N
All Organs: Malignant Mesothelioma			
Overall rate	2/60 (3%)	44/60 (73%)	54/60 (90%)
Adjusted rate	3.7%	80.3%	95.1%
Terminal rate	2/39 (5%)	10/11 (91%)	0/0
First incidence (days)	729 (T)	275	299
Poly-3 test	P<0.001	P<0.001	P<0.001
All Organs: Benign Neoplasms			
Overall rate	60/60 (100%)	57/60 (95%)	56/60 (93%)
Adjusted rate	100.0%	99.5%	98.7%
Terminal rate	39/39 (100%)	11/11 (100%)	0/0
First incidence (days)	441	370	299
Poly-3 test	P=0.337N	P=1.000N	P=0.746N
All Organs: Malignant Neoplasms			
Overall rate	37/60 (62%)	54/60 (90%)	58/60 (97%)
Adjusted rate	63.7%	92.1%	97.9%
Terminal rate	21/39 (54%)	11/11 (100%)	0/0
First incidence (days)	533	260	299
Poly-3 test	P<0.001	P<0.001	P<0.001

TABLE A3b
Statistical Analysis of Primary Neoplasms in Male Rats in the 2-Year Stop-Exposure Feed Study of o-Nitrotoluene

	0 ppm	2,000 ppm	5,000 ppm
All Organs: Benign or Malignant Neoplasms			
Overall rate	60/60 (100%)	60/60 (100%)	60/60 (100%)
Adjusted rate	100.0%	100.0%	100.0%
Terminal rate	39/39 (100%)	11/11 (100%)	0/0
First incidence (days)	441	260	299
Poly-3 test	—	—	—

(T) Terminal sacrifice

- ^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for adrenal gland, liver, lung, pancreas, pituitary gland, preputial gland, testes, and thyroid gland; for other tissues, denominator is number of animals necropsied.
- ^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality
- ^c Observed incidence at terminal kill
- ^d Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by N.
- ^e Not applicable; no neoplasms in animal group
- ^f Value of statistic cannot be computed.

TABLE A4a
Historical Incidence of Malignant Mesothelioma in Control Male F344/N Rats

Study	Incidence in Controls
Historical Incidence in Controls Given NTP-2000 Diet^a	
Citral (feed)	4/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	2/50
Indium phosphide (inhalation)	2/50
60-Hz Magnetic fields (whole body exposure)	4/100
Methacrylonitrile (gavage)	0/50
Naphthalene (inhalation)	0/49
<i>o</i> -Nitrotoluene (feed)	2/60
<i>p</i> -Nitrotoluene (feed)	5/50
Sodium nitrite (drinking water)	3/50
Vanadium pentoxide (inhalation)	1/50
Overall Historical Incidence in Controls Given NTP-2000 Diet	
Total (%)	23/609 (3.8%)
Mean ± standard deviation	3.7% ± 2.9%
Range	0%-10%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b	
Benzyl acetate	0/50
2,2-Bis(bromomethyl)-1,3-propanediol	0/51
Butyl benzyl phthalate	1/50
D&C Yellow No. 11	2/50
Emodin	2/50
<i>o</i> -Nitroanisole	1/50
<i>p</i> -Nitrobenzoic acid	0/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet	
Total (%)	25/1,004 (2.5%)
Mean ± standard deviation	2.5% ± 2.0%
Range	0%-8%

^a Data as of January 18, 2001

^b Data as of December 21, 1999

TABLE A4b
Historical Incidence of Subcutaneous Skin Neoplasms in Control Male F344/N Rats

Study	Incidence in Controls			
	Lipoma	Fibroma	Fibrosarcoma	Fibroma or Fibrosarcoma
Historical Incidence in Controls Given NTP-2000 Diet^a				
Citral (feed)	0/100	2/100	2/100	4/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	1/50	2/50	0/50	2/50
Indium phosphide (inhalation)	1/50	1/50	1/50	2/50
60-Hz Magnetic fields (whole body exposure)	1/100	12/100	1/100	13/100
Methacrylonitrile (gavage)	1/50	3/50	0/50	3/50
Naphthalene (inhalation)	1/49	5/49	2/49	7/49
<i>o</i> -Nitrotoluene (feed)	0/60	5/60	0/60	5/60
<i>p</i> -Nitrotoluene (feed)	0/50	1/50	0/50	1/50
Sodium nitrite (drinking water)	1/50	0/50	1/50	1/50
Vanadium pentoxide (inhalation)	2/50	2/50	1/50	3/50
Overall Historical Incidence in Controls Given NTP-2000 Diet				
Total (%)	8/609 (1.3%)	33/609 (5.4%)	8/609 (1.3%)	41/609 (6.7%)
Mean ± standard deviation	1.5% ± 1.3%	5.1% ± 4.0%	1.3% ± 1.4%	6.4% ± 4.3%
Range	0%-4%	0%-12%	0%-4%	2%-14%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b				
Benzyl acetate	0/50	4/50	0/50	4/50
2,2-Bis(bromomethyl)-1,3-propanediol	0/51	2/51	0/51	2/51
Butyl benzyl phthalate	0/50	5/50	0/50	5/50
D&C Yellow No. 11	0/50	3/50	0/50	3/50
Emodin	1/50	1/50	0/50	1/50
<i>o</i> -Nitroanisole	1/50	1/50	0/50	1/50
<i>p</i> -Nitrobenzoic acid	0/50	4/50	1/50	5/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet				
Total (%)	2/1,004 (0.2%)	56/1,004 (5.6%)	9/1,004 (0.9%)	65/1,004 (6.5%)
Mean ± standard deviation	0.2% ± 0.6%	5.6% ± 3.2%	0.9% ± 1.4%	6.5% ± 3.1%
Range	0%-2%	0%-10%	0%-4%	2%-10%

^a Data as of January 18, 2001

^b Data as of December 21, 1999

TABLE A4c
Historical Incidence of Mammary Gland Fibroadenoma in Control Male F344/N Rats

Study	Incidence in Controls
Historical Incidence in Controls Given NTP-2000 Diet^a	
Citral (feed)	8/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	3/50
Indium phosphide (inhalation)	2/50
60-Hz Magnetic fields (whole body exposure)	6/100
Methacrylonitrile (gavage)	2/50
Naphthalene (inhalation)	0/49
<i>o</i> -Nitrotoluene (feed)	0/60
<i>p</i> -Nitrotoluene (feed)	0/50
Sodium nitrite (drinking water)	3/50
Vanadium pentoxide (inhalation)	2/50
Overall Historical Incidence in Controls Given NTP-2000 Diet	
Total (%)	26/609 (4.3%)
Mean ± standard deviation	3.8% ± 2.9%
Range	0%-8%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b	
Benzyl acetate	4/50
2,2-Bis(bromomethyl)-1,3-propanediol	0/51
Butyl benzyl phthalate	2/50
D&C Yellow No. 11	3/50
Emodin	1/50
<i>o</i> -Nitroanisole	3/50
<i>p</i> -Nitrobenzoic acid	2/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet	
Total (%)	42/1,004 (4.2%)
Mean ± standard deviation	4.2% ± 3.5%
Range	0%-12%

^a Data as of January 18, 2001
^b Data as of December 21, 1999

TABLE A4d
Historical Incidence of Liver Neoplasms in Control Male F344/N Rats

Study	Incidence in Controls				
	Hepatocholangio- carcinoma	Cholangio- carcinoma	Hepatocellular Adenoma	Hepatocellular Carcinoma	Hepatocellular Adenoma or Carcinoma
Historical Incidence in Controls Given NTP-2000 Diet^a					
Citral (feed)	0/100	0/100	0/100	0/100	0/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	0/50	0/50	0/50	2/50	2/50
Indium phosphide (inhalation)	0/50	0/50	0/50	0/50	0/50
60-Hz Magnetic fields (whole body exposure)	0/100	0/100	1/100	0/100	1/100
Methacrylonitrile (gavage)	0/50	0/50	1/50	0/50	1/50
Naphthalene (inhalation)	0/49	0/49	1/49	1/49	2/49
<i>o</i> -Nitrotoluene (feed)	0/60	0/60	2/60	1/60	3/60
<i>p</i> -Nitrotoluene (feed)	0/50	0/50	0/50	1/50	1/50
Sodium nitrite (drinking water)	0/50	0/50	0/50	0/50	0/50
Vanadium pentoxide (inhalation)	0/50	0/50	0/50	0/50	0/50
Overall Historical Incidence in Controls Given NTP-2000 Diet					
Total (%)	0/609	0/609	5/609 (0.8%)	5/609 (0.8%)	10/609 (1.6%)
Mean ± standard deviation			0.8% ± 1.2%	1.0% ± 1.4%	1.8% ± 1.9%
Range			0%-3%	0%-4%	0%-5%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b					
Benzyl acetate	0/50	0/50	5/50	1/50	5/50
2,2-Bis(bromomethyl)-1,3-propanediol	0/51	0/51	0/51	0/51	0/51
Butyl benzyl phthalate	0/50	0/50	2/50	0/50	2/50
D&C Yellow No. 11	0/50	0/50	1/50	0/50	1/50
Emodin	0/50	0/50	1/50	0/50	1/50
<i>o</i> -Nitroanisole	0/50	0/50	0/50	0/50	0/50
<i>p</i> -Nitrobenzoic acid	0/49	0/49	2/49	3/49	4/49
Overall Historical Incidence in Feed Controls Given NIH-07 Diet					
Total (%)	0/1,002	0/1,002	23/1,002 (2.3%)	7/1,002 (0.7%)	28/1,002 (2.8%)
Mean ± standard deviation			2.3% ± 3.0%	0.7% ± 1.5%	2.8% ± 3.3%
Range			0%-10%	0%-6%	0%-10%

^a Data as of January 18, 2001

^b Data as of December 21, 1999

TABLE A4e
Historical Incidence of Alveolar/bronchiolar Neoplasms in Control Male F344/N Rats

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
Historical Incidence in Controls Given NTP-2000 Diet^a			
Citral (feed)	3/100	0/100	3/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	2/50	0/50	2/50
Indium phosphide (inhalation)	6/50	1/50	7/50
60-Hz Magnetic fields (whole body exposure)	3/100	0/100	3/100
Methacrylonitrile (gavage)	0/50	0/50	0/50
Naphthalene (inhalation)	2/49	0/49	2/49
<i>o</i> -Nitrotoluene (feed)	1/60	1/60	2/60
<i>p</i> -Nitrotoluene (feed)	1/50	0/50	1/50
Sodium nitrite (drinking water)	2/50	0/50	2/50
Vanadium pentoxide (inhalation)	4/50	0/50	4/50
Overall Historical Incidence in Controls Given NTP-2000 Diet			
Total (%)	24/609 (3.9%)	2/609 (0.3%)	26/609 (4.3%)
Mean ± standard deviation	4.2% ± 3.5%	0.4% ± 0.8%	4.5% ± 3.9%
Range	0%-12%	0%-2%	0%-14%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b			
Benzyl acetate	1/50	0/50	1/50
2,2-Bis(bromomethyl)-1,3-propanediol	1/51	0/51	1/51
Butyl benzyl phthalate	0/50	0/50	0/50
D&C Yellow No. 11	7/50	1/50	8/50
Emodin	0/50	0/50	0/50
<i>o</i> -Nitroanisole	2/50	1/50	3/50
<i>p</i> -Nitrobenzoic acid	0/50	0/50	0/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet			
Total (%)	24/1,002 (2.4%)	7/1,002 (0.7%)	31/1,002 (3.1%)
Mean ± standard deviation	2.4% ± 3.2%	0.7% ± 1.2%	3.1% ± 3.5%
Range	0%-14%	0%-4%	0%-16%

^a Data as of January 18, 2001

^b Data as of December 21, 1999

TABLE A4f
Historical Incidence of Circulatory System Neoplasms in Control Male F344/N Rats

Study	Incidence in Controls		
	Hemangioma	Hemangiosarcoma	Hemangioma or Hemangiosarcoma
Historical Incidence in Controls Given NTP-2000 Diet^a			
Citral (feed)	1/100	0/100	1/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	0/50	1/50	1/50
Indium phosphide (inhalation)	0/50	1/50	1/50
60-Hz Magnetic fields (whole body exposure)	0/100	0/100	0/100
Methacrylonitrile (gavage)	0/50	0/50	0/50
Naphthalene (inhalation)	0/49	0/49	0/49
<i>o</i> -Nitrotoluene (feed)	0/60	1/60	1/60
<i>p</i> -Nitrotoluene (feed)	2/50	0/50	2/50
Sodium nitrite (drinking water)	0/50	0/50	0/50
Vanadium pentoxide (inhalation)	0/50	0/50	0/50
Overall Historical Incidence in Controls Given NTP-2000 Diet			
Total (%)	3/609 (0.5%)	3/609 (0.5%)	6/609 (1.0%)
Mean ± standard deviation	0.5% ± 1.3%	0.6% ± 0.9%	1.1% ± 1.4%
Range	0%-4%	0%-2%	0%-4%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b			
Benzyl acetate	0/50	0/50	0/50
2,2-Bis(bromomethyl)-1,3-propanediol	0/51	0/51	0/51
Butyl benzyl phthalate	0/50	1/50	1/50
D&C Yellow No. 11	1/50	0/50	1/50
Emodin	0/50	1/50	1/50
<i>o</i> -Nitroanisole	1/50	1/50	2/50
<i>p</i> -Nitrobenzoic acid	0/50	1/50	1/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet			
Total (%)	3/1,004 (0.3%)	7/1,004 (0.7%)	10/1,004 (1.0%)
Mean ± standard deviation	0.3% ± 0.7%	0.7% ± 1.2%	1.0% ± 1.4%
Range	0%-2%	0%-4%	0%-4%

^a Data as of January 18, 2001

^b Data as of December 21, 1999

TABLE A4g
Historical Incidence of Mononuclear Cell Leukemia in Control Male F344/N Rats

Study	Incidence in Controls
Historical Incidence in Controls Given NTP-2000 Diet^a	
Citral (feed)	68/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	27/50
Indium phosphide (inhalation)	16/50
60-Hz Magnetic fields (whole body exposure)	50/100
Methacrylonitrile (gavage)	20/50
Naphthalene (inhalation)	26/49
<i>o</i> -Nitrotoluene (feed)	30/60
<i>p</i> -Nitrotoluene (feed)	24/50
Sodium nitrite (drinking water)	17/50
Vanadium pentoxide (inhalation)	22/50
Overall Historical Incidence in Controls Given NTP-2000 Diet	
Total (%)	300/609 (49.3%)
Mean ± standard deviation	47.3% ± 10.5%
Range	32%-68%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b	
Benzyl acetate	16/50
2,2-Bis(bromomethyl)-1,3-propanediol	27/51
Butyl benzyl phthalate	31/50
D&C Yellow No. 11	37/50
Emodin	28/50
<i>o</i> -Nitroanisole	26/50
<i>p</i> -Nitrobenzoic acid	29/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet	
Total (%)	547/1,004 (54.5%)
Mean ± standard deviation	54.5% ± 10.7%
Range	32%-74%

^a Data as of January 18, 2001; includes data for lymphocytic, monocytic, and undifferentiated leukemia

^b Data as of December 21, 1999; includes data for lymphocytic, monocytic, and undifferentiated leukemia

TABLE A4h
Historical Incidence of Interstitial Cell Adenoma of the Testis in Control Male F344/N Rats

Study	Incidence in Controls
Historical Incidence in Controls Given NTP-2000 Diet^a	
Citral (feed)	96/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	41/50
Indium phosphide (inhalation)	40/50
60-Hz Magnetic fields (whole body exposure)	93/100
Methacrylonitrile (gavage)	40/50
Naphthalene (inhalation)	38/49
<i>o</i> -Nitrotoluene (feed)	55/60
<i>p</i> -Nitrotoluene (feed)	49/50
Sodium nitrite (drinking water)	47/50
Vanadium pentoxide (inhalation)	36/50
Overall Historical Incidence in Controls Given NTP-2000 Diet	
Total (%)	535/609 (87.9%)
Mean ± standard deviation	86.4% ± 9.1%
Range	72%-98%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b	
Benzyl acetate	47/50
2,2-Bis(bromomethyl)-1,3-propanediol	49/51
Butyl benzyl phthalate	44/50
D&C Yellow No. 11	39/49
Emodin	41/50
<i>o</i> -Nitroanisole	48/50
<i>p</i> -Nitrobenzoic acid	44/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet	
Total (%)	889/1,003 (88.6%)
Mean ± standard deviation	88.6% ± 6.0%
Range	74%-96%

^a Data as of January 18, 2001

^b Data as of December 21, 1999

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of o-Nitrotoluene^a

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
Disposition Summary						
Animals initially in study	70	60	60	60	70	70
3-Month interim evaluation	10				10	10
Early deaths						
Accidental deaths			3			
Moribund	18	35	48	53	39	57
Natural deaths	3	7	6	7	10	3
Survivors						
Died last week of study	1					
Terminal sacrifice	38	18	3		11	
Animals examined microscopically	70	60	60	60	70	70
3-Month Interim Evaluation						
Alimentary System						
Liver	(10)				(10)	(10)
Hepatodiaphragmatic nodule					1 (10%)	2 (20%)
Hepatocyte, vacuolization cytoplasmic						10 (100%)
Mesentery						(1)
Accessory spleen						1 (100%)
Pancreas	(10)				(10)	(10)
Atrophy						1 (10%)
Salivary glands	(10)				(10)	(10)
Atrophy					10 (100%)	10 (100%)
Cardiovascular System						
Heart	(10)				(10)	(10)
Cardiomyopathy	5 (50%)				3 (30%)	6 (60%)
Endocrine System						
Adrenal cortex	(10)				(10)	(10)
Accessory adrenal cortical nodule	1 (10%)				2 (20%)	2 (20%)
Islets, pancreatic	(10)				(10)	(10)
Hyperplasia						8 (80%)
Pituitary gland	(10)				(10)	(10)
Pars distalis, cyst					1 (10%)	
Thyroid gland	(10)				(10)	(10)
Ultimobranchial cyst	1 (10%)					2 (20%)
Genital System						
Epididymis	(10)				(10)	(10)
Atrophy						5 (50%)
Atypia cellular					2 (20%)	10 (100%)
Preputial gland	(10)				(10)	(10)
Atrophy					1 (10%)	8 (80%)

^a Number of animals examined microscopically at the site and the number of animals with lesion

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
3-Month Interim Evaluation (continued)						
Genital System (continued)						
Prostate	(10)				(10)	(10)
Inflammation, chronic						1 (10%)
Testes	(10)				(10)	(10)
Mineralization						5 (50%)
Germinal epithelium, atrophy					1 (10%)	9 (90%)
Hematopoietic System						
Lymph node					(1)	(1)
Mediastinal, hemorrhage					1 (100%)	
Mediastinal, pigmentation					1 (100%)	
Renal, hemorrhage						1 (100%)
Renal, pigmentation						1 (100%)
Spleen	(10)				(10)	(10)
Congestion					3 (30%)	10 (100%)
Hematopoietic cell proliferation					2 (20%)	10 (100%)
Pigmentation						1 (10%)
Thymus	(10)				(10)	(10)
Hemorrhage					1 (10%)	
Respiratory System						
Nose	(10)				(10)	(10)
Olfactory epithelium, degeneration					4 (40%)	10 (100%)
Urinary System						
Kidney	(10)				(10)	(10)
Nephropathy					8 (80%)	
Renal tubule, degeneration, hyaline	1 (10%)					10 (100%)
Systems Examined with No Lesions Observed						
General Body System						
Integumentary System						
Musculoskeletal System						
Nervous System						
Special Senses System						
2-Year Study						
Alimentary System						
Intestine large, cecum	(60)	(60)	(60)	(58)	(59)	(60)
Edema	1 (2%)				1 (2%)	
Liver	(60)	(60)	(60)	(60)	(60)	(60)
Angiectasis		1 (2%)	2 (3%)	2 (3%)	3 (5%)	2 (3%)
Atrophy	1 (2%)	1 (2%)	1 (2%)	4 (7%)		3 (5%)
Basophilic focus	38 (63%)	22 (37%)	21 (35%)	25 (42%)	31 (52%)	17 (28%)

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
2-Year Study (continued)						
Alimentary System (continued)						
Liver (continued)	(60)	(60)	(60)	(60)	(60)	(60)
Clear cell focus	29 (48%)	29 (48%)	34 (57%)	31 (52%)	30 (50%)	34 (57%)
Cyst				1 (2%)		
Degeneration, cystic	3 (5%)	3 (5%)	1 (2%)	2 (3%)	6 (10%)	3 (5%)
Eosinophilic focus	7 (12%)	18 (30%)	29 (48%)	24 (40%)	15 (25%)	13 (22%)
Fibrosis		1 (2%)			2 (3%)	
Hematopoietic cell proliferation		6 (10%)	2 (3%)	2 (3%)	11 (18%)	6 (10%)
Hemorrhage	2 (3%)		2 (3%)	2 (3%)	2 (3%)	
Hepatodiaphragmatic nodule	5 (8%)	8 (13%)	8 (13%)	5 (8%)	10 (17%)	4 (7%)
Infiltration cellular, mixed cell	1 (2%)	5 (8%)	11 (18%)	20 (33%)	15 (25%)	33 (55%)
Mixed cell focus	5 (8%)	7 (12%)	12 (20%)	6 (10%)	12 (20%)	8 (13%)
Necrosis, focal	3 (5%)	4 (7%)	6 (10%)	6 (10%)	2 (3%)	3 (5%)
Bile duct, hyperplasia	53 (88%)	37 (62%)	7 (12%)		6 (10%)	1 (2%)
Centrilobular, necrosis	1 (2%)	3 (5%)	8 (13%)	5 (8%)	9 (15%)	3 (5%)
Hepatocyte, vacuolization cytoplasmic	4 (7%)	2 (3%)	1 (2%)	1 (2%)	1 (2%)	6 (10%)
Kupffer cell, pigmentation	4 (7%)		1 (2%)	3 (5%)		2 (3%)
Mesentery	(16)	(6)	(5)	(4)	(1)	(3)
Accessory spleen	3 (19%)			3 (75%)		
Congestion	3 (19%)					
Hemorrhage		1 (17%)				
Fat, necrosis	13 (81%)	3 (50%)	5 (100%)	1 (25%)	1 (100%)	
Oral mucosa			(1)	(1)		
Hyperplasia			1 (100%)			
Pancreas	(60)	(60)	(60)	(60)	(60)	(60)
Atrophy	11 (18%)	14 (23%)	7 (12%)	4 (7%)	11 (18%)	9 (15%)
Acinus, hyperplasia, focal	1 (2%)	2 (3%)	3 (5%)	4 (7%)	6 (10%)	3 (5%)
Salivary glands	(60)	(60)	(59)	(60)	(58)	(59)
Atrophy		2 (3%)	18 (31%)	43 (72%)	16 (28%)	49 (83%)
Stomach, forestomach	(59)	(60)	(60)	(60)	(60)	(60)
Edema	4 (7%)	2 (3%)		1 (2%)	2 (3%)	
Ulcer	2 (3%)	2 (3%)	1 (2%)	1 (2%)	1 (2%)	
Epithelium, hyperplasia	6 (10%)	2 (3%)	1 (2%)	2 (3%)	4 (7%)	1 (2%)
Stomach, glandular	(59)	(60)	(60)	(60)	(60)	(60)
Edema				1 (2%)	1 (2%)	
Erosion	4 (7%)	2 (3%)	7 (12%)	7 (12%)	7 (12%)	3 (5%)
Infiltration cellular, lymphoid	1 (2%)					
Perforation					1 (2%)	
Ulcer	1 (2%)	2 (3%)		3 (5%)	1 (2%)	1 (2%)
Tongue	(1)		(1)			
Epithelium, hyperplasia	1 (100%)					
Tooth				(1)		(1)
Malformation				1 (100%)		
Cardiovascular System						
Heart	(60)	(60)	(60)	(60)	(60)	(60)
Cardiomyopathy	40 (67%)	43 (72%)	43 (72%)	37 (62%)	39 (65%)	46 (77%)
Thrombosis	1 (2%)	1 (2%)	1 (2%)		4 (7%)	1 (2%)

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
2-Year Study (continued)						
Endocrine System						
Adrenal cortex	(60)	(60)	(60)	(60)	(60)	(60)
Accessory adrenal cortical nodule	13 (22%)	12 (20%)	13 (22%)	10 (17%)	14 (23%)	9 (15%)
Degeneration, fatty	16 (27%)	10 (17%)	11 (18%)	4 (7%)	14 (23%)	12 (20%)
Hyperplasia, diffuse			1 (2%)	3 (5%)		2 (3%)
Hyperplasia, focal	6 (10%)	2 (3%)	6 (10%)	3 (5%)	4 (7%)	6 (10%)
Hypertrophy, focal	3 (5%)	2 (3%)	2 (3%)		3 (5%)	2 (3%)
Necrosis						1 (2%)
Adrenal medulla	(60)	(60)	(60)	(59)	(60)	(60)
Hyperplasia	7 (12%)	4 (7%)	2 (3%)		3 (5%)	1 (2%)
Islets, pancreatic	(60)	(60)	(60)	(60)	(60)	(60)
Hyperplasia	2 (3%)			2 (3%)	2 (3%)	10 (17%)
Pigmentation	1 (2%)			2 (3%)		11 (18%)
Parathyroid gland	(58)	(55)	(59)	(58)	(58)	(57)
Hyperplasia		1 (2%)				
Pituitary gland	(59)	(60)	(58)	(59)	(57)	(59)
Pars distalis, angiectasis	3 (5%)		7 (12%)	2 (3%)	4 (7%)	4 (7%)
Pars distalis, cyst	2 (3%)	6 (10%)	3 (5%)	4 (7%)	3 (5%)	6 (10%)
Pars distalis, cytoplasmic alteration	1 (2%)		1 (2%)	4 (7%)	1 (2%)	42 (71%)
Pars distalis, hyperplasia, focal	10 (17%)	13 (22%)	13 (22%)	10 (17%)	9 (16%)	15 (25%)
Pars intermedia, cyst		1 (2%)	1 (2%)		1 (2%)	2 (3%)
Thyroid gland	(59)	(60)	(60)	(60)	(60)	(60)
Ultimobranchial cyst		1 (2%)	2 (3%)	3 (5%)	4 (7%)	3 (5%)
C-cell, hyperplasia	10 (17%)	11 (18%)	9 (15%)	7 (12%)	9 (15%)	6 (10%)
Follicle, cyst	3 (5%)	2 (3%)				
Follicular cell, hyperplasia	1 (2%)					
General Body System						
Peritoneum	(3)	(21)	(33)	(46)	(47)	(54)
Mesothelium, hyperplasia	1 (33%)	1 (5%)	4 (12%)	2 (4%)	3 (6%)	
Genital System						
Epididymis	(60)	(60)	(59)	(60)	(60)	(58)
Atypia cellular	39 (65%)	32 (53%)	27 (46%)	21 (35%)	24 (40%)	28 (48%)
Fibrosis		2 (3%)				
Inflammation, chronic		1 (2%)			2 (3%)	
Preputial gland	(60)	(59)	(58)	(56)	(60)	(59)
Atrophy	7 (12%)	9 (15%)	35 (60%)	41 (73%)	38 (63%)	54 (92%)
Cyst	1 (2%)	4 (7%)	1 (2%)		1 (2%)	1 (2%)
Hyperplasia		1 (2%)				
Inflammation, chronic	40 (67%)	43 (73%)	36 (62%)	27 (48%)	33 (55%)	14 (24%)
Prostate	(60)	(59)	(60)	(60)	(60)	(60)
Inflammation, chronic	24 (40%)	14 (24%)	18 (30%)	9 (15%)	17 (28%)	8 (13%)
Epithelium, hyperplasia					1 (2%)	
Ventral, hyperplasia	18 (30%)	3 (5%)	9 (15%)	1 (2%)	4 (7%)	1 (2%)
Testes	(60)	(60)	(60)	(60)	(60)	(60)
Angiectasis				2 (3%)		1 (2%)
Hemorrhage					2 (3%)	
Germinal epithelium, atrophy	13 (22%)	21 (35%)	12 (20%)	19 (32%)	18 (30%)	53 (88%)
Interstitial cell, hyperplasia	10 (17%)	14 (23%)	13 (22%)	31 (52%)	15 (25%)	4 (7%)

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
2-Year Study (continued)						
Hematopoietic System						
Bone marrow	(60)	(60)	(60)	(60)	(60)	(60)
Hyperplasia	2 (3%)	25 (42%)	43 (72%)	45 (75%)	37 (62%)	33 (55%)
Lymph node	(16)	(14)	(4)	(9)	(18)	(16)
Iliac, hyperplasia, lymphoid				1 (11%)		
Mediastinal, atrophy				1 (11%)		
Mediastinal, ectasia					1 (6%)	
Mediastinal, hemorrhage	2 (13%)	3 (21%)	1 (25%)	2 (22%)	4 (22%)	9 (56%)
Mediastinal, hyperplasia, lymphoid						1 (6%)
Mediastinal, pigmentation	6 (38%)	9 (64%)	3 (75%)	8 (89%)	13 (72%)	13 (81%)
Pancreatic, hyperplasia, histiocytic						2 (13%)
Pancreatic, hyperplasia, lymphoid						1 (6%)
Pancreatic, pigmentation	1 (6%)	1 (7%)				
Renal, ectasia			1 (25%)			
Renal, pigmentation	1 (6%)		1 (25%)			
Lymph node, mandibular	(58)	(59)	(58)	(59)	(58)	(59)
Atrophy				1 (2%)		1 (2%)
Ectasia	2 (3%)				1 (2%)	
Hemorrhage	2 (3%)	2 (3%)	1 (2%)	1 (2%)	4 (7%)	3 (5%)
Hyperplasia, lymphoid	3 (5%)	4 (7%)	3 (5%)	2 (3%)	5 (9%)	3 (5%)
Pigmentation	9 (16%)	2 (3%)	6 (10%)	12 (20%)	5 (9%)	5 (8%)
Lymph node, mesenteric	(60)	(60)	(59)	(58)	(60)	(59)
Atrophy	1 (2%)		2 (3%)	1 (2%)		
Ectasia						2 (3%)
Hemorrhage	2 (3%)	6 (10%)	3 (5%)	2 (3%)	4 (7%)	4 (7%)
Hyperplasia, lymphoid						2 (3%)
Pigmentation	3 (5%)	3 (5%)				
Spleen	(60)	(60)	(60)	(60)	(60)	(60)
Accessory spleen						1 (2%)
Fibrosis	6 (10%)	6 (10%)	9 (15%)	7 (12%)	14 (23%)	27 (45%)
Hematopoietic cell proliferation	7 (12%)	33 (55%)	38 (63%)	47 (78%)	36 (60%)	35 (58%)
Hemorrhage		2 (3%)				
Necrosis						1 (2%)
Pigmentation	9 (15%)	8 (13%)	13 (22%)	16 (27%)	4 (7%)	7 (12%)
Capsule, fibrosis				1 (2%)		
Lymphoid follicle, atrophy					1 (2%)	1 (2%)
Thymus	(56)	(54)	(55)	(55)	(56)	(56)
Cyst					2 (4%)	1 (2%)
Integumentary System						
Mammary gland	(57)	(47)	(46)	(43)	(51)	(50)
Dilatation	3 (5%)	10 (21%)	1 (2%)	1 (2%)	3 (6%)	1 (2%)
Galactocele	2 (4%)					
Hyperplasia	6 (11%)	2 (4%)	2 (4%)	2 (5%)	1 (2%)	4 (8%)
Skin	(60)	(60)	(60)	(60)	(60)	(60)
Cyst		1 (2%)				
Cyst epithelial inclusion						1 (2%)
Inflammation, chronic	1 (2%)		1 (2%)			
Subcutaneous tissue, edema			1 (2%)			
Subcutaneous tissue, inflammation, chronic						1 (2%)

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
2-Year Study (continued)						
Musculoskeletal System						
Bone	(60)	(60)	(60)	(60)	(60)	(60)
Cranium, osteopetrosis		1 (2%)		2 (3%)		1 (2%)
Femur, osteopetrosis						1 (2%)
Skeletal muscle	(1)	(5)	(3)		(6)	(5)
Atrophy	1 (100%)					
Hemorrhage		1 (20%)				
Nervous System						
Brain	(60)	(60)	(60)	(60)	(60)	(60)
Compression	6 (10%)	3 (5%)	2 (3%)	1 (2%)	2 (3%)	1 (2%)
Developmental malformation				1 (2%)		
Gliosis		1 (2%)				
Hemorrhage	1 (2%)				1 (2%)	
Hydrocephalus	3 (5%)	1 (2%)	1 (2%)	2 (3%)	2 (3%)	1 (2%)
Necrosis					1 (2%)	
Spinal cord	(2)	(4)	(1)	(1)	(3)	
Cyst epithelial inclusion					1 (33%)	
Respiratory System						
Lung	(60)	(60)	(60)	(60)	(60)	(60)
Congestion		1 (2%)			2 (3%)	1 (2%)
Hemorrhage	3 (5%)		1 (2%)		3 (5%)	4 (7%)
Infiltration cellular, histiocyte	25 (42%)	42 (70%)	40 (67%)	22 (37%)	23 (38%)	15 (25%)
Metaplasia, osseous	1 (2%)	1 (2%)	1 (2%)			
Alveolar epithelium, hyperplasia	2 (3%)	8 (13%)	3 (5%)	7 (12%)	15 (25%)	29 (48%)
Nose	(60)	(60)	(60)	(60)	(60)	(60)
Foreign body	9 (15%)	13 (22%)	10 (17%)	11 (18%)	14 (23%)	5 (8%)
Inflammation, chronic	9 (15%)	9 (15%)	8 (13%)	9 (15%)	10 (17%)	2 (3%)
Inflammation, suppurative			2 (3%)			
Respiratory epithelium, hyperplasia	6 (10%)	5 (8%)	7 (12%)	8 (13%)	7 (12%)	3 (5%)
Respiratory epithelium, metaplasia, squamous	2 (3%)	1 (2%)	1 (2%)	1 (2%)		
Special Senses System						
Eye	(2)	(1)	(2)	(1)		
Atrophy	1 (50%)					
Cataract		1 (100%)	1 (50%)	1 (100%)		
Inflammation, chronic	1 (50%)					
Retina, degeneration	1 (50%)	1 (100%)	1 (50%)	1 (100%)		
Lacrimal gland		(1)			(1)	
Cyst					1 (100%)	

TABLE A5
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm	2,000 ppm (Stop- Exposure)	5,000 ppm (Stop- Exposure)
2-Year Study (continued)						
Urinary System						
Kidney	(60)	(60)	(60)	(60)	(60)	(60)
Cyst		1 (2%)	3 (5%)	2 (3%)	1 (2%)	2 (3%)
Hydronephrosis						3 (5%)
Infarct				1 (2%)	1 (2%)	1 (2%)
Inflammation, chronic		1 (2%)			2 (3%)	4 (7%)
Mineralization			1 (2%)		1 (2%)	
Nephropathy	56 (93%)	59 (98%)	56 (93%)	56 (93%)	60 (100%)	60 (100%)
Renal tubule, degeneration, hyaline	3 (5%)	3 (5%)		1 (2%)	2 (3%)	1 (2%)
Renal tubule, dilatation	1 (2%)	3 (5%)		2 (3%)	2 (3%)	1 (2%)
Renal tubule, necrosis		1 (2%)	1 (2%)			2 (3%)
Renal tubule, pigmentation	5 (8%)	12 (20%)	14 (23%)	9 (15%)	18 (30%)	6 (10%)
Transitional epithelium, hyperplasia	1 (2%)		1 (2%)		2 (3%)	1 (2%)
Urinary bladder	(60)	(60)	(60)	(59)	(60)	(59)
Fibrosis			1 (2%)	2 (3%)		
Inflammation, chronic			2 (3%)			
Transitional epithelium, hyperplasia		2 (3%)	2 (3%)	3 (5%)	1 (2%)	
Transitional epithelium, inflammation, chronic				1 (2%)		

APPENDIX B
SUMMARY OF LESIONS IN FEMALE RATS
IN THE 2-YEAR FEED STUDY
OF *o*-NITROTOLUENE

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TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of o-Nitrotoluene^a

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Disposition Summary				
Animals initially in study	60	60	60	60
Early deaths				
Moribund	10	11	19	22
Natural deaths	3	2	2	5
Survivors				
Died last week of study	2	1		
Terminal sacrifice	45	46	39	33
Animals examined microscopically	60	60	60	60
Alimentary System				
Intestine large, colon	(58)	(59)	(60)	(59)
Polyp adenomatous		1 (2%)		1 (2%)
Intestine large, rectum	(60)	(60)	(60)	(58)
Polyp adenomatous		1 (2%)		
Intestine small, ileum	(57)	(59)	(59)	(58)
Liver	(60)	(59)	(60)	(60)
Carcinoma, metastatic, adrenal cortex		1 (2%)		
Hepatocellular adenoma	1 (2%)		1 (2%)	5 (8%)
Hepatocellular adenoma, multiple				1 (2%)
Sarcoma		1 (2%)		
Mesentery	(9)	(1)	(3)	(3)
Lipoma				2 (67%)
Pancreas	(60)	(59)	(60)	(60)
Acinus, adenoma		1 (2%)	1 (2%)	
Salivary glands	(60)	(60)	(60)	(60)
Stomach, forestomach	(59)	(59)	(60)	(60)
Squamous cell papilloma			1 (2%)	
Tongue			(1)	(1)
Squamous cell carcinoma				1 (100%)
Squamous cell papilloma			1 (100%)	
Cardiovascular System				
Heart	(60)	(60)	(60)	(60)
Schwannoma malignant				1 (2%)
Endocrine System				
Adrenal cortex	(60)	(60)	(60)	(59)
Adenoma	2 (3%)			1 (2%)
Carcinoma		1 (2%)		
Adrenal medulla	(60)	(60)	(60)	(58)
Pheochromocytoma benign	2 (3%)			1 (2%)
Islets, pancreatic	(60)	(59)	(60)	(60)
Adenoma	1 (2%)	1 (2%)	1 (2%)	
Carcinoma			1 (2%)	
Pituitary gland	(59)	(60)	(60)	(60)
Pars distalis, adenoma	19 (32%)	30 (50%)	26 (43%)	20 (33%)
Pars distalis, adenoma, multiple		2 (3%)		
Pars intermedia, adenoma	1 (2%)	1 (2%)		

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Endocrine System (continued)				
Thyroid gland	(60)	(60)	(60)	(60)
Bilateral, C-cell, adenoma		1 (2%)	1 (2%)	
C-cell, adenoma	9 (15%)	5 (8%)	2 (3%)	5 (8%)
C-cell, carcinoma	1 (2%)	3 (5%)	3 (5%)	1 (2%)
Follicular cell, adenoma			1 (2%)	
Follicular cell, carcinoma			1 (2%)	1 (2%)
General Body System				
None				
Genital System				
Clitoral gland	(59)	(57)	(54)	(53)
Adenoma	12 (20%)	8 (14%)	3 (6%)	3 (6%)
Carcinoma	2 (3%)	4 (7%)	3 (6%)	
Bilateral, adenoma		1 (2%)		
Ovary	(60)	(59)	(60)	(60)
Granulosa cell tumor malignant				1 (2%)
Granulosa-theca tumor malignant		1 (2%)		
Uterus	(60)	(59)	(60)	(60)
Hemangioma				1 (2%)
Polyp stromal	11 (18%)	7 (12%)	15 (25%)	16 (27%)
Sarcoma stromal		1 (2%)		1 (2%)
Vagina	(2)	(1)	(3)	(6)
Fibroma			1 (33%)	
Granular cell tumor benign				1 (17%)
Hemangioma				1 (17%)
Leiomyoma	1 (50%)			
Leiomyosarcoma				1 (17%)
Squamous cell papilloma			1 (33%)	1 (17%)
Hematopoietic System				
Bone marrow	(60)	(60)	(60)	(60)
Lymph node	(8)	(4)	(4)	(4)
Deep cervical, carcinoma, metastatic, thyroid gland			1 (25%)	
Lymph node, mandibular	(60)	(60)	(59)	(59)
Lymph node, mesenteric	(59)	(58)	(59)	(59)
Spleen	(60)	(59)	(60)	(59)
Carcinoma, metastatic, adrenal cortex		1 (2%)		
Thymus	(57)	(55)	(58)	(58)
Integumentary System				
Mammary gland	(60)	(60)	(59)	(60)
Adenoma				1 (2%)
Carcinoma			1 (2%)	1 (2%)
Fibroadenoma	17 (28%)	12 (20%)	13 (22%)	14 (23%)
Fibroadenoma, multiple	6 (10%)	35 (58%)	39 (66%)	42 (70%)

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Integumentary System (continued)				
Skin	(60)	(60)	(60)	(60)
Basal cell adenoma		1 (2%)		
Pinna, melanoma malignant		1 (2%)		
Pinna, neural crest tumor				1 (2%)
Sebaceous gland, adenoma				1 (2%)
Subcutaneous tissue, fibroma	3 (5%)	3 (5%)	18 (30%)	19 (32%)
Subcutaneous tissue, fibroma, multiple				1 (2%)
Subcutaneous tissue, fibrosarcoma			4 (7%)	2 (3%)
Subcutaneous tissue, fibrosarcoma, multiple				1 (2%)
Subcutaneous tissue, hemangiosarcoma			1 (2%)	
Subcutaneous tissue, neural crest tumor			1 (2%)	
Musculoskeletal System				
Skeletal muscle	(1)	(1)	(1)	(1)
Lipoma	1 (100%)			
Rhabdomyosarcoma			1 (100%)	
Nervous System				
Brain	(60)	(60)	(60)	(60)
Astrocytoma malignant				1 (2%)
Respiratory System				
Lung	(60)	(60)	(60)	(60)
Alveolar/bronchiolar adenoma	1 (2%)	1 (2%)		3 (5%)
Alveolar/bronchiolar adenoma, multiple		1 (2%)		
Alveolar/bronchiolar carcinoma				1 (2%)
Sarcoma, metastatic, liver		1 (2%)		
Special Senses System				
Zymbal's gland	(2)	(1)		(3)
Adenoma	1 (50%)			1 (33%)
Carcinoma	1 (50%)	1 (100%)		2 (67%)
Urinary System				
Kidney	(60)	(59)	(60)	(60)
Urinary bladder	(60)	(59)	(60)	(60)
Papilloma			1 (2%)	1 (2%)
Squamous cell papilloma, multiple				1 (2%)
Systemic Lesions				
Multiple organs ^b	(60)	(60)	(60)	(60)
Leukemia mononuclear	21 (35%)	6 (10%)	4 (7%)	5 (8%)
Lymphoma malignant			1 (2%)	1 (2%)

TABLE B1
Summary of the Incidence of Neoplasms in Female Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Neoplasm Summary				
Total animals with primary neoplasms ^c	54	58	60	60
Total primary neoplasms	113	131	147	165
Total animals with benign neoplasms	47	57	59	59
Total benign neoplasms	88	112	126	143
Total animals with malignant neoplasms	24	16	18	20
Total malignant neoplasms	25	19	20	21
Total animals with metastatic neoplasms		2	1	
Total metastatic neoplasms		3	1	
Total animals with uncertain neoplasms- benign or malignant			1	1
Total uncertain neoplasms			1	1

^a Number of animals examined microscopically at the site and the number of animals with neoplasm
^b Number of animals with any tissue examined microscopically
^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE B3
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Clitoral Gland: Adenoma				
Overall rate ^a	12/59 (20%)	9/57 (16%)	3/54 (6%)	3/53 (6%)
Adjusted rate ^b	21.5%	17.0%	6.2%	6.3%
Terminal rate ^c	11/46 (24%)	9/45 (20%)	3/36 (8%)	3/32 (9%)
First incidence (days) ^d	726	729 (T)	729 (T)	729 (T)
Poly-3 test	P=0.005N	P=0.366N	P=0.025N	P=0.027N
Clitoral Gland: Carcinoma				
Overall rate	2/59 (3%)	4/57 (7%)	3/54 (6%)	0/53 (0%)
Adjusted rate	3.6%	7.5%	6.2%	0.0%
Terminal rate	1/46 (2%)	2/45 (4%)	2/36 (6%)	0/32 (0%)
First incidence (days)	719	505	706	— ^e
Poly-3 test	P=0.229N	P=0.319	P=0.434	P=0.275N
Clitoral Gland: Adenoma or Carcinoma				
Overall rate	14/59 (24%)	13/57 (23%)	6/54 (11%)	3/53 (6%)
Adjusted rate	25.0%	24.3%	12.4%	6.3%
Terminal rate	12/46 (26%)	11/45 (24%)	5/36 (14%)	3/32 (9%)
First incidence (days)	719	505	706	729 (T)
Poly-3 test	P=0.003N	P=0.550N	P=0.083N	P=0.009N
Liver: Hepatocellular Adenoma				
Overall rate	1/60 (2%)	0/59 (0%)	1/60 (2%)	6/60 (10%)
Adjusted rate	1.8%	0.0%	1.9%	11.2%
Terminal rate	1/47 (2%)	0/47 (0%)	1/39 (3%)	4/33 (12%)
First incidence (days)	729 (T)	—	729 (T)	687
Poly-3 test	P=0.005	P=0.507N	P=0.748	P=0.048
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	1/60 (2%)	2/60 (3%)	0/60 (0%)	3/60 (5%)
Adjusted rate	1.8%	3.6%	0.0%	5.6%
Terminal rate	1/47 (2%)	1/47 (2%)	0/39 (0%)	3/33 (9%)
First incidence (days)	729 (T)	705	—	729 (T)
Poly-3 test	P=0.258	P=0.493	P=0.511N	P=0.283
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	1/60 (2%)	2/60 (3%)	0/60 (0%)	4/60 (7%)
Adjusted rate	1.8%	3.6%	0.0%	7.5%
Terminal rate	1/47 (2%)	1/47 (2%)	0/39 (0%)	4/33 (12%)
First incidence (days)	729 (T)	705	—	729 (T)
Poly-3 test	P=0.126	P=0.493	P=0.511N	P=0.161
Mammary Gland: Fibroadenoma				
Overall rate	23/60 (38%)	47/60 (78%)	52/60 (87%)	56/60 (93%)
Adjusted rate	40.0%	82.0%	91.7%	96.2%
Terminal rate	18/47 (38%)	39/47 (83%)	36/39 (92%)	33/33 (100%)
First incidence (days)	620	505	525	464
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
Mammary Gland: Fibroadenoma or Carcinoma				
Overall rate	23/60 (38%)	47/60 (78%)	52/60 (87%)	57/60 (95%) ^f
Adjusted rate	40.0%	82.0%	91.7%	97.1%
Terminal rate	18/47 (38%)	39/47 (83%)	36/39 (92%)	33/33 (100%)
First incidence (days)	620	505	525	464
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001

TABLE B3
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Pituitary Gland (Pars Distalis): Adenoma				
Overall rate	19/59 (32%)	32/60 (53%)	26/60 (43%)	20/60 (33%)
Adjusted rate	33.8%	55.7%	47.4%	36.2%
Terminal rate	16/46 (35%)	25/47 (53%)	19/39 (49%)	10/33 (30%)
First incidence	691	531	636	569
Poly-3 test	P=0.484N	P=0.014	P=0.100	P=0.473
Skin (Subcutaneous Tissue): Fibroma				
Overall rate	3/60 (5%)	3/60 (5%)	18/60 (30%)	20/60 (33%)
Adjusted rate	5.3%	5.4%	32.7%	36.9%
Terminal rate	3/47 (6%)	3/47 (6%)	15/39 (39%)	14/33 (42%)
First incidence (days)	729 (T)	729 (T)	432	663
Poly-3 test	P<0.001	P=0.652	P<0.001	P<0.001
Skin (Subcutaneous Tissue): Fibrosarcoma				
Overall rate	0/60 (0%)	0/60 (0%)	4/60 (7%)	3/60 (5%)
Adjusted rate	0.0%	0.0%	7.3%	5.6%
Terminal rate	0/47 (0%)	0/47 (0%)	2/39 (5%)	3/33 (9%)
First incidence (days)	—	— ^g	569	729 (T)
Poly-3 test	P=0.018	— ^g	P=0.056	P=0.109
Skin (Subcutaneous Tissue): Fibroma or Fibrosarcoma				
Overall rate	3/60 (5%)	3/60 (5%)	21/60 (35%)	22/60 (37%)
Adjusted rate	5.3%	5.4%	37.6%	40.6%
Terminal rate	3/47 (6%)	3/47 (6%)	16/39 (41%)	16/33 (49%)
First incidence (days)	729 (T)	729 (T)	432	663
Poly-3 test	P<0.001	P=0.652	P<0.001	P<0.001
Thyroid Gland (C-Cell): Adenoma				
Overall rate	9/60 (15%)	6/60 (10%)	3/60 (5%)	5/60 (8%)
Adjusted rate	15.8%	10.6%	5.4%	9.3%
Terminal rate	9/47 (19%)	5/47 (11%)	0/39 (0%)	3/33 (9%)
First incidence (days)	729 (T)	495	370	663
Poly-3 test	P=0.109N	P=0.296N	P=0.068N	P=0.227N
Thyroid Gland (C-Cell): Carcinoma				
Overall rate	1/60 (2%)	3/60 (5%)	3/60 (5%)	1/60 (2%)
Adjusted rate	1.8%	5.4%	5.6%	1.9%
Terminal rate	1/47 (2%)	3/47 (6%)	3/39 (8%)	0/33 (0%)
First incidence (days)	729 (T)	729 (T)	729 (T)	708
Poly-3 test	P=0.556	P=0.299	P=0.286	P=0.746
Thyroid Gland (C-Cell): Adenoma or Carcinoma				
Overall rate	10/60 (17%)	9/60 (15%)	6/60 (10%)	6/60 (10%)
Adjusted rate	17.6%	16.0%	10.8%	11.1%
Terminal rate	10/47 (21%)	8/47 (17%)	3/39 (8%)	3/33 (9%)
First incidence (days)	729 (T)	495	370	663
Poly-3 test	P=0.142N	P=0.508N	P=0.226N	P=0.244N
Uterus: Stromal Polyp				
Overall rate	11/60 (18%)	7/60 (12%)	15/60 (25%)	16/60 (27%)
Adjusted rate	19.3%	12.5%	27.5%	29.2%
Terminal rate	11/47 (23%)	6/47 (13%)	13/39 (33%)	9/33 (27%)
First incidence (days)	729 (T)	582	523	586
Poly-3 test	P=0.043	P=0.229N	P=0.212	P=0.159

TABLE B3
Statistical Analysis of Primary Neoplasms in Female Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Uterus: Stromal Polyp or Stromal Sarcoma				
Overall rate	11/60 (18%)	8/60 (13%)	15/60 (25%)	17/60 (28%)
Adjusted rate	19.3%	14.2%	27.5%	31.0%
Terminal rate	11/47 (23%)	7/47 (15%)	13/39 (33%)	10/33 (30%)
First incidence (days)	729 (T)	582	523	586
Poly-3 test	P=0.032	P=0.318N	P=0.212	P=0.113
Zymbal's Gland: Adenoma or Carcinoma				
Overall rate	2/60 (3%)	1/60 (2%)	0/60 (0%)	3/60 (5%)
Adjusted rate	3.5%	1.8%	0.0%	5.6%
Terminal rate	1/47 (2%)	1/47 (2%)	0/39 (0%)	2/33 (6%)
First incidence (days)	719	729 (T)	—	632
Poly-3 test	P=0.388	P=0.507N	P=0.251N	P=0.474
All Organs: Mononuclear Cell Leukemia				
Overall rate	21/60 (35%)	6/60 (10%)	4/60 (7%)	5/60 (8%)
Adjusted rate	35.6%	10.6%	7.3%	9.1%
Terminal rate	11/47 (23%)	3/47 (6%)	1/39 (3%)	1/33 (3%)
First incidence (days)	453	429	523	488
Poly-3 test	P=0.001N	P=0.001N	P=0.001N	P=0.001N
All Organs: Benign Neoplasms				
Overall rate	47/60 (78%)	57/60 (95%)	59/60 (98%)	59/60 (98%)
Adjusted rate	81.5%	96.3%	99.2%	99.5%
Terminal rate	41/47 (87%)	45/47 (96%)	39/39 (100%)	33/33 (100%)
First incidence (days)	620	495	370	464
Poly-3 test	P<0.001	P=0.008	P<0.001	P<0.001
All Organs: Malignant Neoplasms				
Overall rate	24/60 (40%)	16/60 (27%)	18/60 (30%)	20/60 (33%)
Adjusted rate	40.6%	27.9%	32.1%	35.5%
Terminal rate	13/47 (28%)	12/47 (26%)	11/39 (28%)	10/33 (30%)
First incidence (days)	453	429	523	488
Poly-3 test	P=0.369N	P=0.104N	P=0.224N	P=0.353N
All Organs: Benign or Malignant Neoplasms				
Overall rate	54/60 (90%)	58/60 (97%)	60/60 (100%)	60/60 (100%)
Adjusted rate	91.3%	96.7%	100.0%	100.0%
Terminal rate	42/47 (89%)	45/47 (96%)	39/39 (100%)	33/33 (100%)
First incidence (days)	453	429	370	464
Poly-3 test	P=0.003	P=0.195	P=0.026	P=0.026

(T) Terminal sacrifice

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for clitoral gland, liver, lung, pituitary gland, and thyroid gland; for other tissues, denominator is number of animals necropsied.

^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by N.

^e Not applicable; no neoplasms in animal group

^f A single adenoma occurred in an animal that also had a fibroadenoma.

^g Value of statistic cannot be computed.

TABLE B4a
Historical Incidence of Subcutaneous Skin Neoplasms in Control Female F344/N Rats

Study	Incidence in Controls		
	Fibroma	Fibrosarcoma	Fibroma or Fibrosarcoma
Historical Incidence in Controls Given NTP-2000 Diet^a			
Citral (feed)	1/100	0/100	1/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	0/50	1/50	1/50
Indium phosphide (inhalation)	3/50	0/50	3/50
60-Hz Magnetic fields (whole body exposure)	4/100	1/100	5/100
Methacrylonitrile (gavage)	0/50	0/50	0/50
Naphthalene (inhalation)	0/49	0/49	0/49
<i>o</i> -Nitrotoluene (feed)	3/60	0/60	3/60
<i>p</i> -Nitrotoluene (feed)	0/50	1/50	1/50
Riddelliine (gavage)	1/50	1/50	2/50
Sodium nitrite (drinking water)	1/50	0/50	1/50
Vanadium pentoxide (inhalation)	1/50	0/50	1/50
Overall Historical Incidence in Controls Given NTP-2000 Diet			
Total (%)	14/659 (2.1%)	4/659 (0.6%)	18/659 (2.7%)
Mean ± standard deviation	2.0% ± 2.1%	0.6% ± 0.9%	2.6% ± 2.1%
Range	0%-6%	0%-2%	0%-6%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b			
Benzyl acetate	1/50	0/50	1/50
2,2-Bis(bromomethyl)-1,3-propanediol	1/50	0/50	1/50
Butyl benzyl phthalate	1/50	0/50	1/50
D&C Yellow No. 11	1/50	0/50	1/50
Emodin	2/50	0/50	2/50
<i>o</i> -Nitroanisole	0/50	0/50	0/50
<i>p</i> -Nitrobenzoic acid	2/50	0/50	2/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet			
Total (%)	15/1,001 (1.5%)	4/1,001 (0.4%)	19/1,001 (1.9%)
Mean ± standard deviation	1.5% ± 1.6%	0.4% ± 0.8%	1.9% ± 2.0%
Range	0%-4%	0%-2%	0%-6%

^a Data as of January 18, 2001
^b Data as of December 21, 1999

TABLE B4b
Historical Incidence of Mammary Gland Neoplasms in Control Female F344/N Rats

Study	Incidence in Controls		
	Fibroadenoma	Carcinoma	Fibroadenoma or Carcinoma
Historical Incidence in Controls Given NTP-2000 Diet^a			
Citral (feed)	53/100	0/100	53/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	15/50	1/50	15/50
Indium phosphide (inhalation)	20/50	0/50	20/50
60-Hz Magnetic fields (whole body exposure)	56/100	2/100	58/100
Methacrylonitrile (gavage)	21/50	3/50	23/50
Naphthalene (inhalation)	17/49	3/49	18/49
<i>o</i> -Nitrotoluene (feed)	23/60	0/60	23/60
<i>p</i> -Nitrotoluene (feed)	14/50	1/50	14/50
Riddelliine (gavage)	28/50	2/50	30/50
Sodium nitrite (drinking water)	21/50	1/50	22/50
Vanadium pentoxide (inhalation)	16/50	2/50	18/50
Overall Historical Incidence in Controls Given NTP-2000 Diet			
Total (%)	284/659 (43.1%)	15/659 (2.3%)	294/659 (44.6%)
Mean ± standard deviation	41.1% ± 10.1%	2.6% ± 2.2%	42.9% ± 10.7%
Range	28%-56%	0% ± 6%	28%-60%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b			
Benzyl acetate	12/50	0/50	12/50
2,2-Bis(bromomethyl)-1,3-propanediol	25/50	4/50	27/50
Butyl benzyl phthalate	28/50	2/50	29/50
D&C Yellow No. 11	21/50	4/50	24/50
Emodin	23/50	0/50	23/50
<i>o</i> -Nitroanisole	17/50	2/50	18/50
<i>p</i> -Nitrobenzoic acid	22/50	2/50	24/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet			
Total (%)	431/1,001 (43.1%)	32/1,001 (3.2%)	453/1,001 (45.3%)
Mean ± standard deviation	43.1% ± 10.7%	3.2% ± 2.6%	45.3% ± 11.4%
Range	24%-60%	0%-8%	24%-62%

^a Data as of January 18, 2001
^b Data as of December 21, 1999

TABLE B4c
Historical Incidence of Hepatocellular Adenoma in Control Female F344/N Rats

Study	Incidence in Controls
Historical Incidence in Controls Given NTP-2000 Diet^a	
Citral (feed)	0/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	0/50
Indium phosphide (inhalation)	0/50
60-Hz Magnetic fields (whole body exposure)	0/100
Methacrylonitrile (gavage)	1/50
Naphthalene (inhalation)	0/49
<i>o</i> -Nitrotoluene (feed)	1/60
<i>p</i> -Nitrotoluene (feed)	0/50
Riddelliine (gavage)	1/50
Sodium nitrite (drinking water)	0/50
Vanadium pentoxide (inhalation)	1/50
Overall Historical Incidence in Controls Given NTP-2000 Diet	
Total (%)	4/659 (0.6%)
Mean ± standard deviation	0.7% ± 1.0%
Range	0%-2%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b	
Benzyl acetate	1/50
2,2-Bis(bromomethyl)-1,3-propanediol	0/50
Butyl benzyl phthalate	0/50
D&C Yellow No. 11	0/50
Emodin	0/49
<i>o</i> -Nitroanisole	0/50
<i>p</i> -Nitrobenzoic acid	2/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet	
Total (%)	4/1,000 (0.4%)
Mean ± standard deviation	0.4% ± 1.1%
Range	0%-4%

^a Data as of January 18, 2001
^b Data as of December 21, 1999

TABLE B4d
Historical Incidence of Mononuclear Cell Leukemia in Control Female F344/N Rats

Study	Incidence in Controls
Historical Incidence in Controls Given NTP-2000 Diet^a	
Citral (feed)	24/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	8/50
Indium phosphide (inhalation)	14/50
60-Hz Magnetic fields (whole body exposure)	20/100
Methacrylonitrile (gavage)	21/50
Naphthalene (inhalation)	16/49
<i>o</i> -Nitrotoluene (feed)	21/60
<i>p</i> -Nitrotoluene (feed)	13/50
Riddelliine (gavage)	12/50
Sodium nitrite (drinking water)	15/50
Vanadium pentoxide (inhalation)	21/50
Overall Historical Incidence in Controls Given NTP-2000 Diet	
Total (%)	185/659 (28.1%)
Mean ± standard deviation	29.1% ± 8.4%
Range	16%-42%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b	
Benzyl acetate	9/50
2,2-Bis(bromomethyl)-1,3-propanediol	15/50
Butyl benzyl phthalate	21/50
D&C Yellow No. 11	16/50
Emodin	14/50
<i>o</i> -Nitroanisole	14/50
<i>p</i> -Nitrobenzoic acid	17/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet	
Total (%)	293/1,001 (29.3%)
Mean ± standard deviation	29.3% ± 7.6%
Range	16%-42%

^a Data as of January 18, 2001; includes data for lymphocytic, monocytic, and undifferentiated leukemia

^b Data as of December 21, 1999; includes data for lymphocytic, monocytic, and undifferentiated leukemia

TABLE B5
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of o-Nitrotoluene^a

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Disposition Summary				
Animals initially in study	60	60	60	60
Early deaths				
Moribund	10	11	19	22
Natural deaths	3	2	2	5
Survivors				
Died last week of study	2	1		
Terminal sacrifice	45	46	39	33
Animals examined microscopically	60	60	60	60
Alimentary System				
Intestine large, colon	(58)	(59)	(60)	(59)
Crypt, diverticulum	1 (2%)			
Intestine large, rectum	(60)	(60)	(60)	(58)
Fibrosis			1 (2%)	
Intestine large, cecum	(58)	(59)	(60)	(59)
Edema		1 (2%)	1 (2%)	
Liver	(60)	(59)	(60)	(60)
Angiectasis	2 (3%)	3 (5%)	4 (7%)	4 (7%)
Basophilic focus	51 (85%)	56 (95%)	60 (100%)	54 (90%)
Clear cell focus	16 (27%)	30 (51%)	28 (47%)	33 (55%)
Degeneration, cystic			1 (2%)	2 (3%)
Eosinophilic focus	5 (8%)	12 (20%)	25 (42%)	32 (53%)
Hematopoietic cell proliferation		2 (3%)	1 (2%)	3 (5%)
Hemorrhage		1 (2%)	1 (2%)	
Hepatodiaphragmatic nodule	9 (15%)	13 (22%)	10 (17%)	7 (12%)
Infiltration cellular, mixed cell	15 (25%)	11 (19%)	6 (10%)	12 (20%)
Mixed cell focus	6 (10%)	9 (15%)	11 (18%)	28 (47%)
Necrosis, focal		1 (2%)	3 (5%)	5 (8%)
Regeneration, diffuse				1 (2%)
Regeneration, focal	1 (2%)	2 (3%)		
Bile duct, hyperplasia	7 (12%)	2 (3%)		
Centrilobular, necrosis	3 (5%)		2 (3%)	2 (3%)
Hepatocyte, vacuolization cytoplasmic	4 (7%)	2 (3%)	1 (2%)	
Kupffer cell, pigmentation	3 (5%)			
Mesentery	(9)	(1)	(3)	(3)
Accessory spleen	2 (22%)		1 (33%)	1 (33%)
Fat, necrosis	7 (78%)	1 (100%)	2 (67%)	
Pancreas	(60)	(59)	(60)	(60)
Atrophy	13 (22%)	10 (17%)	7 (12%)	4 (7%)
Acinus, hyperplasia, focal	1 (2%)	1 (2%)	2 (3%)	2 (3%)
Salivary glands	(60)	(60)	(60)	(60)
Atrophy	2 (3%)	3 (5%)	9 (15%)	48 (80%)
Stomach, forestomach	(59)	(59)	(60)	(60)
Erosion	1 (2%)		1 (2%)	
Perforation		1 (2%)		
Ulcer	1 (2%)	1 (2%)		1 (2%)
Epithelium, hyperplasia	5 (8%)	3 (5%)	1 (2%)	
Stomach, glandular	(60)	(59)	(60)	(60)
Erosion	1 (2%)	1 (2%)	1 (2%)	3 (5%)
Ulcer	2 (3%)		1 (2%)	1 (2%)
Tooth	(2)			
Inflammation, chronic	1 (50%)			
Malformation	2 (100%)			

^a Number of animals examined microscopically at the site and the number of animals with lesion

TABLE B5
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Cardiovascular System				
Heart	(60)	(60)	(60)	(60)
Cardiomyopathy	22 (37%)	16 (27%)	13 (22%)	13 (22%)
Thrombosis			1 (2%)	
Myocardium, fibrosis	1 (2%)	1 (2%)		1 (2%)
Endocrine System				
Adrenal cortex	(60)	(60)	(60)	(59)
Accessory adrenal cortical nodule	8 (13%)	10 (17%)	8 (13%)	10 (17%)
Degeneration, fatty	13 (22%)	11 (18%)	17 (28%)	11 (19%)
Hyperplasia, diffuse	3 (5%)	1 (2%)		1 (2%)
Hyperplasia, focal	9 (15%)	6 (10%)	14 (23%)	15 (25%)
Hypertrophy, focal	6 (10%)	7 (12%)	6 (10%)	7 (12%)
Necrosis	1 (2%)			
Adrenal medulla	(60)	(60)	(60)	(58)
Hyperplasia	8 (13%)	2 (3%)	3 (5%)	3 (5%)
Pituitary gland	(59)	(60)	(60)	(60)
Pars distalis, angiectasis	6 (10%)	5 (8%)	4 (7%)	4 (7%)
Pars distalis, cyst	22 (37%)	20 (33%)	22 (37%)	24 (40%)
Pars distalis, hyperplasia, focal	12 (20%)	6 (10%)	12 (20%)	9 (15%)
Pars intermedia, angiectasis		2 (3%)	1 (2%)	
Pars intermedia, cyst	3 (5%)	1 (2%)	1 (2%)	1 (2%)
Thyroid gland	(60)	(60)	(60)	(60)
Ultimobranchial cyst	1 (2%)	1 (2%)	1 (2%)	
C-cell, hyperplasia	22 (37%)	24 (40%)	22 (37%)	9 (15%)
Follicle, cyst		2 (3%)	1 (2%)	1 (2%)
Follicular cell, hyperplasia		1 (2%)		
General Body System				
None				
Genital System				
Clitoral gland	(59)	(57)	(54)	(53)
Atrophy	1 (2%)	3 (5%)	6 (11%)	25 (47%)
Cyst	2 (3%)	4 (7%)	4 (7%)	2 (4%)
Hyperplasia	5 (8%)	1 (2%)	4 (7%)	1 (2%)
Inflammation, chronic	4 (7%)	4 (7%)	6 (11%)	5 (9%)
Ovary	(60)	(59)	(60)	(60)
Angiectasis				2 (3%)
Cyst	9 (15%)	8 (14%)	11 (18%)	6 (10%)
Uterus	(60)	(59)	(60)	(60)
Angiectasis	1 (2%)			
Cyst				1 (2%)
Hydrometra	7 (12%)	6 (10%)	1 (2%)	3 (5%)
Hyperplasia, cystic	8 (13%)	6 (10%)	9 (15%)	8 (13%)
Inflammation, chronic	2 (3%)	1 (2%)	1 (2%)	
Epithelium, hyperplasia				1 (2%)

TABLE B5
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Hematopoietic System				
Bone marrow	(60)	(60)	(60)	(60)
Hyperplasia	2 (3%)	7 (12%)	15 (25%)	24 (40%)
Infiltration cellular, histiocyte	2 (3%)	1 (2%)	1 (2%)	
Myelofibrosis	1 (2%)			2 (3%)
Lymph node	(8)	(4)	(4)	(4)
Mediastinal, hemorrhage	1 (13%)	1 (25%)	1 (25%)	3 (75%)
Mediastinal, pigmentation	6 (75%)	1 (25%)	1 (25%)	3 (75%)
Renal, hemorrhage				1 (25%)
Renal, pigmentation				1 (25%)
Lymph node, mandibular	(60)	(60)	(59)	(59)
Ectasia	1 (2%)	3 (5%)		4 (7%)
Hemorrhage	4 (7%)	4 (7%)	1 (2%)	4 (7%)
Hyperplasia, lymphoid	3 (5%)	5 (8%)	6 (10%)	15 (25%)
Pigmentation	31 (52%)	33 (55%)	30 (51%)	30 (51%)
Lymph node, mesenteric	(59)	(58)	(59)	(59)
Hemorrhage	5 (8%)	3 (5%)	4 (7%)	5 (8%)
Hyperplasia, lymphoid				1 (2%)
Pigmentation		4 (7%)	1 (2%)	3 (5%)
Spleen	(60)	(59)	(60)	(59)
Congestion		1 (2%)		
Fibrosis	1 (2%)		1 (2%)	
Hematopoietic cell proliferation	22 (37%)	38 (64%)	48 (80%)	48 (81%)
Necrosis	2 (3%)			
Pigmentation	36 (60%)	44 (75%)	43 (72%)	46 (78%)
Lymphoid follicle, atrophy		1 (2%)		1 (2%)
Thymus	(57)	(55)	(58)	(58)
Cyst	1 (2%)		2 (3%)	2 (3%)
Integumentary System				
Mammary gland	(60)	(60)	(59)	(60)
Dilatation	40 (67%)	30 (50%)	29 (49%)	24 (40%)
Galactocele		1 (2%)		
Hyperplasia	14 (23%)	36 (60%)	30 (51%)	19 (32%)
Skin	(59)	(60)	(60)	(60)
Angiectasis			1 (2%)	
Ulcer	1 (2%)			
Subcutaneous tissue, edema			1 (2%)	
Musculoskeletal System				
Bone	(60)	(60)	(60)	(60)
Cranium, osteopetrosis	2 (3%)	6 (10%)	6 (10%)	10 (17%)
Femur, osteopetrosis	8 (13%)	9 (15%)	13 (22%)	16 (27%)
Nervous System				
Brain	(60)	(60)	(60)	(60)
Compression	6 (10%)	10 (17%)	5 (8%)	3 (5%)
Hydrocephalus	2 (3%)	4 (7%)		3 (5%)

TABLE B5
Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Respiratory System				
Lung	(60)	(60)	(60)	(60)
Hemorrhage	2 (3%)	2 (3%)	2 (3%)	1 (2%)
Infiltration cellular, histiocyte	52 (87%)	54 (90%)	53 (88%)	55 (92%)
Alveolar epithelium, hyperplasia	6 (10%)	14 (23%)	16 (27%)	9 (15%)
Alveolar epithelium, hyperplasia, atypical				1 (2%)
Nose	(60)	(60)	(60)	(60)
Foreign body	2 (3%)			7 (12%)
Inflammation, chronic	2 (3%)	1 (2%)	2 (3%)	8 (13%)
Respiratory epithelium, hyperplasia	2 (3%)			3 (5%)
Respiratory epithelium, metaplasia, squamous			1 (2%)	1 (2%)
Special Senses System				
Eye	(3)	(3)	(2)	(1)
Cataract	1 (33%)	2 (67%)	1 (50%)	1 (100%)
Hemorrhage	1 (33%)			
Inflammation, chronic	1 (33%)	1 (33%)		
Retina, degeneration	2 (67%)	2 (67%)	1 (50%)	1 (100%)
Urinary System				
Kidney	(60)	(59)	(60)	(60)
Cyst		1 (2%)	1 (2%)	
Hydronephrosis			1 (2%)	
Infarct	2 (3%)		1 (2%)	
Inflammation, chronic	2 (3%)	1 (2%)	1 (2%)	1 (2%)
Nephropathy	53 (88%)	45 (76%)	48 (80%)	52 (87%)
Renal tubule, cytoplasmic alteration		1 (2%)	1 (2%)	
Renal tubule, degeneration, hyaline	9 (15%)	10 (17%)	4 (7%)	11 (18%)
Renal tubule, necrosis	2 (3%)			
Renal tubule, pigmentation	9 (15%)	10 (17%)	7 (12%)	17 (28%)
Transitional epithelium, hyperplasia	1 (2%)		1 (2%)	1 (2%)
Urinary bladder	(60)	(59)	(60)	(60)
Transitional epithelium, hyperplasia			1 (2%)	

APPENDIX C
SUMMARY OF LESIONS IN MALE MICE
IN THE 2-YEAR FEED STUDY
OF *o*-NITROTOLUENE

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TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Feed Study of o-Nitrotoluene^a

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Disposition Summary				
Animals initially in study	60	60	60	60
Early deaths				
Moribund	3	5	21	31
Natural deaths	5	21	39	29
Survivors				
Terminal sacrifice	52	34		
Animals examined microscopically	60	60	60	60
Alimentary System				
Gallbladder	(54)	(42)	(31)	(35)
Intestine large, colon	(56)	(52)	(46)	(54)
Muscularis, serosa, hemangiosarcoma			1 (2%)	
Intestine large, cecum	(56)	(49)	(36)	(44)
Carcinoma		12 (24%)	9 (25%)	
Intestine small, duodenum	(55)	(45)	(40)	(42)
Hemangiosarcoma	1 (2%)			
Intestine small, jejunum	(56)	(43)	(35)	(40)
Adenocarcinoma	1 (2%)			
Carcinoma	1 (2%)			
Intestine small, ileum	(55)	(40)	(35)	(39)
Carcinoma		1 (3%)		
Liver	(60)	(59)	(57)	(60)
Hemangiosarcoma	1 (2%)	1 (2%)	1 (2%)	
Hemangiosarcoma, multiple		3 (5%)		
Hemangiosarcoma, metastatic, skeletal muscle			1 (2%)	
Hepatoblastoma	1 (2%)	1 (2%)		
Hepatocellular carcinoma	9 (15%)	9 (15%)	4 (7%)	2 (3%)
Hepatocellular carcinoma, multiple	3 (5%)	7 (12%)	1 (2%)	
Hepatocellular adenoma	15 (25%)	12 (20%)	1 (2%)	
Hepatocellular adenoma, multiple	3 (5%)	6 (10%)	2 (4%)	
Histiocytic sarcoma		1 (2%)		
Mesentery	(12)	(12)	(42)	(38)
Carcinoma, metastatic, intestine large, cecum		1 (8%)	1 (2%)	
Hemangiosarcoma		8 (67%)	30 (71%)	24 (63%)
Hemangiosarcoma, multiple			8 (19%)	14 (37%)
Hemangiosarcoma, metastatic, skeletal muscle			1 (2%)	
Oral mucosa		(1)		
Pharyngeal, squamous cell carcinoma		1 (100%)		
Pancreas	(59)	(59)	(59)	(58)
Hemangiosarcoma				1 (2%)
Acinus, hemangiosarcoma			1 (2%)	
Salivary glands	(60)	(60)	(60)	(60)
Stomach, forestomach	(60)	(60)	(58)	(60)
Squamous cell papilloma				2 (3%)
Stomach, glandular	(57)	(54)	(53)	(55)
Cardiovascular System				
Heart	(60)	(60)	(60)	(60)
Hemangiosarcoma		2 (3%)		2 (3%)

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Endocrine System				
Adrenal cortex	(60)	(60)	(58)	(60)
Adenoma	1 (2%)			
Capsule, adenoma	1 (2%)			
Pituitary gland	(53)	(50)	(50)	(46)
Pars distalis, adenoma	1 (2%)			
Thyroid gland	(59)	(59)	(59)	(59)
C-cell, carcinoma		1 (2%)		
Follicular cell, adenoma	1 (2%)			
General Body System				
Tissue NOS	(2)		(2)	(3)
Hemangiosarcoma				1 (33%)
Pelvic, hemangiosarcoma			1 (50%)	1 (33%)
Thoracic, sarcoma	1 (50%)			
Thoracic, sarcoma, multiple	1 (50%)			
Genital System				
Coagulating gland			(2)	(1)
Hemangiosarcoma			1 (50%)	
Preputial gland	(60)	(59)	(59)	(60)
Histiocytic sarcoma	1 (2%)			
Prostate	(60)	(59)	(57)	(58)
Hemangiosarcoma			4 (7%)	
Hemangiosarcoma, metastatic, mesentery				1 (2%)
Testes	(60)	(60)	(60)	(60)
Interstitial cell, adenoma		1 (2%)		
Hematopoietic System				
Bone marrow	(60)	(60)	(60)	(60)
Hemangiosarcoma		1 (2%)		
Mast cell tumor malignant	1 (2%)			
Lymph node	(3)	(4)	(7)	(1)
Inguinal, hemangiosarcoma, metastatic, skeletal muscle				1 (100%)
Mediastinal, hemangiosarcoma, metastatic, mesentery			1 (14%)	
Mediastinal, sarcoma, metastatic, tissue NOS	1 (33%)			
Pancreatic, histiocytic sarcoma		1 (25%)		
Lymph node, mandibular	(59)	(55)	(50)	(54)
Hemangioma			1 (2%)	
Lymph node, mesenteric	(60)	(55)	(56)	(53)
Carcinoma, metastatic, intestine large, cecum		1 (2%)	3 (5%)	
Hemangiosarcoma, metastatic, spleen	1 (2%)			
Histiocytic sarcoma		1 (2%)		
Spleen	(60)	(60)	(58)	(60)
Hemangioma	1 (2%)			
Hemangiosarcoma	2 (3%)	3 (5%)		
Histiocytic sarcoma		1 (2%)		
Thymus	(57)	(52)	(45)	(54)

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Integumentary System				
Skin	(60)	(60)	(60)	(60)
Sebacous gland, carcinoma		1 (2%)		
Subcutaneous tissue, fibrosarcoma			1 (2%)	
Subcutaneous tissue, hemangioma		1 (2%)		
Subcutaneous tissue, hemangiosarcoma		4 (7%)	8 (13%)	12 (20%)
Subcutaneous tissue, hemangiosarcoma, multiple				8 (13%)
Musculoskeletal System				
Bone	(60)	(60)	(60)	(60)
Cranium, osteoma		1 (2%)		
Skeletal muscle	(1)	(6)	(35)	(47)
Hemangiosarcoma		6 (100%)	25 (71%)	19 (40%)
Hemangiosarcoma, multiple			8 (23%)	26 (55%)
Nervous System				
Brain	(60)	(60)	(60)	(60)
Respiratory System				
Lung	(60)	(60)	(60)	(60)
Alveolar/bronchiolar adenoma	9 (15%)	6 (10%)	4 (7%)	
Alveolar/bronchiolar carcinoma	3 (5%)	1 (2%)	2 (3%)	
Alveolar/bronchiolar carcinoma, multiple	2 (3%)			
Carcinoma, metastatic, intestine large, cecum		1 (2%)		
Hemangiosarcoma, metastatic, mesentery			3 (5%)	1 (2%)
Hemangiosarcoma, metastatic, skin			3 (5%)	4 (7%)
Hemangiosarcoma, metastatic, uncertain primary site		1 (2%)	1 (2%)	2 (3%)
Hemangiosarcoma, metastatic, skeletal muscle			2 (3%)	8 (13%)
Hepatocellular carcinoma, metastatic, liver		2 (3%)	1 (2%)	
Histiocytic sarcoma		1 (2%)		
Mediastinum, hemangiosarcoma				4 (7%)
Nose	(60)	(60)	(60)	(60)
Special Senses System				
Ear	(1)			
External ear, neural crest tumor, malignant	1 (100%)			
Harderian gland	(5)	(3)	(3)	
Adenoma	4 (80%)	1 (33%)	1 (33%)	
Carcinoma	1 (20%)	1 (33%)	1 (33%)	
Urinary System				
Kidney	(58)	(59)	(58)	(60)
Hemangiosarcoma, metastatic, mesentery			1 (2%)	
Histiocytic sarcoma		1 (2%)		
Capsule, hemangiosarcoma				1 (2%)
Urinary bladder	(59)	(59)	(59)	(60)
Hemangiosarcoma			1 (2%)	

TABLE C1
Summary of the Incidence of Neoplasms in Male Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Systemic Lesions				
Multiple organs ^b	(60)	(60)	(60)	(60)
Histiocytic sarcoma	1 (2%)	1 (2%)		
Lymphoma malignant	3 (5%)		1 (2%)	
Neoplasm Summary				
Total animals with primary neoplasms ^c	45	50	59	60
Total primary neoplasms	69	92	117	117
Total animals with benign neoplasms	30	24	8	2
Total benign neoplasms	36	28	9	2
Total animals with malignant neoplasms	26	41	59	60
Total malignant neoplasms	33	64	108	115
Total animals with metastatic neoplasms	1	5	13	17
Total metastatic neoplasms	2	6	18	17
Total animals with malignant neoplasms of uncertain primary site		1	1	2

^a Number of animals examined microscopically at the site and the number of animals with neoplasm

^b Number of animals with any tissue examined microscopically

^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE C2
Individual Animal Tumor Pathology of Male Mice in the 2-Year Feed Study of *o*-Nitrotoluene: 2,500 ppm

Number of Days on Study	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	6	6	6	7																		
	4	4	4	4	5	5	5	5	5	6	6	6	6	6	6	6	6	6	6	7	7	8	8	9	9	9	1	2	4	0																
	0	4	8	8	3	4	6	6	7	0	0	3	4	6	6	7	9	9	9	2	2	8	8	0	2	6	9	4	5	6																
Carcass ID Number	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	Total Tissues/ Tumors															
	5	5	5	7	6	3	2	2	3	3	5	6	2	3	4	7	3	6	6	2	7	4	5	3	7	2	7	7	6	4																
	9	8	6	0	8	1	5	8	5	7	0	5	9	0	7	8	3	2	7	3	3	6	2	9	2	1	9	4	6	2																
Genital System																																														
Coagulating gland																														2																
Hemangiosarcoma																														1																
Epididymis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	60														
Preputial gland	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	M	+	+	+	+	+	+	+	+	+	+	+	+	+	59														
Prostate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	M	+	+	+	+	+	+	+	I	+	+	+	+	M	57															
Hemangiosarcoma																														4																
Seminal vesicle	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	60															
Testes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	60															
Hematopoietic System																																														
Bone marrow	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	60															
Lymph node																														7																
Mediastinal, hemangiosarcoma,																														1																
metastatic, mesentery																														1																
Lymph node, mandibular	M	+	+	M	+	+	+	+	+	+	+	+	+	+	+	M	+	+	+	+	+	+	+	+	+	M	+	+	+	+	50															
Hemangioma																														1																
Lymph node, mesenteric	+	M	M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	56															
Carcinoma, metastatic,																														3																
intestine large, cecum																														3																
Spleen	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	58															
Thymus	+	M	+	+	I	+	+	+	+	I	+	+	+	+	M	+	M	+	+	+	I	+	+	M	+	+	+	M	+	+	45															
Integumentary System																																														
Mammary gland	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M																
Skin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	60															
Subcutaneous tissue, fibrosarcoma																														1																
Subcutaneous tissue, hemangiosarcoma	X	X																					X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	8
Musculoskeletal System																																														
Bone	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	60															
Skeletal muscle	+			+	+	+			+	+	+			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	35																
Hemangiosarcoma	X					X	X			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	25																	
Hemangiosarcoma, multiple			X					X			X					X					X	X	X	X	X	X	X	X	8																	
Nervous System																																														
Brain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	60															
Peripheral nerve																														3																
Spinal cord																														3																

TABLE C3
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Harderian Gland: Adenoma				
Overall rate ^a	4/60 (7%)	1/60 (2%)	1/60 (2%)	0/60 (0%)
Adjusted rate ^b	7.0%	2.0%	4.2%	0.0%
Terminal rate ^c	4/52 (8%)	1/34 (3%)	0/0	0/0 ^e
First incidence (days) ^d	729 (T)	729 (T)	645	—
Poly-3 test ^d	P=0.196N	P=0.225N	P=0.509N	P=0.483N
Harderian Gland: Adenoma or Carcinoma				
Overall rate	5/60 (8%)	2/60 (3%)	2/60 (3%)	0/60 (0%)
Adjusted rate	8.8%	4.0%	8.2%	0.0%
Terminal rate	5/52 (10%)	1/34 (3%)	0/0	0/0
First incidence (days)	729 (T)	641	560	—
Poly-3 test	P=0.298N	P=0.274N	P=0.617N	P=0.443N
Large Intestine (Cecum): Carcinoma				
Overall rate	0/60 (0%)	12/60 (20%)	9/60 (15%)	0/60 (0%)
Adjusted rate	0.0%	22.7%	31.6%	0.0%
Terminal rate	0/52 (0%)	4/34 (12%)	0/0	0/0
First incidence (days)	—	485	270	— ^f
Poly-3 test	P<0.001	P<0.001	P<0.001	—
Liver: Hemangiosarcoma				
Overall rate	1/60 (2%)	4/59 (7%)	1/57 (2%)	0/60 (0%)
Adjusted rate	1.8%	8.0%	4.3%	0.0%
Terminal rate	1/52 (2%)	1/34 (3%)	0/0	0/0
First incidence (days)	729 (T)	641	588	—
Poly-3 test	P=0.257	P=0.142	P=0.542	P=0.721N
Liver: Hepatocellular Adenoma				
Overall rate	18/60 (30%)	18/59 (31%)	3/57 (5%)	0/60 (0%)
Adjusted rate	31.4%	35.0%	12.3%	0.0%
Terminal rate	17/52 (33%)	14/34 (41%)	0/0	0/0
First incidence (days)	637	451	470	—
Poly-3 test	P=0.118N	P=0.421	P=0.087N	P=0.183N
Liver: Hepatocellular Carcinoma				
Overall rate	12/60 (20%)	16/59 (27%)	5/57 (9%)	2/60 (3%)
Adjusted rate	20.5%	31.4%	20.1%	18.5%
Terminal rate	7/52 (14%)	10/34 (29%)	0/0	0/0
First incidence (days)	596	451	556	350
Poly-3 test	P=0.354	P=0.138	P=0.587N	P=0.562N
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	27/60 (45%)	28/59 (47%)	7/57 (12%)	2/60 (3%)
Adjusted rate	46.1%	53.7%	26.7%	18.5%
Terminal rate	22/52 (42%)	19/34 (56%)	0/0	0/0
First incidence (days)	596	451	470	350
Poly-3 test	P=0.149N	P=0.271	P=0.093N	P=0.198N
Liver: Hepatocellular Carcinoma or Hepatoblastoma				
Overall rate	13/60 (22%)	17/59 (29%)	5/57 (9%)	2/60 (3%)
Adjusted rate	22.2%	33.4%	20.1%	18.5%
Terminal rate	7/52 (14%)	10/34 (29%)	0/0	0/0
First incidence (days)	596	451	556	350
Poly-3 test	P=0.399	P=0.136	P=0.526N	P=0.531N

TABLE C3
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Liver: Hepatocellular Adenoma, Hepatocellular Carcinoma, or Hepatoblastoma				
Overall rate	28/60 (47%)	29/59 (49%)	7/57 (12%)	2/60 (3%)
Adjusted rate	47.8%	55.6%	26.7%	18.5%
Terminal rate	22/52 (42%)	19/34 (56%)	0/0	0/0
First incidence (days)	596	451	470	350
Poly-3 test	P=0.125N	P=0.262	P=0.073N	P=0.181N
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	9/60 (15%)	6/60 (10%)	4/60 (7%)	0/60 (0%)
Adjusted rate	15.8%	11.8%	15.4%	0.0%
Terminal rate	9/52 (17%)	3/34 (9%)	0/0	0/0
First incidence (days)	729 (T)	469	434	—
Poly-3 test	P=0.323N	P=0.372N	P=0.595N	P=0.332N
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	5/60 (8%)	1/60 (2%)	2/60 (3%)	0/60 (0%)
Adjusted rate	8.7%	2.0%	8.1%	0.0%
Terminal rate	4/52 (8%)	1/34 (3%)	0/0	0/0
First incidence (days)	649	729 (T)	544	—
Poly-3 test	P=0.219N	P=0.140N	P=0.614N	P=0.444N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	14/60 (23%)	7/60 (12%)	6/60 (10%)	0/60 (0%)
Adjusted rate	24.4%	13.7%	22.1%	0.0%
Terminal rate	13/52 (25%)	4/34 (12%)	0/0	0/0
First incidence (days)	649	469	434	—
Poly-3 test	P=0.136N	P=0.121N	P=0.513N	P=0.241N
Lung: Hemangiosarcoma				
Overall rate	0/60 (0%)	0/60 (0%)	0/60 (0%)	4/60 (7%)
Adjusted rate	0.0%	0.0%	0.0%	32.1%
Terminal rate	0/52 (0%)	0/34 (0%)	0/0	0/0
First incidence (days)	—	—	—	358
Poly-3 test	P=0.014	—	—	P=0.006
Mesentery: Hemangiosarcoma				
Overall rate	0/60 (0%)	8/60 (13%)	38/60 (63%)	38/60 (63%)
Adjusted rate	0.0%	15.8%	83.0%	92.8%
Terminal rate	0/52 (0%)	5/34 (15%)	0/0	0/0
First incidence (days)	—	572	410	297
Poly-3 test	P<0.001	P=0.002	P<0.001	P<0.001
Prostate Gland: Hemangiosarcoma				
Overall rate	0/60 (0%)	0/59 (0%)	4/57 (7%)	0/58 (0%)
Adjusted rate	0.0%	0.0%	16.8%	0.0%
Terminal rate	0/52 (0%)	0/34 (0%)	0/0	0/0
First incidence (days)	—	—	476	—
Poly-3 test	P=0.029	—	P=0.011	—
Skeletal Muscle: Hemangiosarcoma				
Overall rate	0/60 (0%)	6/60 (10%)	33/60 (55%)	45/60 (75%)
Adjusted rate	0.0%	11.7%	79.1%	95.3%
Terminal rate	0/52 (0%)	1/34 (3%)	0/0	0/0
First incidence (days)	—	542	365	277
Poly-3 test	P<0.001	P=0.011	P<0.001	P<0.001

TABLE C3
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Skin (Subcutaneous Tissue): Hemangiosarcoma				
Overall rate	0/60 (0%)	4/60 (7%)	8/60 (13%)	20/60 (33%)
Adjusted rate	0.0%	8.0%	29.4%	77.0%
Terminal rate	0/52 (0%)	2/34 (6%)	0/0	0/0
First incidence (days)	—	542	544	326
Poly-3 test	P<0.001	P=0.047	P<0.001	P<0.001
Spleen: Hemangiosarcoma				
Overall rate	2/60 (3%)	3/60 (5%)	0/58 (0%)	0/60 (0%)
Adjusted rate	3.5%	6.0%	0.0%	0.0%
Terminal rate	1/52 (2%)	1/34 (3%)	0/0	0/0
First incidence (days)	710	641	—	—
Poly-3 test	P=0.589N	P=0.441	P=0.462N	P=0.602N
All Organs: Hemangiosarcoma				
Overall rate	4/60 (7%)	17/60 (28%)	55/60 (92%)	60/60 (100%)
Adjusted rate	7.0%	32.7%	97.9%	100.0%
Terminal rate	3/52 (6%)	9/34 (27%)	0/0	0/0
First incidence (days)	710	542	365	277
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	5/60 (8%)	18/60 (30%)	55/60 (92%)	60/60 (100%)
Adjusted rate	8.8%	34.7%	97.9%	100.0%
Terminal rate	4/52 (8%)	10/34 (29%)	0/0	0/0
First incidence (days)	710	542	365	277
Poly-3 test	P<0.001	P<0.001	P<0.001	P<0.001
All Organs: Malignant Lymphoma				
Overall rate	3/60 (5%)	0/60 (0%)	1/60 (2%)	0/60 (0%)
Adjusted rate	5.3%	0.0%	4.1%	0.0%
Terminal rate	3/52 (6%)	0/34 (0%)	0/0	0/0
First incidence (days)	729 (T)	—	434	—
Poly-3 test	P=0.220N	P=0.146N	P=0.614N	P=0.533N
All Organs: Benign Neoplasms				
Overall rate	30/60 (50%)	24/60 (40%)	8/60 (13%)	2/60 (3%)
Adjusted rate	52.3%	45.3%	28.6%	18.6%
Terminal rate	29/52 (56%)	17/34 (50%)	0/0	0/0
First incidence (days)	637	451	434	391
Poly-3 test	P=0.016N	P=0.288N	P=0.040N	P=0.137N
All Organs: Malignant Neoplasms				
Overall rate	26/60 (43%)	41/60 (68%)	59/60 (98%)	60/60 (100%)
Adjusted rate	44.4%	71.0%	99.9%	100.0%
Terminal rate	20/52 (39%)	19/34 (56%)	0/0	0/0
First incidence (days)	596	451	270	277
Poly-3 test	P<0.001	P=0.002	P<0.001	P<0.001

TABLE C3
Statistical Analysis of Primary Neoplasms in Male Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
All Organs: Benign or Malignant Neoplasms				
Overall rate	45/60 (75%)	50/60 (83%)	59/60 (98%)	60/60 (100%)
Adjusted rate	76.8%	85.5%	99.9%	100.0%
Terminal rate	39/52 (75%)	27/34 (79%)	0/0	0/0
First incidence (days)	596	451	270	277
Poly-3 test	P<0.001	P=0.164	P<0.001	P<0.001

(T) Terminal sacrifice

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for liver, lung, prostate gland, and spleen; for other tissues, denominator is number of animals necropsied.

^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by N.

^e Not applicable; no neoplasms in animal group

^f Value of statistic cannot be computed.

TABLE C4a
Historical Incidence of Hemangiosarcoma (All Sites) in Control Male B6C3F₁ Mice

Study	Incidence in Controls
Historical Incidence in Controls Given NTP-2000 Diet^a	
Acrylonitrile (gavage)	3/50
Citral (feed)	3/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	3/50
Indium phosphide (inhalation)	3/50
60-Hz Magnetic fields (whole body exposure)	6/100
Methacrylonitrile (gavage)	3/49
<i>o</i> -Nitrotoluene (feed)	4/60
<i>p</i> -Nitrotoluene (feed)	1/50
Riddelliine (gavage)	3/50
Sodium nitrite (drinking water)	7/50
Vanadium pentoxide (inhalation)	1/50
Overall Historical Incidence in Controls Given NTP-2000 Diet	
Total (%)	37/659 (5.6%)
Mean ± standard deviation	5.8% ± 3.2%
Range	2%-14%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b	
Benzyl acetate	2/50
2,2-Bis(bromomethyl)-1,3-propanediol	2/50
<i>t</i> -Butylhydroquinone	7/50
Emodin	3/50
<i>o</i> -Nitroanisole	3/50
<i>p</i> -Nitrobenzoic acid	6/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet	
Total (%)	53/952 (5.6%)
Mean ± standard deviation	5.6% ± 3.5%
Range	2%-14%

^a Data as of December 22, 2000

^b Data as of December 23, 1999

TABLE C4b
Historical Incidence of Large Intestine (Cecum) Carcinoma in Control Male B6C3F₁ Mice

Study	Incidence in Controls
Historical Incidence in Controls Given NTP-2000 Diet^a	
Acrylonitrile (gavage)	0/50
Citral (feed)	0/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	0/50
Indium phosphide (inhalation)	0/50
60-Hz Magnetic fields (whole body exposure)	0/100
Methacrylonitrile (gavage)	0/49
<i>o</i> -Nitrotoluene (feed)	0/60
<i>p</i> -Nitrotoluene (feed)	1/50
Riddelliine (gavage)	0/50
Sodium nitrite (drinking water)	0/50
Vanadium pentoxide (inhalation)	0/50
Overall Historical Incidence in Controls Given NTP-2000 Diet	
Total (%)	1/659 (0.2%)
Mean ± standard deviation	0.2% ± 0.6%
Range	0%-2%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b	
Benzyl acetate	0/50
2,2-Bis(bromomethyl)-1,3-propanediol	0/50
<i>t</i> -Butylhydroquinone	0/50
Emodin	0/50
<i>o</i> -Nitroanisole	0/50
<i>p</i> -Nitrobenzoic acid	0/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet	
Total	0/952

^a Data as of December 22, 2000

^b Data as of December 23, 1999

TABLE C4c
Historical Incidence of Hepatocellular Neoplasms in Control Male B6C3F₁ Mice

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
Historical Incidence in Controls Given NTP-2000 Diet^a			
Acrylonitrile (gavage)	23/50	14/50	32/50
Citral (feed)	20/100	13/100	28/100
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	6/50	9/50	15/50
Indium phosphide (inhalation)	17/50	11/50	26/50
60-Hz Magnetic fields (whole body exposure)	30/100	19/100	46/100
Methacrylonitrile (gavage)	17/49	13/49	24/49
<i>o</i> -Nitrotoluene (feed)	18/60	12/60	27/60
<i>p</i> -Nitrotoluene (feed)	14/50	8/50	20/50
Riddelliine (gavage)	16/50	23/50	36/50
Sodium nitrite (drinking water)	19/50	9/50	24/50
Vanadium pentoxide (inhalation)	15/50	14/50	26/50
Overall Historical Incidence in Controls Given NTP-2000 Diet			
Total (%)	195/659 (29.6%)	145/659 (22.0%)	304/659 (46.1%)
Mean ± standard deviation	30.4% ± 8.9%	23.1% ± 9.0%	47.8% ± 12.9%
Range	12%-46%	13%-46%	28%-72%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b			
Benzyl acetate	10/50	12/50	22/50
2,2-Bis(bromomethyl)-1,3-propanediol	18/49	11/49	27/49
<i>t</i> -Butylhydroquinone	28/50	8/50	31/50
Emodin	9/50	20/50	28/50
<i>o</i> -Nitroanisole	14/50	7/50	21/50
<i>p</i> -Nitrobenzoic acid	17/50	8/50	22/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet			
Total (%)	363/950 (38.2%)	194/950 (20.4%)	493/950 (51.9%)
Mean ± standard deviation	38.2% ± 10.7%	20.4% ± 6.7%	51.9% ± 7.9%
Range	18%-60%	10%-40%	40%-68%

^a Data as of December 22, 2000

^b Data as of December 23, 1999

TABLE C5
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Feed Study of o-Nitrotoluene^a

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Disposition Summary				
Animals initially in study	60	60	60	60
Early deaths				
Moribund	3	5	21	31
Natural deaths	5	21	39	29
Survivors				
Terminal sacrifice	52	34		
Animals examined microscopically	60	60	60	60
Alimentary System				
Gallbladder	(54)	(42)	(31)	(35)
Fibrosis		1 (2%)		
Intestine large, cecum	(56)	(49)	(36)	(44)
Epithelium, degeneration, mucoid		1 (2%)		
Intestine small, jejunum	(56)	(43)	(35)	(40)
Inflammation, focal		1 (2%)		
Ulcer		1 (2%)		
Epithelium, degeneration, cystic, focal	1 (2%)			
Epithelium, hyperplasia, focal, adenomatous	1 (2%)			
Peyer's patch, hyperplasia, lymphoid		1 (2%)		
Peyer's patch, inflammation, chronic		1 (2%)		
Liver	(60)	(59)	(57)	(60)
Amyloid deposition		1 (2%)		
Angiectasis	1 (2%)		2 (4%)	1 (2%)
Basophilic focus		6 (10%)	4 (7%)	
Clear cell focus	3 (5%)	2 (3%)		
Congestion, focal	2 (3%)	1 (2%)		
Eosinophilic focus	3 (5%)	14 (24%)	1 (2%)	1 (2%)
Fibrosis, focal		1 (2%)	1 (2%)	
Infiltration cellular, mixed cell	46 (77%)	40 (68%)	24 (42%)	29 (48%)
Inflammation, chronic, focal		1 (2%)	1 (2%)	
Inflammation, focal, suppurative		1 (2%)		
Mineralization, focal		1 (2%)		
Mixed cell focus	2 (3%)			
Necrosis	1 (2%)	15 (25%)	27 (47%)	30 (50%)
Artery, angiectasis				1 (2%)
Artery, inflammation, chronic				1 (2%)
Hepatocyte, fatty change, diffuse	2 (3%)			
Hepatocyte, necrosis, focal	2 (3%)			
Hepatocyte, syncytial alteration, focal	16 (27%)	26 (44%)	43 (75%)	39 (65%)
Hepatocyte, tension lipidosis	1 (2%)			
Hepatocyte, vacuolization cytoplasmic, diffuse	10 (17%)	4 (7%)		
Hepatocyte, vacuolization cytoplasmic, focal	4 (7%)	5 (8%)	2 (4%)	1 (2%)
Hepatocyte, periportal, atrophy				1 (2%)
Hepatocyte, centrilobular, depletion glycogen		1 (2%)		3 (5%)
Hepatocyte, centrilobular, fatty change	1 (2%)	2 (3%)	5 (9%)	1 (2%)
Hepatocyte, midzonal, fatty change				1 (2%)
Oval cell, hyperplasia				2 (3%)

^a Number of animals examined microscopically at the site and the number of animals with lesion

TABLE C5
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Alimentary System (continued)				
Mesentery	(12)	(12)	(42)	(38)
Inflammation, chronic	3 (25%)	2 (17%)	2 (5%)	
Inflammation, chronic, focal			1 (2%)	
Artery, inflammation, chronic	3 (25%)	1 (8%)		
Fat, necrosis	6 (50%)	1 (8%)	1 (2%)	
Pancreas	(59)	(59)	(59)	(58)
Amyloid deposition		1 (2%)		
Acinus, atrophy, focal	1 (2%)	1 (2%)	1 (2%)	
Duct, cyst	1 (2%)	1 (2%)		
Duct, inflammation, chronic, focal		1 (2%)		
Stomach, forestomach	(60)	(60)	(58)	(60)
Inflammation, focal		1 (2%)	3 (5%)	1 (2%)
Necrosis, focal			1 (2%)	
Ulcer		1 (2%)	3 (5%)	1 (2%)
Epithelium, cyst	1 (2%)			
Epithelium, hyperplasia	2 (3%)	5 (8%)	6 (10%)	3 (5%)
Stomach, glandular	(57)	(54)	(53)	(55)
Erosion			2 (4%)	
Inflammation, chronic, focal		1 (2%)		
Inflammation, focal		1 (2%)		1 (2%)
Pigmentation, focal			1 (2%)	
Tooth	(14)	(9)	(2)	
Malformation	5 (36%)			
Peridental tissue, inflammation, chronic	10 (71%)	9 (100%)	1 (50%)	
Cardiovascular System				
Heart	(60)	(60)	(60)	(60)
Infiltration cellular, mixed cell		1 (2%)	3 (5%)	1 (2%)
Inflammation, chronic, focal	1 (2%)		4 (7%)	1 (2%)
Mineralization			2 (3%)	1 (2%)
Thrombosis			1 (2%)	
Endocrine System				
Adrenal cortex	(60)	(60)	(58)	(60)
Accessory adrenal cortical nodule		1 (2%)		
Cyst		2 (3%)		
Cytoplasmic alteration, focal	7 (12%)	2 (3%)		
Fibrosis			1 (2%)	
Hypertrophy, focal		1 (2%)		
Inflammation, chronic, focal				1 (2%)
Parathyroid gland	(55)	(54)	(57)	(56)
Amyloid deposition		1 (2%)		
Cyst			1 (2%)	
Pituitary gland	(53)	(50)	(50)	(46)
Pars distalis, cyst	2 (4%)	1 (2%)		
Pars distalis, hyperplasia, focal				1 (2%)
Rathke's cleft, cyst		1 (2%)		
Thyroid gland	(59)	(59)	(59)	(59)
Degeneration, cystic, focal	10 (17%)	11 (19%)	3 (5%)	
Follicular cell, hyperplasia		1 (2%)		

TABLE C5
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
General Body System				
Tissue NOS	(2)		(2)	(3)
Pelvic, inflammation, chronic, focal				1 (33%)
Genital System				
Coagulating gland			(2)	(1)
Inflammation, chronic			1 (50%)	1 (100%)
Epididymis	(60)	(60)	(60)	(60)
Inflammation, chronic	1 (2%)			
Preputial gland	(60)	(59)	(59)	(60)
Degeneration, cystic	28 (47%)	27 (46%)	16 (27%)	1 (2%)
Inflammation, chronic	1 (2%)	5 (8%)	1 (2%)	
Prostate	(60)	(59)	(57)	(58)
Inflammation, chronic		1 (2%)	7 (12%)	4 (7%)
Seminal vesicle	(60)	(60)	(60)	(60)
Congestion			1 (2%)	1 (2%)
Dilatation	2 (3%)	1 (2%)	1 (2%)	
Inflammation, chronic			1 (2%)	
Inflammation, suppurative				1 (2%)
Hematopoietic System				
Bone marrow	(60)	(60)	(60)	(60)
Depletion cellular		1 (2%)		
Hemorrhage, focal		2 (3%)	1 (2%)	
Hyperplasia		3 (5%)	2 (3%)	
Thrombosis		1 (2%)		
Erythroid cell, depletion cellular			1 (2%)	
Lymph node	(3)	(4)	(7)	(1)
Iliac, hematopoietic cell proliferation			1 (14%)	
Iliac, hyperplasia			1 (14%)	
Mediastinal, hemorrhage	1 (33%)	1 (25%)	3 (43%)	
Mediastinal, hyperplasia		1 (25%)	1 (14%)	
Mediastinal, pigmentation		1 (25%)		
Pancreatic, hemorrhage		1 (25%)		
Lymph node, mandibular	(59)	(55)	(50)	(54)
Hyperplasia, lymphoid	1 (2%)			
Lymph node, mesenteric	(60)	(55)	(56)	(53)
Hematopoietic cell proliferation		1 (2%)		
Hemorrhage	1 (2%)	2 (4%)		
Hyperplasia			1 (2%)	
Hyperplasia, lymphoid		1 (2%)		
Spleen	(60)	(60)	(58)	(60)
Amyloid deposition		1 (2%)		
Depletion cellular	1 (2%)	7 (12%)	2 (3%)	
Hematopoietic cell proliferation	13 (22%)	24 (40%)	49 (84%)	60 (100%)
Hyperplasia, lymphoid	2 (3%)	3 (5%)		
Pigmentation, focal	1 (2%)			
Thymus	(57)	(52)	(45)	(54)
Atrophy		3 (6%)	3 (7%)	6 (11%)
Cyst	4 (7%)	3 (6%)	1 (2%)	
Hyperplasia, lymphoid	1 (2%)			
Epithelial cell, hyperplasia	1 (2%)			

TABLE C5
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Integumentary System				
Skin	(60)	(60)	(60)	(60)
Ulcer, chronic, focal	1 (2%)			
Artery, subcutaneous tissue, inflammation, chronic	2 (3%)			
Prepuce, subcutaneous tissue, inflammation, suppurative			1 (2%)	
Subcutaneous tissue, edema		3 (5%)	14 (23%)	22 (37%)
Subcutaneous tissue, hemorrhage, focal			1 (2%)	2 (3%)
Subcutaneous tissue, inflammation, chronic, focal		1 (2%)	1 (2%)	2 (3%)
Subcutaneous tissue, necrosis			1 (2%)	
Musculoskeletal System				
Bone	(60)	(60)	(60)	(60)
Fracture		1 (2%)		
Skeletal muscle	(1)	(6)	(35)	(47)
Hemorrhage, focal			2 (6%)	2 (4%)
Nervous System				
None				
Respiratory System				
Lung	(60)	(60)	(60)	(60)
Congestion		1 (2%)	1 (2%)	1 (2%)
Cyst			1 (2%)	
Hemorrhage	1 (2%)		1 (2%)	5 (8%)
Hyperplasia, histiocytic		2 (3%)		1 (2%)
Infiltration cellular, polymorphonuclear			1 (2%)	
Infiltration cellular, mixed cell		4 (7%)	9 (15%)	3 (5%)
Inflammation, chronic, focal		3 (5%)		
Alveolar epithelium, hyperplasia	1 (2%)	1 (2%)	1 (2%)	1 (2%)
Bronchus, glands, cyst	1 (2%)			
Nose	(60)	(60)	(60)	(60)
Mucosa, glands, dilatation, focal		1 (2%)		
Nasolacrimal duct, inflammation		1 (2%)		
Olfactory epithelium, degeneration		36 (60%)	60 (100%)	60 (100%)
Sinus, foreign body	1 (2%)			
Sinus, inflammation, chronic, suppurative	1 (2%)			
Special Senses System				
Eye		(1)	(1)	
Atrophy		1 (100%)		
Inflammation, chronic			1 (100%)	
Cornea, necrosis, focal			1 (100%)	

TABLE C5
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Urinary System				
Kidney	(58)	(59)	(58)	(60)
Amyloid deposition		1 (2%)		
Atrophy		1 (2%)	1 (2%)	
Atrophy, focal		2 (3%)		
Congestion	5 (9%)	3 (5%)		
Cyst	2 (3%)	4 (7%)	1 (2%)	1 (2%)
Fibrosis, focal		2 (3%)		
Hyperplasia, lymphoid	1 (2%)	3 (5%)		
Infarct		1 (2%)		
Infiltration cellular, mixed cell			1 (2%)	
Inflammation, chronic		1 (2%)	1 (2%)	
Inflammation, chronic, focal		1 (2%)		
Inflammation, focal, suppurative		1 (2%)		
Inflammation, suppurative		1 (2%)	3 (5%)	
Metaplasia, focal, osseous			1 (2%)	
Nephropathy	56 (97%)	53 (90%)	34 (59%)	18 (30%)
Artery, inflammation, chronic	2 (3%)			
Papilla, necrosis			1 (2%)	
Pelvis, dilatation			2 (3%)	
Renal tubule, accumulation, hyaline droplet	1 (2%)	2 (3%)	5 (9%)	3 (5%)
Renal tubule, dilatation		1 (2%)	4 (7%)	
Renal tubule, necrosis		1 (2%)		2 (3%)
Renal tubule, pigmentation	1 (2%)	6 (10%)	32 (55%)	35 (58%)
Renal tubule, vacuolization cytoplasmic	1 (2%)		1 (2%)	
Urethra			(1)	(2)
Inflammation, focal			1 (100%)	2 (100%)
Urinary bladder	(59)	(59)	(59)	(60)
Calculus, microscopic observation only			1 (2%)	
Inflammation, chronic			1 (2%)	
Transitional epithelium, hyperplasia			1 (2%)	

APPENDIX D
SUMMARY OF LESIONS IN FEMALE MICE
IN THE 2-YEAR FEED STUDY
OF *o*-NITROTOLUENE

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TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Feed Study of o-Nitrotoluene^a

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Disposition Summary				
Animals initially in study	60	60	60	60
Early deaths				
Moribund	4	6	7	22
Natural deaths	4	8	6	33
Survivors				
Terminal sacrifice	52	46	47	5
Animals examined microscopically	60	60	60	60
Alimentary System				
Esophagus	(60)	(60)	(59)	(57)
Gallbladder	(54)	(53)	(54)	(31)
Intestine large, colon	(56)	(55)	(57)	(41)
Hemangiosarcoma, metastatic, mesentery				1 (2%)
Intestine large, cecum	(56)	(53)	(54)	(32)
Carcinoma		1 (2%)	4 (7%)	3 (9%)
Sarcoma			1 (2%)	
Intestine small, jejunum	(56)	(53)	(56)	(34)
Carcinoma				1 (3%)
Intestine small, ileum	(57)	(53)	(56)	(34)
Liver	(60)	(59)	(59)	(60)
Cholangioma				1 (2%)
Hemangiosarcoma			1 (2%)	
Hemangiosarcoma, metastatic, skin				1 (2%)
Hepatocellular carcinoma	2 (3%)	3 (5%)	4 (7%)	9 (15%)
Hepatocellular carcinoma, multiple		1 (2%)	2 (3%)	7 (12%)
Hepatocellular adenoma	7 (12%)	5 (8%)	16 (27%)	9 (15%)
Hepatocellular adenoma, multiple			3 (5%)	20 (33%)
Hepatocholangiocarcinoma				1 (2%)
Histiocytic sarcoma		2 (3%)	1 (2%)	
Osteosarcoma, metastatic, bone		1 (2%)		
Sarcoma, metastatic, uncertain primary site			1 (2%)	
Mesentery	(11)	(9)	(9)	(34)
Carcinoma, metastatic, uncertain primary site		1 (11%)		
Carcinoma, metastatic, intestine large, cecum			1 (11%)	
Hemangiosarcoma				25 (74%)
Hemangiosarcoma, multiple				7 (21%)
Histiocytic sarcoma		1 (11%)		
Sarcoma				1 (3%)
Sarcoma, multiple			1 (11%)	
Sarcoma, metastatic, skin			1 (11%)	
Sarcoma, metastatic, skeletal muscle			1 (11%)	
Pancreas	(60)	(57)	(58)	(57)
Hemangiosarcoma, metastatic, mesentery				1 (2%)
Sarcoma			1 (2%)	
Sarcoma, metastatic, mesentery				1 (2%)
Salivary glands	(59)	(59)	(58)	(49)
Histiocytic sarcoma			1 (2%)	
Stomach, forestomach	(60)	(58)	(58)	(59)
Hemangioma	1 (2%)			
Squamous cell papilloma	1 (2%)	2 (3%)	2 (3%)	1 (2%)
Stomach, glandular	(58)	(57)	(57)	(52)
Sarcoma			1 (2%)	

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Cardiovascular System				
Heart	(60)	(60)	(59)	(58)
Carcinoma, metastatic, uncertain primary site		1 (2%)		
Endocrine System				
Adrenal cortex	(59)	(59)	(58)	(60)
Capsule, adenoma	1 (2%)			
Adrenal medulla	(59)	(57)	(58)	(59)
Pheochromocytoma benign		1 (2%)		
Islets, pancreatic	(60)	(57)	(60)	(56)
Carcinoma		1 (2%)		
Pituitary gland	(58)	(58)	(53)	(53)
Adenoma	1 (2%)			
Pars distalis, adenoma	2 (3%)	4 (7%)	3 (6%)	
Pars intermedia, adenoma			1 (2%)	
Thyroid gland	(60)	(59)	(58)	(53)
Follicular cell, adenoma		1 (2%)		
General Body System				
Tissue NOS	(1)		(3)	(3)
Abdominal, hemangiosarcoma				1 (33%)
Abdominal, hemangiosarcoma, multiple				1 (33%)
Thoracic, sarcoma				1 (33%)
Genital System				
Ovary	(59)	(56)	(56)	(56)
Carcinoma		1 (2%)		
Cystadenoma	1 (2%)		2 (4%)	
Hemangiosarcoma		1 (2%)		
Histiocytic sarcoma		1 (2%)		
Luteoma	2 (3%)		1 (2%)	1 (2%)
Teratoma malignant			1 (2%)	
Tubulostromal adenoma		1 (2%)		
Oviduct	(1)			
Uterus	(60)	(59)	(60)	(59)
Hemangioma	1 (2%)	2 (3%)		
Hemangiosarcoma		1 (2%)		2 (3%)
Histiocytic sarcoma		1 (2%)	1 (2%)	
Leiomyoma		1 (2%)		
Leiomyosarcoma			1 (2%)	
Cervix, hemangiosarcoma				1 (2%)
Endometrium, carcinoma			1 (2%)	
Endometrium, polyp stromal	2 (3%)	2 (3%)	2 (3%)	
Vagina		(1)		
Carcinoma, metastatic, uncertain primary site		1 (100%)		
Hematopoietic System				
Bone marrow	(60)	(58)	(60)	(60)
Carcinoma, metastatic, uncertain primary site		1 (2%)		
Histiocytic sarcoma		1 (2%)	1 (2%)	

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Hematopoietic System (continued)				
Lymph node	(6)	(3)	(10)	(11)
Iliac, sarcoma, metastatic, skin				1 (9%)
Inguinal, sarcoma, metastatic, skin			1 (10%)	
Mediastinal, sarcoma, metastatic, uncertain primary site				1 (9%)
Pancreatic, sarcoma			1 (10%)	
Renal, hemangiosarcoma, metastatic, mesentery				1 (9%)
Renal, sarcoma, metastatic, skin			1 (10%)	1 (9%)
Lymph node, mandibular	(58)	(55)	(55)	(48)
Histiocytic sarcoma			1 (2%)	
Lymph node, mesenteric	(53)	(58)	(57)	(57)
Histiocytic sarcoma		1 (2%)	1 (2%)	
Sarcoma	1 (2%)			
Sarcoma, metastatic, mesentery				1 (2%)
Spleen	(59)	(57)	(58)	(57)
Histiocytic sarcoma			1 (2%)	
Thymus	(57)	(56)	(55)	(44)
Thymoma malignant			1 (2%)	
Integumentary System				
Skin	(60)	(60)	(60)	(60)
Basal cell carcinoma				1 (2%)
Basosquamous tumor malignant		1 (2%)		
Subcutaneous tissue, carcinoma, multiple		1 (2%)		
Subcutaneous tissue, hemangiosarcoma			2 (3%)	16 (27%)
Subcutaneous tissue, hemangiosarcoma, multiple				3 (5%)
Subcutaneous tissue, sarcoma	1 (2%)	1 (2%)	5 (8%)	2 (3%)
Subcutaneous tissue, sarcoma, multiple			1 (2%)	
Musculoskeletal System				
Bone	(60)	(60)	(60)	(60)
Osteosarcoma		1 (2%)		
Femur, osteosarcoma			1 (2%)	
Skeletal muscle	(2)	(3)	(3)	(18)
Carcinoma, metastatic, uncertain primary site		1 (33%)		
Hemangioma	1 (50%)			
Hemangiosarcoma				14 (78%)
Hemangiosarcoma, multiple				2 (11%)
Sarcoma		1 (33%)	2 (67%)	1 (6%)
Sarcoma, multiple			1 (33%)	1 (6%)
Nervous System				
None				
Respiratory System				
Lung	(60)	(60)	(59)	(57)
Alveolar/bronchiolar adenoma	2 (3%)	3 (5%)	4 (7%)	4 (7%)
Alveolar/bronchiolar carcinoma	3 (5%)	1 (2%)		
Alveolar/bronchiolar carcinoma, multiple		2 (3%)		2 (4%)
Carcinoma, metastatic, harderian gland		1 (2%)		1 (2%)
Carcinoma, metastatic, uncertain primary site		1 (2%)		
Hemangiosarcoma, metastatic, skin				1 (2%)

TABLE D1
Summary of the Incidence of Neoplasms in Female Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Respiratory System (continued)				
Lung (continued)	(60)	(60)	(59)	(57)
Hemangiosarcoma, metastatic, uncertain primary site				1 (2%)
Hemangiosarcoma, metastatic, skeletal muscle				2 (4%)
Hepatocellular carcinoma, metastatic, liver		1 (2%)		1 (2%)
Histiocytic sarcoma		1 (2%)	1 (2%)	
Osteosarcoma, metastatic, bone		1 (2%)	1 (2%)	
Sarcoma, metastatic, mesentery				1 (2%)
Sarcoma, metastatic, skeletal muscle			1 (2%)	
Teratoma malignant, metastatic, ovary			1 (2%)	
Mediastinum, alveolar/bronchiolar carcinoma, metastatic, lung		1 (2%)		
Mediastinum, carcinoma		1 (2%)		
Mediastinum, carcinoma, metastatic, harderian gland		1 (2%)		
Mediastinum, histiocytic sarcoma			1 (2%)	
Mediastinum, sarcoma, metastatic, uncertain primary site			1 (2%)	
Mediastinum, sarcoma, metastatic, skeletal muscle			1 (2%)	
Nose	(60)	(60)	(59)	(57)
Special Senses System				
Harderian gland	(5)	(1)	(4)	(4)
Adenoma	4 (80%)		1 (25%)	2 (50%)
Carcinoma	1 (20%)	1 (100%)	3 (75%)	2 (50%)
Urinary System				
Kidney	(59)	(56)	(58)	(59)
Carcinoma		1 (2%)		
Histiocytic sarcoma		1 (2%)		
Urinary bladder	(59)	(54)	(57)	(54)
Hemangiosarcoma				1 (2%)
Systemic Lesions				
Multiple organs ^b	(60)	(60)	(60)	(60)
Histiocytic sarcoma		2 (3%)	1 (2%)	
Lymphoma malignant	8 (13%)	7 (12%)	5 (8%)	3 (5%)
Neoplasm Summary				
Total animals with primary neoplasms ^c	33	37	44	56
Total primary neoplasms	42	51	76	146
Total animals with benign neoplasms	21	20	26	33
Total benign neoplasms	26	22	35	38
Total animals with malignant neoplasms	16	23	27	55
Total malignant neoplasms	16	29	41	108
Total animals with metastatic neoplasms		4	6	12
Total metastatic neoplasms		12	11	16
Total animals with malignant neoplasms of uncertain primary site		1	1	2

^a Number of animals examined microscopically at the site and the number of animals with neoplasm

^b Number of animals with any tissue examined microscopically

^c Primary neoplasms: all neoplasms except metastatic neoplasms

TABLE D3
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Harderian Gland: Adenoma				
Overall rate ^a	4/60 (7%)	0/60 (0%)	1/60 (2%)	2/60 (3%)
Adjusted rate ^b	7.1%	0.0%	1.8%	5.0%
Terminal rate ^c	4/52 (8%)	0/46 (0%)	1/47 (2%)	1/5 (20%)
First incidence (days) ^d	729 (T)	—	729 (T)	630
Poly-3 test	P=0.378N	P=0.067N	P=0.175N	P=0.501N
Harderian Gland: Carcinoma				
Overall rate	1/60 (2%)	1/60 (2%)	3/60 (5%)	2/60 (3%)
Adjusted rate	1.8%	1.8%	5.2%	5.0%
Terminal rate	1/52 (2%)	0/46 (0%)	2/47 (4%)	1/5 (20%)
First incidence (days)	729 (T)	644	672	548
Poly-3 test	P=0.190	P=0.752	P=0.313	P=0.388
Harderian Gland: Adenoma or Carcinoma				
Overall rate	5/60 (8%)	1/60 (2%)	4/60 (7%)	4/60 (7%)
Adjusted rate	8.9%	1.8%	7.0%	9.8%
Terminal rate	5/52 (10%)	0/46 (0%)	3/47 (6%)	2/5 (40%)
First incidence (days)	729 (T)	644	672	548
Poly-3 test	P=0.397	P=0.112N	P=0.488N	P=0.575
Large Intestine (Cecum): Carcinoma				
Overall rate	0/60 (0%)	1/60 (2%)	4/60 (7%)	3/60 (5%)
Adjusted rate	0.0%	1.9%	7.0%	7.4%
Terminal rate	0/52 (0%)	1/46 (2%)	3/47 (6%)	1/5 (20%)
First incidence (days)	—	729 (T)	715	619
Poly-3 test	P=0.024	P=0.492	P=0.064	P=0.072
Liver: Hepatocellular Adenoma				
Overall rate	7/60 (12%)	5/59 (8%)	19/59 (32%)	29/60 (48%)
Adjusted rate	12.4%	9.4%	33.5%	62.0%
Terminal rate	7/52 (14%)	5/46 (11%)	17/47 (36%)	5/5 (100%)
First incidence (days)	729 (T)	729 (T)	673	567
Poly-3 test	P<0.001	P=0.419N	P=0.006	P<0.001
Liver: Hepatocellular Carcinoma				
Overall rate	2/60 (3%)	4/59 (7%)	6/59 (10%)	16/60 (27%)
Adjusted rate	3.5%	7.5%	10.6%	36.2%
Terminal rate	1/52 (2%)	4/46 (9%)	4/47 (9%)	1/5 (20%)
First incidence (days)	362	729 (T)	661	567
Poly-3 test	P<0.001	P=0.307	P=0.132	P<0.001
Liver: Hepatocellular Adenoma or Carcinoma				
Overall rate	9/60 (15%)	9/59 (15%)	24/59 (41%)	39/60 (65%)
Adjusted rate	15.7%	16.9%	42.1%	79.1%
Terminal rate	8/52 (15%)	9/46 (20%)	20/47 (43%)	5/5 (100%)
First incidence (days)	362	729 (T)	661	567
Poly-3 test	P<0.001	P=0.538	P<0.001	P<0.001
Lung: Alveolar/bronchiolar Adenoma				
Overall rate	2/60 (3%)	3/60 (5%)	4/59 (7%)	4/57 (7%)
Adjusted rate	3.6%	5.6%	7.1%	10.3%
Terminal rate	2/52 (4%)	3/46 (7%)	3/47 (6%)	0/5 (0%)
First incidence (days)	729 (T)	729 (T)	701	516
Poly-3 test	P=0.126	P=0.481	P=0.339	P=0.190

TABLE D3
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Lung: Alveolar/bronchiolar Carcinoma				
Overall rate	3/60 (5%)	3/60 (5%)	0/59 (0%)	2/57 (4%)
Adjusted rate	5.3%	5.5%	0.0%	5.2%
Terminal rate	3/52 (6%)	2/46 (4%)	0/47 (0%)	0/5 (0%)
First incidence (days)	729 (T)	553	—	488
Poly-3 test	P=0.358N	P=0.647	P=0.120N	P=0.666N
Lung: Alveolar/bronchiolar Adenoma or Carcinoma				
Overall rate	5/60 (8%)	6/60 (10%)	4/59 (7%)	6/57 (11%)
Adjusted rate	8.9%	11.0%	7.1%	15.1%
Terminal rate	5/52 (10%)	5/46 (11%)	3/47 (6%)	0/5 (0%)
First incidence (days)	729 (T)	553	701	488
Poly-3 test	P=0.293	P=0.478	P=0.499N	P=0.273
Mesentery: Hemangiosarcoma				
Overall rate	0/60 (0%)	0/60 (0%)	0/60 (0%)	32/60 (53%)
Adjusted rate	0.0%	0.0%	0.0%	65.5%
Terminal rate	0/52 (0%)	0/46 (0%)	0/47 (0%)	4/5 (80%)
First incidence (days)	—	— ^f	—	507
Poly-3 test	P<0.001	—	—	P<0.001
Pituitary Gland (Pars Distalis or Unspecified Site): Adenoma				
Overall rate	3/58 (5%)	4/58 (7%)	3/53 (6%)	0/53 (0%)
Adjusted rate	5.4%	7.5%	6.0%	0.0%
Terminal rate	2/51 (4%)	3/46 (7%)	3/42 (7%)	0/5 (0%)
First incidence (days)	299	644	729 (T)	—
Poly-3 test	P=0.201N	P=0.474	P=0.611	P=0.221N
Skeletal Muscle: Hemangiosarcoma				
Overall rate	0/60 (0%)	0/60 (0%)	0/60 (0%)	16/60 (27%)
Adjusted rate	0.0%	0.0%	0.0%	35.6%
Terminal rate	0/52 (0%)	0/46 (0%)	0/47 (0%)	0/5 (0%)
First incidence (days)	—	—	—	444
Poly-3 test	P<0.001	—	—	P<0.001
Skeletal Muscle: Sarcoma				
Overall rate	0/60 (0%)	1/60 (2%)	3/60 (5%)	2/60 (3%)
Adjusted rate	0.0%	1.9%	5.2%	5.0%
Terminal rate	0/52 (0%)	1/46 (2%)	0/47 (0%)	0/5 (0%)
First incidence (days)	—	729 (T)	553	583
Poly-3 test	P=0.074	P=0.492	P=0.125	P=0.173
Skin (Subcutaneous Tissue): Hemangiosarcoma				
Overall rate	0/60 (0%)	0/60 (0%)	2/60 (3%)	19/60 (32%)
Adjusted rate	0.0%	0.0%	3.5%	43.2%
Terminal rate	0/52 (0%)	0/46 (0%)	1/47 (2%)	2/5 (40%)
First incidence (days)	—	—	624	409
Poly-3 test	P<0.001	—	P=0.243	P<0.001
Skin (Subcutaneous Tissue): Sarcoma				
Overall rate	1/60 (2%)	1/60 (2%)	6/60 (10%)	2/60 (3%)
Adjusted rate	1.8%	1.9%	10.4%	5.0%
Terminal rate	0/52 (0%)	1/46 (2%)	2/47 (4%)	0/5 (0%)
First incidence (days)	710	729 (T)	661	696
Poly-3 test	P=0.112	P=0.751	P=0.062	P=0.384

TABLE D3
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Uterus: Hemangiosarcoma				
Overall rate	0/60 (0%)	1/60 (2%)	0/60 (0%)	3/60 (5%)
Adjusted rate	0.0%	1.9%	0.0%	7.3%
Terminal rate	0/52 (0%)	1/46 (2%)	0/47 (0%)	0/5 (0%)
First incidence (days)	—	729 (T)	—	522
Poly-3 test	P=0.033	P=0.492	—	P=0.074
All Organs: Hemangioma				
Overall rate	3/60 (5%)	2/60 (3%)	0/60 (0%)	0/60 (0%)
Adjusted rate	5.3%	3.7%	0.0%	0.0%
Terminal rate	3/52 (6%)	2/46 (4%)	0/47 (0%)	0/5 (0%)
First incidence (days)	729 (T)	729 (T)	—	—
Poly-3 test	P=0.043N	P=0.519N	P=0.117N	P=0.195N
All Organs: Hemangiosarcoma				
Overall rate	0/60 (0%)	2/60 (3%)	3/60 (5%)	50/60 (83%)
Adjusted rate	0.0%	3.6%	5.2%	90.2%
Terminal rate	0/52 (0%)	1/46 (2%)	2/47 (4%)	4/5 (80%)
First incidence (days)	—	343	624	409
Poly-3 test	P<0.001	P=0.232	P=0.124	P<0.001
All Organs: Hemangioma or Hemangiosarcoma				
Overall rate	3/60 (5%)	4/60 (7%)	3/60 (5%)	50/60 (83%)
Adjusted rate	5.3%	7.3%	5.2%	90.2%
Terminal rate	3/52 (6%)	3/46 (7%)	2/47 (4%)	4/5 (80%)
First incidence (days)	729 (T)	343	624	409
Poly-3 test	P<0.001	P=0.486	P=0.651N	P<0.001
All Organs: Malignant Lymphoma				
Overall rate	8/60 (13%)	7/60 (12%)	5/60 (8%)	3/60 (5%)
Adjusted rate	14.2%	12.6%	8.7%	7.4%
Terminal rate	7/52 (14%)	5/46 (11%)	3/47 (6%)	0/5 (0%)
First incidence (days)	725	266	671	661
Poly-3 test	P=0.148N	P=0.514N	P=0.266N	P=0.244N
All Organs: Benign Neoplasms				
Overall rate	21/60 (35%)	20/60 (33%)	26/60 (43%)	33/60 (55%)
Adjusted rate	36.4%	36.8%	45.1%	68.5%
Terminal rate	18/52 (35%)	19/46 (41%)	23/47 (49%)	5/5 (100%)
First incidence (days)	299	644	673	516
Poly-3 test	P<0.001	P=0.557	P=0.221	P<0.001
All Organs: Malignant Neoplasms				
Overall rate	16/60 (27%)	23/60 (38%)	27/60 (45%)	55/60 (92%)
Adjusted rate	27.9%	39.3%	45.2%	96.1%
Terminal rate	13/52 (25%)	14/46 (30%)	15/47 (32%)	5/5 (100%)
First incidence (days)	362	266	553	409
Poly-3 test	P<0.001	P=0.136	P=0.039	P<0.001

TABLE D3
Statistical Analysis of Primary Neoplasms in Female Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
All Organs: Benign or Malignant Neoplasms				
Overall rate	33/60 (55%)	37/60 (62%)	44/60 (73%)	56/60 (93%)
Adjusted rate	56.2%	63.2%	73.3%	97.6%
Terminal rate	28/52 (54%)	28/46 (61%)	31/47 (66%)	5/5 (100%)
First incidence (days)	299	266	553	409
Poly-3 test	P<0.001	P=0.281	P=0.038	P<0.001

(T) Terminal sacrifice

^a Number of neoplasm-bearing animals/number of animals examined. Denominator is number of animals examined microscopically for liver, lung, and pituitary gland; for other tissues, denominator is number of animals necropsied.

^b Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality

^c Observed incidence at terminal kill

^d Beneath the control incidence is the P value associated with the trend test. Beneath the exposed group incidence are the P values corresponding to pairwise comparisons between the controls and that exposed group. The Poly-3 test accounts for the differential mortality in animals that do not reach terminal sacrifice. A negative trend or a lower incidence in an exposure group is indicated by N.

^e Not applicable; no neoplasms in animal group

^f Value of statistic cannot be computed.

TABLE D4a
Historical Incidence of Hemangiosarcoma (All Sites) in Control Female B6C3F₁ Mice

Study	Incidence in Controls
Historical Incidence in Controls Given NTP-2000 Diet^a	
Acrylonitrile (gavage)	4/50
Citral (feed)	0/99
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	0/50
Indium phosphide (inhalation)	3/50
60-Hz Magnetic fields (whole body exposure)	2/100
Methacrylonitrile (gavage)	2/50
<i>o</i> -Nitrotoluene (feed)	0/60
<i>p</i> -Nitrotoluene (feed)	1/50
Riddelline (gavage)	0/50
Sodium nitrite (drinking water)	1/50
Vanadium pentoxide (inhalation)	2/50
Overall Historical Incidence in Controls Given NTP-2000 Diet	
Total (%)	15/659 (2.3%)
Mean ± standard deviation	2.6% ± 2.7%
Range	0%-8%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b	
Benzyl acetate	0/50
2,2-Bis(bromomethyl)-1,3-propanediol	0/52
<i>t</i> -Butylhydroquinone	2/51
Emodin	1/50
<i>o</i> -Nitroanisole	1/50
<i>p</i> -Nitrobenzoic acid	4/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet	
Total (%)	31/953 (3.3%)
Mean ± standard deviation	3.3% ± 2.4%
Range	0%-8%

^a Data as of December 22, 2000

^b Data as of December 23, 1999

TABLE D4b
Historical Incidence of Hepatocellular Neoplasms in Control Female B6C3F₁ Mice

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
Historical Incidence in Controls Given NTP-2000 Diet^a			
Acrylonitrile (gavage)	14/50	7/50	20/50
Citral (feed)	8/99	4/99	12/99
<i>p,p'</i> -Dichlorodiphenyl sulfone (feed)	4/50	3/50	6/50
Indium phosphide (inhalation)	12/50	6/50	18/50
60-Hz Magnetic fields (whole body exposure)	17/98	6/98	22/98
Methacrylonitrile (gavage)	9/50	2/50	10/50
<i>o</i> -Nitrotoluene (feed)	7/60	2/60	9/60
<i>p</i> -Nitrotoluene (feed)	6/49	3/49	8/49
Riddelliine (gavage)	9/49	8/49	16/49
Sodium nitrite (drinking water)	9/50	2/50	10/50
Vanadium pentoxide (inhalation)	6/50	6/50	12/50
Overall Historical Incidence in Controls Given NTP-2000 Diet			
Total (%)	101/655 (15.4%)	49/655 (7.5%)	143/655 (21.8%)
Mean ± standard deviation	16.0% ± 6.3%	8.0% ± 4.7%	22.8% ± 9.6%
Range	8%-28%	3%-16%	12%-40%
Historical Incidence in Feed Controls Given NIH-07 Diet at Southern Research Institute^b			
Benzyl acetate	6/50	10/50	14/50
2,2-Bis(bromomethyl)-1,3-propanediol	16/51	5/51	20/51
<i>t</i> -Butylhydroquinone	9/51	8/51	17/51
Emodin	9/50	4/50	13/50
<i>o</i> -Nitroanisole	14/50	5/50	17/50
<i>p</i> -Nitrobenzoic acid	11/50	4/50	15/50
Overall Historical Incidence in Feed Controls Given NIH-07 Diet			
Total (%)	218/951 (22.9%)	104/951 (10.9%)	292/951 (30.7%)
Mean ± standard deviation	22.9% ± 9.9%	10.9% ± 4.6%	30.7% ± 10.2%
Range	12%-50%	4%-20%	12%-56%

^a Data as of December 22, 2000

^b Data as of December 23, 1999

TABLE D5
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Feed Study of o-Nitrotoluene^a

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Disposition Summary				
Animals initially in study	60	60	60	60
Early deaths				
Moribund	4	6	7	22
Natural deaths	4	8	6	33
Survivors				
Terminal sacrifice	52	46	47	5
Animals examined microscopically	60	60	60	60
Alimentary System				
Gallbladder	(54)	(53)	(54)	(31)
Cyst	1 (2%)	1 (2%)		
Intestine large, cecum	(56)	(53)	(54)	(32)
Edema			1 (2%)	
Inflammation, chronic				1 (3%)
Intestine small, jejunum	(56)	(53)	(56)	(34)
Peyer's patch, hyperplasia, lymphoid	1 (2%)		1 (2%)	
Peyer's patch, inflammation, chronic, focal, suppurative		1 (2%)		
Liver	(60)	(59)	(59)	(60)
Angiectasis		1 (2%)		3 (5%)
Basophilic focus	1 (2%)	6 (10%)	2 (3%)	6 (10%)
Clear cell focus	1 (2%)		2 (3%)	4 (7%)
Congestion, focal	1 (2%)	2 (3%)	1 (2%)	
Cyst		1 (2%)		
Eosinophilic focus	2 (3%)	3 (5%)	6 (10%)	28 (47%)
Hyperplasia, focal, lymphoid	2 (3%)	5 (8%)	2 (3%)	1 (2%)
Infiltration cellular, mixed cell	45 (75%)	38 (64%)	37 (63%)	43 (72%)
Inflammation, focal				1 (2%)
Mixed cell focus			1 (2%)	3 (5%)
Necrosis	3 (5%)		2 (3%)	13 (22%)
Tension lipidosis	1 (2%)	1 (2%)		
Bile duct, cyst		1 (2%)		
Hepatocyte, fatty change		1 (2%)		
Hepatocyte, fatty change, diffuse		2 (3%)		
Hepatocyte, necrosis, focal				6 (10%)
Hepatocyte, tension lipidosis				1 (2%)
Hepatocyte, vacuolization cytoplasmic, diffuse		2 (3%)		
Hepatocyte, vacuolization cytoplasmic, focal	1 (2%)	2 (3%)	2 (3%)	9 (15%)
Hepatocyte, periportal, depletion glycogen				1 (2%)
Hepatocyte, centrilobular, fatty change	2 (3%)		2 (3%)	
Hepatocyte, centrilobular, necrosis			1 (2%)	
Oval cell, hyperplasia				4 (7%)
Mesentery	(11)	(9)	(9)	(34)
Hemorrhage			1 (11%)	
Inflammation, chronic	1 (9%)			2 (6%)
Artery, inflammation, chronic			1 (11%)	
Fat, necrosis	6 (55%)	5 (56%)	4 (44%)	
Pancreas	(60)	(57)	(58)	(57)
Acinus, atrophy, diffuse		2 (4%)	1 (2%)	1 (2%)
Acinus, atrophy, focal	1 (2%)			1 (2%)
Duct, cyst	2 (3%)	1 (2%)		
Duct, inflammation, chronic, focal	2 (3%)			

^a Number of animals examined microscopically at the site and the number of animals with lesion

TABLE D5
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Alimentary System (continued)				
Stomach, forestomach	(60)	(58)	(58)	(59)
Diverticulum	1 (2%)			
Edema		1 (2%)		
Inflammation, focal	1 (2%)	2 (3%)	1 (2%)	
Ulcer	1 (2%)	1 (2%)	1 (2%)	
Epithelium, hyperplasia	1 (2%)	4 (7%)	6 (10%)	1 (2%)
Stomach, glandular	(58)	(57)	(57)	(52)
Erosion	1 (2%)			
Inflammation, focal				2 (4%)
Ulcer		1 (2%)	2 (4%)	1 (2%)
Glands, degeneration, cystic, focal			2 (4%)	
Tooth	(8)	(4)		(2)
Peridental tissue, inflammation, chronic	8 (100%)	4 (100%)		2 (100%)
Cardiovascular System				
Blood vessel		(2)	(1)	(1)
Aorta, mineralization		1 (50%)	1 (100%)	
Heart	(60)	(60)	(59)	(58)
Angiectasis				1 (2%)
Infiltration cellular, mixed cell		1 (2%)	1 (2%)	
Inflammation, chronic, focal				2 (3%)
Endocrine System				
Adrenal cortex	(59)	(59)	(58)	(60)
Accessory adrenal cortical nodule	2 (3%)		1 (2%)	
Cyst			2 (3%)	
Cytoplasmic alteration, focal		1 (2%)		
Hyperplasia, focal				1 (2%)
Adrenal medulla	(59)	(57)	(58)	(59)
Hyperplasia	2 (3%)			
Islets, pancreatic	(60)	(57)	(60)	(56)
Atrophy, diffuse		1 (2%)		
Parathyroid gland	(57)	(55)	(57)	(50)
Cyst	1 (2%)	1 (2%)		1 (2%)
Pituitary gland	(58)	(58)	(53)	(53)
Pars distalis, cyst		1 (2%)		
Pars distalis, cytoplasmic alteration, focal	1 (2%)	4 (7%)	3 (6%)	
Pars distalis, hyperplasia, focal	1 (2%)	1 (2%)	2 (4%)	
Thyroid gland	(60)	(59)	(58)	(53)
Degeneration, cystic, focal	14 (23%)	16 (27%)	16 (28%)	13 (25%)
Hyperplasia, lymphoid			1 (2%)	
Inflammation, chronic, focal	2 (3%)	1 (2%)		
Follicle, cyst		1 (2%)		2 (4%)
Follicular cell, hyperplasia	1 (2%)	1 (2%)		

TABLE D5
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
General Body System				
Tissue NOS	(1)		(3)	(3)
Abdominal, cyst			1 (33%)	
Genital System				
Clitoral gland	(58)	(57)	(57)	(58)
Degeneration, cystic	11 (19%)	4 (7%)	5 (9%)	2 (3%)
Inflammation, chronic	3 (5%)	2 (4%)		
Pigmentation	3 (5%)	2 (4%)		1 (2%)
Ovary	(59)	(56)	(56)	(56)
Angiectasis	3 (5%)	2 (4%)	1 (2%)	
Cyst	11 (19%)	16 (29%)	12 (21%)	11 (20%)
Cyst, multiple	1 (2%)	2 (4%)	1 (2%)	
Hemorrhage	1 (2%)	2 (4%)		1 (2%)
Hyperplasia, tubular			1 (2%)	
Mineralization			1 (2%)	
Interstitial cell, hyperplasia			2 (4%)	
Periovarian tissue, cyst		1 (2%)		
Periovarian tissue, inflammation, chronic			1 (2%)	
Uterus	(60)	(59)	(60)	(59)
Angiectasis	1 (2%)		2 (3%)	1 (2%)
Angiectasis, focal		1 (2%)		
Atrophy			1 (2%)	
Congestion		1 (2%)		
Cyst	1 (2%)	1 (2%)	3 (5%)	
Hemorrhage		1 (2%)	1 (2%)	1 (2%)
Hydrometra	24 (40%)	24 (41%)	22 (37%)	15 (25%)
Hyperplasia, focal, histiocytic			1 (2%)	
Inflammation, suppurative	2 (3%)	1 (2%)	1 (2%)	2 (3%)
Pigmentation, focal			1 (2%)	
Endometrium, hyperplasia, cystic	54 (90%)	51 (86%)	53 (88%)	37 (63%)
Hematopoietic System				
Bone marrow	(60)	(58)	(60)	(60)
Hemorrhage, focal			1 (2%)	
Hyperplasia	1 (2%)		2 (3%)	2 (3%)
Inflammation, chronic, focal	1 (2%)			
Myelofibrosis		2 (3%)		
Myelofibrosis, focal	1 (2%)			
Lymph node	(6)	(3)	(10)	(11)
Iliac, hematopoietic cell proliferation				1 (9%)
Iliac, hemorrhage			1 (10%)	
Iliac, hyperplasia			1 (10%)	
Iliac, hyperplasia, lymphoid			2 (20%)	
Inguinal, hyperplasia				1 (9%)
Mediastinal, hematopoietic cell proliferation				1 (9%)
Mediastinal, hemorrhage				3 (27%)
Mediastinal, hyperplasia				1 (9%)
Mediastinal, hyperplasia, lymphoid				2 (18%)
Pancreatic, hyperplasia, lymphoid			1 (10%)	
Renal, hemorrhage			1 (10%)	
Renal, hyperplasia			2 (20%)	1 (9%)
Renal, hyperplasia, lymphoid			1 (10%)	

TABLE D5
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Hematopoietic System (continued)				
Lymph node, mandibular	(58)	(55)	(55)	(48)
Hyperplasia				1 (2%)
Hyperplasia, lymphoid		1 (2%)	1 (2%)	1 (2%)
Lymph node, mesenteric	(53)	(58)	(57)	(57)
Hemorrhage			1 (2%)	1 (2%)
Hyperplasia	2 (4%)	1 (2%)	2 (4%)	4 (7%)
Hyperplasia, lymphoid	6 (11%)	2 (3%)	2 (4%)	2 (4%)
Hyperplasia, mast cell	1 (2%)			
Spleen	(59)	(57)	(58)	(57)
Congestion			2 (3%)	
Depletion cellular				1 (2%)
Fibrosis	1 (2%)			
Hematopoietic cell proliferation	11 (19%)	19 (33%)	21 (36%)	54 (95%)
Hyperplasia, lymphoid	8 (14%)	5 (9%)	4 (7%)	2 (4%)
Pigmentation, focal			1 (2%)	
Thymus	(57)	(56)	(55)	(44)
Angiectasis	1 (2%)		1 (2%)	1 (2%)
Atrophy	1 (2%)			1 (2%)
Cyst	1 (2%)			
Hyperplasia, lymphoid	5 (9%)		2 (4%)	1 (2%)
Integumentary System				
Mammary gland	(60)	(59)	(60)	(59)
Ectasia	1 (2%)		1 (2%)	
Skin	(60)	(60)	(60)	(60)
Subcutaneous tissue, angiectasis, focal			1 (2%)	
Subcutaneous tissue, cyst	1 (2%)			
Subcutaneous tissue, edema		1 (2%)	2 (3%)	4 (7%)
Subcutaneous tissue, hemorrhage, focal	1 (2%)			
Subcutaneous tissue, inflammation, chronic, focal	2 (3%)			1 (2%)
Musculoskeletal System				
Bone	(60)	(60)	(60)	(60)
Callus			1 (2%)	
Fibrous osteodystrophy				1 (2%)
Hyperostosis				2 (3%)
Femur, callus				1 (2%)
Maxilla, hyperostosis, focal	1 (2%)			
Nervous System				
Brain	(60)	(60)	(59)	(58)
Atrophy, focal	1 (2%)		1 (2%)	
Compression, focal			1 (2%)	

TABLE D5
Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the 2-Year Feed Study of o-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Respiratory System				
Lung	(60)	(60)	(59)	(57)
Congestion	1 (2%)	2 (3%)	1 (2%)	1 (2%)
Edema, focal			1 (2%)	
Hemorrhage	2 (3%)	1 (2%)	1 (2%)	2 (4%)
Hyperplasia, focal, histiocytic		2 (3%)		
Hyperplasia, histiocytic	1 (2%)			
Hyperplasia, lymphoid	1 (2%)	1 (2%)	2 (3%)	1 (2%)
Infiltration cellular, mixed cell				9 (16%)
Metaplasia, osseous		1 (2%)		
Alveolar epithelium, hyperplasia		1 (2%)		
Artery, mediastinum, inflammation, chronic				1 (2%)
Bronchus, glands, cyst				1 (2%)
Nose	(60)	(60)	(59)	(57)
Hemorrhage				1 (2%)
Mucosa, glands, dilatation, focal			3 (5%)	1 (2%)
Olfactory epithelium, degeneration		28 (47%)	59 (100%)	57 (100%)
Special Senses System				
None				
Urinary System				
Kidney	(59)	(56)	(58)	(59)
Atrophy, focal	2 (3%)			
Congestion	2 (3%)	3 (5%)	3 (5%)	1 (2%)
Cyst	2 (3%)		2 (3%)	1 (2%)
Hydronephrosis			1 (2%)	
Hyperplasia, lymphoid		4 (7%)	1 (2%)	
Infiltration cellular, mixed cell			1 (2%)	
Inflammation, suppurative				1 (2%)
Mineralization, focal				1 (2%)
Nephropathy	9 (15%)	14 (25%)	10 (17%)	11 (19%)
Pigmentation				1 (2%)
Artery, inflammation, chronic				1 (2%)
Bilateral, atrophy, focal		1 (2%)		
Bilateral, fibrosis, focal		1 (2%)		
Bilateral, inflammation, chronic, focal		1 (2%)		
Pelvis, dilatation		1 (2%)		1 (2%)
Pelvis, hemorrhage				1 (2%)
Renal tubule, accumulation, hyaline droplet	1 (2%)	3 (5%)	2 (3%)	10 (17%)
Renal tubule, casts protein	6 (10%)	8 (14%)	7 (12%)	6 (10%)
Renal tubule, dilatation				1 (2%)
Renal tubule, necrosis			1 (2%)	
Renal tubule, pigmentation		1 (2%)	3 (5%)	35 (59%)
Renal tubule, vacuolization cytoplasmic			1 (2%)	
Urinary bladder	(59)	(54)	(57)	(54)
Transitional epithelium, hyperplasia, focal			1 (2%)	

APPENDIX E

GENETIC TOXICOLOGY

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GENETIC TOXICOLOGY

***SALMONELLA TYPHIMURIUM* MUTAGENICITY TEST PROTOCOL**

Testing was performed as reported by Haworth *et al.* (1983). *o*-Nitrotoluene was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537 either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and five doses of *o*-nitrotoluene. The high dose was limited by toxicity. All trials were repeated.

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

CHINESE HAMSTER OVARY CELL CYTOGENETICS PROTOCOLS

Testing was performed as reported by Galloway *et al.* (1987). *o*-Nitrotoluene was sent to the laboratory as a coded aliquot by Radian Corporation. It was tested in cultured Chinese hamster ovary (CHO) cells for induction of sister chromatid exchanges (SCEs) and chromosomal aberrations (Abs), both in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 and cofactor mix. Cultures were handled under gold lights to prevent photolysis of bromodeoxyuridine-substituted DNA. Each test consisted of concurrent solvent and positive controls and of at least three doses of *o*-nitrotoluene; the high dose was limited by toxicity. A single flask per dose was used, and all tests were repeated.

Sister Chromatid Exchange Test: In the SCE test without S9, CHO cells were incubated for 25.5 hours with *o*-nitrotoluene in supplemented McCoy's 5A medium. Bromodeoxyuridine (BrdU) was added 2 hours after culture initiation. After 25.5 hours, the medium containing *o*-nitrotoluene was removed and replaced with fresh medium plus BrdU and Colcemid, and incubation was continued for 2 hours. Cells were then harvested by mitotic shake-off, fixed, and stained with Hoechst 33258 and Giemsa. In the SCE test with S9, cells were incubated with *o*-nitrotoluene, serum-free medium, and S9 for 2 hours. The medium was then removed and replaced with medium containing serum and BrdU and no *o*-nitrotoluene. Incubation proceeded for an additional 25.5 hours, with Colcemid present for the final 2 hours. Harvesting and staining were the same as for cells treated without S9. All slides were scored blind, and those from a single test were read by the same person. Fifty second-division metaphase cells were scored for frequency of SCEs/cell from each dose level. Because significant chemical-induced cell cycle delay was seen in the trial without S9, incubation time was lengthened 8.5 hours to ensure a sufficient number of scorable (second-division metaphase) cells.

Statistical analyses were conducted on the slopes of the dose-response curves and the individual dose points (Galloway *et al.*, 1987). An SCE frequency 20% above the concurrent solvent control value was chosen as a statistically conservative positive response. The probability of this level of difference occurring by chance at one dose point is less than 0.01; the probability for such a chance occurrence at two dose points is less than 0.001. An increase of 20% or greater at any single dose was considered weak evidence of activity; increases at two or more doses resulted in a determination that the trial was positive. A statistically significant trend ($P < 0.005$) in the absence of any responses reaching 20% above background led to a call of equivocal.

Chromosomal Aberrations Test: In the Abs test without S9, cells were incubated in McCoy's 5A medium with o-nitrotoluene for 8.5 hours; Colcemid was added and incubation continued for 2 hours. The cells were then harvested by mitotic shake-off, fixed, and stained with Giemsa. For the Abs test with S9, cells were treated with o-nitrotoluene and S9 for 2 hours, after which the treatment medium was removed and the cells were incubated for 8.5 hours in fresh medium, with Colcemid present for the final 2 hours. Cells were harvested in the same manner as for the treatment without S9. The harvest time for the Abs test was based on the cell cycle information obtained in the SCE test.

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

Chromosomal aberration data are presented as percentage of cells with aberrations. To arrive at a statistical call for a trial, analyses were conducted on both the dose response curve and individual dose points. For a single trial, a statistically significant ($P \leq 0.05$) difference for one dose point and a significant trend ($P \leq 0.015$) were considered weak evidence for a positive response; significant differences for two or more doses indicated the trial was positive. A positive trend test in the absence of a statistically significant increase at any one dose resulted in an equivocal call (Galloway *et al.*, 1987). Ultimately, the trial calls were based on a consideration of the statistical analyses as well as the biological information available to the reviewers.

RAT AND MOUSE BONE MARROW MICRONUCLEUS TEST PROTOCOL

Preliminary range-finding studies were performed. Factors affecting dose selection included chemical solubility and toxicity and the extent of cell cycle delay induced by o-nitrotoluene exposure. Male F344/N rats were given a single intraperitoneal injection of o-nitrotoluene dissolved in corn oil followed by bone marrow analysis 24 (trial 1) or 48 (trial 2) hours after the injection. The standard three-exposure protocol used for mice is described in detail by Shelby *et al.* (1993). Male B6C3F₁ mice were injected intraperitoneally (three times at 24-hour intervals) with o-nitrotoluene dissolved in corn oil. The mice were killed 24 hours after the third injection. Vehicle control rats and mice were injected with corn oil only. The positive control animals received injections of cyclophosphamide. Blood smears were prepared from bone marrow cells obtained from the femurs. Air-dried smears were fixed and stained; 2,000 polychromatic erythrocytes (PCEs) were scored in up to five animals per dose group.

The results were tabulated as the mean of the pooled results from all animals within a treatment group plus or minus the standard error of the mean. The frequency of micronucleated cells among PCEs was analyzed by a statistical software package that tested for increasing trend over dose groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each dosed group and the control group (ILS, 1990). In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single dosed group is less than or equal to 0.025 divided by the number of dosed groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitudes of those effects.

MOUSE PERIPHERAL BLOOD MICRONUCLEUS TEST PROTOCOL

A detailed discussion of this assay is presented by MacGregor *et al.* (1990). At the end of the 13-week toxicity study, peripheral blood samples were obtained from male and female mice. Smears were immediately prepared

and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange dye and coded. Slides were scanned to determine the frequency of micronuclei in 2,000 normochromatic (mature) erythrocytes (NCEs) in each of 10 animals per dose group.

The results for NCEs were tabulated and analyzed as described for PCEs in the bone marrow micronucleus test. In addition, the percentage of PCEs among the total erythrocyte population in the peripheral blood was scored for each exposure group as a measure of toxicity.

EVALUATION PROTOCOL

These are the basic guidelines for arriving at an overall assay result for assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple aliquots of a chemical were tested in the same assay, and different results were obtained among aliquots and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the *in vitro* assays have another variable that must be considered in arriving at an overall test result. *In vitro* assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The summary table in the Abstract of this Technical Report presents a result that represents a scientific judgement of the overall evidence for activity of the chemical in an assay.

RESULTS

o-Nitrotoluene (3.0 to 1,000 µg/plate) was not mutagenic in *S. typhimurium* strain TA98, TA100, TA1535, or TA1537, with or without Aroclor-induced rat or hamster liver S9 (Table E1; Haworth *et al.*, 1983). Significantly increased SCE frequencies were observed in cultured CHO cells treated with *o*-nitrotoluene with S9; an equivocal response was seen without S9 (Table E2; Galloway *et al.*, 1987). Due to *o*-nitrotoluene-induced cell cycle delay in the trial without S9, an extended culture time was employed to permit accumulation of sufficient second-division metaphase cells for analysis. *o*-Nitrotoluene did not induce Abs in cultured CHO cells with or without S9 (Table E3; Galloway *et al.*, 1987). No increases in the frequencies of micronucleated polychromatic erythrocytes were observed *in vivo* in the bone marrow of male rats or male mice treated with *o*-nitrotoluene. In male F344/N rats, an acute micronucleus test was performed using two protocols (Table E4). The initial test used a single intraperitoneal injection of *o*-nitrotoluene followed by bone marrow analysis 24 hours later; in the second test, bone marrow was harvested for analysis 48 hours after a single intraperitoneal injection of *o*-nitrotoluene. In neither test was a positive response observed. In male mice, a three-intraperitoneal injection protocol also yielded negative results, although a small increase in the frequency of micronucleated PCEs was observed at all doses tested (Table E5). *o*-Nitrotoluene, administered in feed for 13 weeks, did not increase the frequency of micronucleated NCEs in peripheral blood of female mice. However, a small increase in the frequency of micronucleated NCEs was noted in male mice at the highest dose tested, 10,000 ppm (Table E6). The increase in male mice was sufficient to generate a significant trend test ($P=0.003$), but because none of the frequencies in individual dose groups were significantly increased over the corresponding control group and because the increase in the frequency of micronuclei in the 10,000 ppm group was small, the test in male mice was judged to be equivocal.

TABLE E1
Mutagenicity of *o*-Nitrotoluene in *Salmonella typhimurium*^a

Strain	Dose (µg/plate)	Revertants/Plate ^b					
		-S9		+10% hamster S9		+10% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Study performed at SRI International							
TA100	0.0	145 ± 1.5	120 ± 4.7	160 ± 11.8	126 ± 9.1	154 ± 16.5	117 ± 7.8
	3.0	151 ± 6.1	118 ± 8.5				
	10.0	142 ± 7.8	105 ± 4.7	149 ± 5.4	133 ± 4.7	150 ± 7.6	130 ± 3.8
	33.0	139 ± 12.7	110 ± 7.5	148 ± 15.8	125 ± 0.9	126 ± 12.7	133 ± 7.5
	100.0	146 ± 12.4	104 ± 11.2	154 ± 5.5	125 ± 13.9	134 ± 7.4	147 ± 5.3
	333.0	45 ± 5.9 ^c	Toxic	156 ± 7.4	125 ± 7.0	111 ± 6.3	114 ± 1.9
	666.0				137 ± 5.7		119 ± 2.5
	1,000.0			Toxic		Toxic	
	Trial summary	Negative	Negative	Negative	Negative	Negative	Negative
	Positive control ^d	321 ± 11.7	424 ± 16.2	2,113 ± 4.8	1,895 ± 83.7	1,055 ± 61.4	900 ± 15.3
TA1535	0.0	21 ± 3.7	15 ± 1.7	10 ± 1.7	12 ± 2.1	7 ± 2.5	10 ± 1.9
	3.0	22 ± 3.7	10 ± 1.5				
	10.0	22 ± 3.0	11 ± 1.2	7 ± 2.0	6 ± 1.7	9 ± 1.5	10 ± 2.8
	33.0	20 ± 5.5	14 ± 1.5	9 ± 3.2	10 ± 2.6	8 ± 2.5	10 ± 2.1
	100.0	20 ± 3.9	18 ± 5.2	11 ± 3.5	10 ± 1.5	7 ± 2.3	7 ± 2.9
	333.0	2 ± 1.2 ^c	0 ± 0.0 ^c	7 ± 0.9	8 ± 0.7	11 ± 1.9	11 ± 1.5
	666.0				Toxic		9 ± 3.0
	1,000.0			3 ± 1.8 ^c		Toxic	
	Trial summary	Negative	Negative	Negative	Negative	Negative	Negative
	Positive control	384 ± 17.9	396 ± 2.3	429 ± 31.8	507 ± 35.4	255 ± 18.4	313 ± 77.0
TA1537	0.0	8 ± 0.6	5 ± 2.6	5 ± 1.9	8 ± 2.6	13 ± 2.9	6 ± 0.9
	3.0	12 ± 3.0	4 ± 0.6				
	10.0	9 ± 2.6	3 ± 0.7	7 ± 2.5	9 ± 2.3	11 ± 2.5	5 ± 1.3
	33.0	9 ± 0.0	4 ± 0.3	6 ± 0.9	6 ± 1.2	9 ± 1.5	6 ± 0.7
	100.0	5 ± 0.6 ^c	3 ± 0.9	7 ± 1.0	8 ± 2.6	8 ± 0.7	8 ± 0.7
	333.0	0 ± 0.0 ^c	Toxic	8 ± 2.6	6 ± 1.5	6 ± 1.5	6 ± 1.0
	666.0				Toxic		6 ± 1.3
	1,000.0			Toxic		0 ± 0.0 ^c	
	Trial summary	Negative	Negative	Negative	Negative	Negative	Negative
	Positive control	99 ± 3.5	100 ± 18.2	385 ± 63.6	353 ± 32.0	238 ± 4.4	283 ± 14.2
TA98	0.0	25 ± 0.9	22 ± 2.7	28 ± 1.9	28 ± 4.6	38 ± 6.7	24 ± 2.6
	3.0	25 ± 0.6	24 ± 1.9				
	10.0	24 ± 3.7	16 ± 0.9	22 ± 2.2	35 ± 1.7	28 ± 4.3	29 ± 2.3
	33.0	24 ± 3.3	17 ± 1.5	25 ± 4.1	29 ± 1.8	26 ± 4.1	30 ± 0.9
	100.0	19 ± 3.5	36 ± 22.3	25 ± 2.1	27 ± 1.3	27 ± 1.2	34 ± 2.7
	333.0	Toxic	0 ± 0.0 ^c	27 ± 0.7	28 ± 2.6 ^c	21 ± 3.7	31 ± 3.8
	666.0				0 ± 0.0 ^c		32 ± 0.5
	1,000.0			10 ± 5.4 ^c		0 ± 0.0 ^c	
	Trial summary	Negative	Negative	Negative	Negative	Negative	Negative
	Positive control	830 ± 33.4	760 ± 8.0	1,845 ± 75.2	1,761 ± 147.7	613 ± 12.5	640 ± 8.7

TABLE E1
Mutagenicity of o-Nitrotoluene in *Salmonella typhimurium*

Strain	Dose (µg/plate)	Revertants/Plate					
		-S9		+10% hamster S9		+10% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Study performed at EG&G Mason Research Institute							
TA100	0.0	122 ± 9.1	138 ± 16.3	79 ± 2.6	134 ± 6.2	86 ± 10.1	123 ± 7.9
	3.3		135 ± 4.3		127 ± 2.3		149 ± 7.8
	10.0	125 ± 3.5	139 ± 5.8	89 ± 3.5	118 ± 7.6	78 ± 3.2	128 ± 3.2
	33.0	104 ± 1.2	122 ± 6.9	76 ± 3.7	134 ± 2.5	109 ± 6.2	147 ± 21.7
	100.0	113 ± 3.7	121 ± 11.1	89 ± 3.5 ^c	135 ± 9.3	118 ± 6.1	142 ± 5.2
	333.0	83 ± 1.9 ^c	132 ± 9.2 ^c	90 ± 7.3 ^c	148 ± 3.3	111 ± 7.2 ^c	115 ± 11.0
	666.0	Toxic		Toxic		Toxic	
	Trial summary	Negative	Negative	Negative	Negative	Negative	Negative
Positive control	2,037 ± 65.0	2,103 ± 44.5	1,741 ± 95.2	1,900 ± 92.3	1,045 ± 81.2	991 ± 44.5	
TA1535	0.0	22 ± 2.0	25 ± 3.8	9 ± 1.3	14 ± 1.7	15 ± 1.5	10 ± 0.6
	3.3		26 ± 2.5		15 ± 3.2		10 ± 1.2
	10.0	37 ± 3.6	23 ± 3.3	10 ± 1.8	17 ± 2.5	12 ± 2.3	9 ± 1.7
	33.0	28 ± 3.2	24 ± 1.7	10 ± 1.9	20 ± 1.5	12 ± 0.6	10 ± 3.3
	100.0	28 ± 1.7	22 ± 4.9	8 ± 3.0	16 ± 1.5	13 ± 1.5	19 ± 2.0
	333.0	29 ± 2.9 ^c	23 ± 2.8 ^c	11 ± 2.0 ^c	16 ± 2.5	12 ± 3.8 ^c	12 ± 1.2
	666.0	Toxic		Toxic		Toxic	
	Trial summary	Negative	Negative	Negative	Negative	Negative	Equivocal
Positive control	1,380 ± 77.8	1,320 ± 43.1	74 ± 5.6	128 ± 6.3	110 ± 7.0	108 ± 5.5	
TA1537	0.0	6 ± 0.7	8 ± 1.5	6 ± 0.6	13 ± 1.2	8 ± 1.0	10 ± 1.5
	3.3		8 ± 2.1		9 ± 2.1		8 ± 0.9
	10.0	5 ± 1.2	5 ± 1.8	6 ± 0.6	12 ± 0.0	8 ± 0.3	6 ± 1.2
	33.0	6 ± 1.0	6 ± 1.2	13 ± 0.9	9 ± 0.6	8 ± 1.3	7 ± 2.1
	100.0	6 ± 1.3	8 ± 1.3	9 ± 3.1	8 ± 1.2	6 ± 1.2	6 ± 0.9
	333.0	7 ± 1.7 ^c	6 ± 0.3 ^c	10 ± 2.7 ^c	11 ± 0.9	5 ± 0.9 ^c	7 ± 0.9
	666.0	Toxic		6 ± 2.1 ^c		6 ± 0.3 ^c	
	Trial summary	Negative	Negative	Negative	Negative	Negative	Negative
Positive control	361 ± 85.0	901 ± 105.9	118 ± 12.0	173 ± 7.2	123 ± 2.3	71 ± 4.9	
TA98	0.0	16 ± 1.2	14 ± 2.7	31 ± 1.5	28 ± 1.2	20 ± 2.3	25 ± 0.7
	3.3		20 ± 3.8		31 ± 1.9		29 ± 1.2
	10.0	20 ± 2.7	16 ± 1.9	29 ± 2.6	31 ± 7.1	22 ± 3.5	27 ± 0.3
	33.0	18 ± 3.2	22 ± 1.2	32 ± 3.1	32 ± 1.9	23 ± 1.2	27 ± 5.2
	100.0	18 ± 4.4	20 ± 1.2	26 ± 6.4	34 ± 2.3	24 ± 4.0	25 ± 1.9
	333.0	14 ± 0.6 ^c	12 ± 1.8 ^c	27 ± 2.3	36 ± 3.5	27 ± 3.5 ^c	27 ± 3.7
	666.0	Toxic		Toxic		Toxic	
	Trial summary	Negative	Negative	Negative	Negative	Negative	Negative
Positive control	1,661 ± 63.7	2,119 ± 51.4	1,189 ± 54.6	1,454 ± 95.4	1,173 ± 31.0	874 ± 29.2	

^a The detailed protocol and these data are presented by Haworth *et al.* (1983).

^b Revertants are presented as mean ± standard error from three plates.

^c Slight toxicity

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

TABLE E2
Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by *o*-Nitrotoluene^a

Compound	Dose (µg/mL)	Total Cells Scored	No. of Chromosomes	No. of SCEs	SCEs/Chromosome	SCEs/Cell	Hrs in BrdU	Relative Change of SCEs/Chromosome ^b (%)
-S9								
Summary: Equivocal								
Dimethylsulfoxide ^c		50	1,035	485	0.46	9.7	25.5	
<i>o</i> -Nitrotoluene	117.000	50	1,039	569	0.54	11.4	34.0 ^d	16.87
	176.000	50	1,021	557	0.54	11.1	34.0 ^d	16.42
	218.000	50	1,035	576	0.55	11.5	34.0 ^d	18.76
	282.000	0						
					P=0.005 ^e			
Mitomycin-C ^f	0.005	50	1,037	2,236	2.15	44.7	25.5	360.15
+S9								
Summary: Positive								
Dimethylsulfoxide		50	1,050	380	0.36	7.6	25.5	
<i>o</i> -Nitrotoluene	354.83	50	1,050	499	0.47	10.0	25.5	31.32*
	380.95	50	1,050	467	0.44	9.3	25.5	22.90*
	423.28	50	1,047	488	0.46	9.8	25.5	28.79*
					P=0.001			
Cyclophosphamide ^f	1.50	50	1,049	1,651	1.57	33.0	25.5	334.89

* Positive (≥20% increase over solvent control)

^a Study was performed at Litton Bionetics, Inc. The detailed protocol and these data are presented by Galloway *et al.* (1987). SCE=sister chromatid exchange; BrdU=bromodeoxyuridine

^b SCEs/chromosome in treated cells versus SCEs/chromosome in solvent control cells

^c Solvent control

^d Due to cell cycle delay, harvest time was extended to maximize the proportion of second-division metaphase cells available for analysis.

^e Significance of SCEs/chromosome tested by the linear regression trend test versus log of the dose

^f Positive control

TABLE E3
Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by o-Nitrotoluene^a

Compound	Dose (µg/mL)	Total Cells Scored	Number of Abberations	Aberrations/Cell	Cells with Aberrations (%)
-S9					
Harvest time: 10.5 hours					
Summary: Negative					
Dimethylsulfoxide ^b		100	3	0.03	3.0
o-Nitrotoluene	200.7	100	2	0.02	2.0
	252.5	100	1	0.01	1.0
	393.6	100	1	0.01	1.0
					P=0.873 ^c
Mitomycin-C ^d	0.5	100	20	0.20	16.0
+S9					
Harvest time: 10.5 hours					
Summary: Negative					
Dimethylsulfoxide		100	5	0.05	4.0
o-Nitrotoluene	375.36	100	3	0.03	2.0
	398.82	100	9	0.09	8.0
	422.28	100	5	0.05	5.0
					P=0.168
Cyclophosphamide ^d	50	100	30	0.30	20.0

^a Study was performed at Litton Bionetics, Inc. The detailed protocol and these data are presented by Galloway *et al.* (1987).

^b Solvent control

^c Significance of percent cells with aberrations tested by the linear regression trend test versus log of the dose

^d Positive control

TABLE E4
Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Rats
Treated with *o*-Nitrotoluene by Intraperitoneal Injection^a

Compound	Dose (mg/kg)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs ^b	P Value ^c
Trial 1 (24-hour sample)				
Corn oil ^d	0	5	0.60 ± 0.19	
<i>o</i> -Nitrotoluene	625	5	1.50 ± 0.76	0.0247
	1,250	3	0.33 ± 0.33	0.7675
	2,500	5	0.80 ± 0.25	0.2964
			P=0.609 ^e	
Cyclophosphamide ^f	25	5	9.80 ± 2.18	0.0000
Trial 2 (48-hour sample)				
Corn oil	0	5	1.10 ± 0.19	
<i>o</i> -Nitrotoluene	625	5	1.30 ± 0.41	0.3415
	2,500	4	0.75 ± 0.25	0.7763
			P=0.814	
Cyclophosphamide	25	5	13.40 ± 1.76	0.0000

^a Study was performed at Integrated Laboratory Systems, Inc. PCE=polychromatic erythrocyte

^b Mean ± standard error

^c Pairwise comparison with the vehicle control. Dosed group values are significant at P≤0.008 (trial 1) or P≤0.013 (trial 2); positive control values are significant at P≤0.05 (ILS, 1990).

^d Vehicle control

^e Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test; significant at P≤0.025 (ILS, 1990)

^f Positive control

TABLE E5
Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Mice
Treated with o-Nitrotoluene by Intraperitoneal Injection^a

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated PCEs/1,000 PCEs ^b	P Value ^c
Corn oil ^d	0	5	0.90 ± 0.10	
o-Nitrotoluene	100	5	1.50 ± 0.16	0.1102
	200	5	1.30 ± 0.60	0.1968
	300	5	1.60 ± 0.37	0.0806
	400	5	1.80 ± 0.30	0.0415
			P=0.055 ^e	
Cyclophosphamide ^f	25	5	6.20 ± 1.15	0.0000

^a Study was performed at Integrated Laboratory Systems, Inc. The detailed protocol is presented by Shelby *et al.* (1993).

PCE=polychromatic erythrocyte

^b Mean ± standard error

^c Pairwise comparison with the vehicle control. Dosed group values are significant at $P \leq 0.006$; positive control value is significant at $P \leq 0.05$ (ILS, 1990).

^d Vehicle control

^e Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test; significant at $P \leq 0.025$ (ILS, 1990)

^f Positive control

TABLE E6
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Mice
Following Administration of *o*-Nitrotoluene in Feed for 13 Weeks^a

Concentration (ppm)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/1,000 NCEs ^b	P Value ^c	PCEs (%)
Male				
0	10	0.70 ± 0.21		1.6
625	10	0.60 ± 0.15	0.6526	1.5
1,250	10	0.90 ± 0.18	0.2397	1.4
2,500	10	0.85 ± 0.24	0.2949	1.3
5,000	10	0.90 ± 0.15	0.2397	1.7
10,000	10	1.40 ± 0.23	0.0153	1.1
		P=0.003 ^d		
Female				
0	10	0.40 ± 0.12		1.5
625	10	0.55 ± 0.17	0.2456	1.6
1,250	10	0.15 ± 0.08	0.9342	1.5
2,500	10	0.40 ± 0.16	0.5000	1.6
5,000	10	0.30 ± 0.11	0.7035	1.4
10,000	10	0.30 ± 0.11	0.7035	1.0
		P=0.750		

^a Study was performed at SITEK Research Laboratories, Inc. The detailed protocol is presented by MacGregor *et al.* (1990).

NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte

^b Mean ± standard error

^c Pairwise comparison with the controls, significant at P≤0.005 (ILS, 1990)

^d Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test; significant at P≤0.025 (ILS, 1990)

APPENDIX F
***o*-NITROBENZOIC ACID,**
***o*-NITROBENZYL MERCAPTURIC ACID,**
AND *o*-AMINOBENZOIC ACID —
BIOMARKERS OF EXPOSURE

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***o*-NITROBENZOIC ACID, *o*-NITROBENZYL MERCAPTURIC ACID, AND *o*-AMINO BENZOIC ACID — BIOMARKERS OF EXPOSURE**

INTRODUCTION

Biotransformation studies of *o*-nitrotoluene in rats indicate only one major route of metabolism: oxidation of the methyl group and further modification of the benzyl alcohol thus formed. A small percentage (less than 2%) of the urinary metabolites resulted from nitro-group reduction (Chism *et al.*, 1984). To determine how the relative contribution of these pathways may change with exposure concentration and age, three urinary metabolites were followed during the 2-year *o*-nitrotoluene studies, *o*-nitrobenzoic acid, *o*-nitrobenzylmercapturic acid and *o*-aminobenzoic acid.

MATERIALS AND METHODS

Urinary metabolites were quantitated by high-performance liquid chromatography using a C₁₈ column. For *o*-nitrobenzoic acid and *o*-nitrobenzylmercapturic acid, the solvent system was A) water:methanol:trifluoroacetic acid (850:150:1) and B) water:methanol:trifluoroacetic acid (500:500:1) at a flow rate of 1 mL per minute; the pH was adjusted to 2.3 with triethylamine. The solvent system program was 50% A:50% B for 1 minute, followed by a linear increase to 23% A:77% B over 19 minutes. Detection was by ultraviolet absorption (266 nm for *o*-nitrobenzoic acid and *o*-nitrobenzylmercapturic acid; 254 nm for *o*-aminobenzoic acid). The limits of quantitation were 0.0321 mg/mL for *o*-nitrobenzoic acid, 0.0438 mg/mL for *o*-nitrobenzylmercapturic acid, and 0.0517 mg/mL for *o*-aminobenzoic acid.

RESULTS AND DISCUSSION

Comparisons among the metabolite data were made using the metabolite/creatinine ratio obtained by dividing the metabolite concentration by the creatinine concentration. Creatinine excretion is considered related to muscle mass. Thus, normalizing the metabolite data to creatinine in effect normalizes the metabolite to body weight. This step was considered necessary because comparisons are being made across time as the animals' weight changes and because males are generally heavier than females. The efficiency of urine collection in a metabolism cage is not 100%, so calculation of total metabolite based on the amount of urine collected has some uncertainty. Basing the comparison on concentrations of creatinine and metabolite in measure aliquots eliminates the need to know the total urine output and the associated uncertainties.

The ratios of *o*-nitrobenzoic acid to creatinine excreted in urine by rats are shown in Table F1. With only one exception, the ratios for male rats were larger at the 2-week time point than at later time points. In females, by contrast, the difference between the time points was generally not significant. The ratios were generally larger for females than males. The ratios of *o*-nitrobenzoic acid to creatinine were linear with exposure concentration.

In contrast to the *o*-nitrobenzoic acid/creatinine ratios, larger ratios of *o*-nitrobenzylmercapturic acid to creatinine were seen in urine at week 2 compared to later time points in all exposed groups, with one exception (Table F1). The ratios were significantly smaller for females than males. The *o*-nitrobenzylmercapturic acid/creatinine ratios were linear with exposure concentration.

The ratios of *o*-aminobenzoic acid to creatinine excreted in urine by control rats were similar to ratios seen in exposed groups of animals in many cases (Table F1). *o*-Aminobenzoic acid is a product of catabolism of

tryptophan (White *et al.*, 1978) and is a relatively minor metabolite of *o*-nitrotoluene (Chism *et al.*, 1984). No further analyses of these data were performed.

The concentrations of *o*-aminobenzoic acid and *o*-nitrobenzylmercapturic acid in urine of mice were generally below the limit of quantitation (Table F2). At time points with sufficient data to make determinations, the ratios of urinary *o*-nitrobenzoic acid to creatinine appeared to increase with increasing exposure concentration. Because of the large standard errors, no statistical analyses were attempted.

There appeared to be a change in metabolism of *o*-nitrotoluene to *o*-nitrobenzoic acid in male, but not female, rats after week 2 (Table F1). The relative concentrations of *o*-nitrobenzoic acid were generally greater for females. There was no evidence for nonlinear metabolism with respect to exposure concentration.

In contrast to *o*-nitrobenzoic acid formation, there appeared to be a change in metabolism of *o*-nitrotoluene with respect to formation of *o*-nitrobenzylmercapturic acid after week 2 in both sexes of rats. Because the first step in the formation of either metabolite is oxidation of the methyl group to a benzyl alcohol, metabolic differences must be either in further oxidation to the carboxylic acid or in formation of conjugates of the alcohol and further reaction with reduced glutathione. The relative concentrations of the mercapturic acid were lower for female rats than for males. The relative concentrations of *o*-nitrobenzoic acid, by contrast, were higher for females than for males. Again, because the first step in formation of both metabolites is oxidation of the methyl group to a benzylic alcohol, these observations are consistent with a sex-related difference in one of the later metabolic steps that favors formation of the mercapturic acid in males.

TABLE F1
Urinary Biomarker Data for Rats in the 2-Year Feed Study of o-Nitrotoluene^a

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Male				
n	5	5	5	5
Volume (mL/24 hours)				
Week 2	3.2 ± 0.4	4.2 ± 0.6	3.0 ± 0.4	4.2 ± 0.4
Month 3	5.7 ± 1.7	5.0 ± 0.8	3.8 ± 0.4	4.5 ± 0.4
Month 12	3.4 ± 0.3	2.9 ± 0.4	4.5 ± 1.6	3.2 ± 0.6
Month 18	5.6 ± 1.2	3.8 ± 0.3	5.8 ± 1.3	5.2 ± 1.6
Creatinine (mg/dL)				
Week 2	186 ± 15	210 ± 58	212 ± 23	196 ± 25
Month 3	244 ± 43	280 ± 25	274 ± 36	265 ± 37
Month 12	356 ± 35	401 ± 19	326 ± 36	375 ± 59
Month 18	215 ± 45	266 ± 34	224 ± 12	190 ± 18
o-Nitrobenzoic acid (mg/24 hours)				
Week 2	0.131 ± 0.017	2.24 ± 0.17	3.66 ± 0.51	7.61 ± 0.66
Month 3	0 ± 0	2.77 ± 0.43	4.65 ± 0.63	6.05 ± 0.42
Month 12	0 ± 0	1.96 ± 0.27	4.05 ± 0.80	5.74 ± 0.37
Month 18	0 ± 0	1.75 ± 0.30	3.67 ± 0.71	7.06 ± 1.96
o-Nitrobenzoic acid/creatinine ratio				
Week 2	0.0222 ± 0.0010	0.316 ± 0.051	0.595 ± 0.046	0.965 ± 0.075
Month 3	0 ± 0	0.204 ± 0.019*	0.451 ± 0.038*	0.543 ± 0.059*
Month 12	0 ± 0	0.173 ± 0.018*	0.315 ± 0.015*	0.534 ± 0.031*
Month 18	0 ± 0	0.175 ± 0.011*	0.289 ± 0.021*	0.842 ± 0.129
o-Nitrobenzylmercapturic acid (mg/24 hours)				
Week 2	0 ± 0	0.739 ± 0.102	1.23 ± 0.06	2.13 ± 0.12
Month 3	0 ± 0	1.12 ± 0.16	1.55 ± 0.28	2.07 ± 0.19
Month 12	0 ± 0	0.628 ± 0.113	1.34 ± 0.31	1.81 ± 0.13
Month 18	0 ± 0	0.337 ± 0.095	0.749 ± 0.119	1.65 ± 0.45
o-Nitrobenzylmercapturic acid/creatinine ratio				
Week 2	0 ± 0	0.101 ± 0.014	0.206 ± 0.013	0.281 ± 0.018
Month 3	0 ± 0	0.0824 ± 0.0056	0.152 ± 0.015*	0.182 ± 0.014*
Month 12	0 ± 0	0.0543 ± 0.0071*	0.103 ± 0.014*	0.164 ± 0.014*
Month 18	0 ± 0	0.0299 ± 0.0076*	0.0653 ± 0.0099*	0.163 ± 0.014*
o-Aminobenzoic acid (mg/24 hours)				
Week 2	0.221 ± 0.139	0.0527 ± 0.0323	0.0349 ± 0.0349	0.258 ± 0.122
Month 3	0.131 ± 0.094	0.304 ± 0.183	0.227 ± 0.092	0.277 ± 0.158
Month 12	0.110 ± 0.049	0.191 ± 0.031	0.241 ± 0.047	0.143 ± 0.059
Month 18	0 ± 0	0 ± 0	0 ± 0	0 ± 0
o-Aminobenzoic acid/creatinine ratio				
Week 2	0.0291 ± 0.0178	0.00810 ± 0.00532	0.00600 ± 0.00600	0.0321 ± 0.0136
Month 3	0.0115 ± 0.0081	0.0256 ± 0.0135	0.0218 ± 0.0098	0.0269 ± 0.0167
Month 12	0.00935 ± 0.00427	0.0168 ± 0.0031	0.0196 ± 0.0036	0.0128 ± 0.0049
Month 18	0 ± 0	0 ± 0	0 ± 0	0 ± 0

TABLE F1
Urinary Biomarker Data for Rats in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	625 ppm	1,250 ppm	2,000 ppm
Female				
n	5	5	5	5
Volume (mL/24 hours)				
Week 2	3.4 ± 0.2	4.6 ± 0.5	3.0 ± 0.6	4.6 ± 0.7
Month 3	3.4 ± 0.5	3.3 ± 0.5	3.9 ± 0.8	4.2 ± 0.7
Month 12	3.6 ± 0.4	3.3 ± 0.4	3.6 ± 1.4	2.0 ± 0.5
Month 18	5.5 ± 1.0	4.4 ± 0.8	6.5 ± 0.7	4.9 ± 1.0
Creatinine (mg/dL)				
Week 2	137 ± 20	146 ± 17	159 ± 14	103 ± 4
Month 3	169 ± 21	206 ± 17	182 ± 8	154 ± 20
Month 12	213 ± 46	247 ± 17	170 ± 25	245 ± 38
Month 18	170 ± 16	186 ± 14	198 ± 8	177 ± 16
<i>o</i> -Nitrobenzoic acid (mg/24 hours)				
Week 2	0.140 ± 0.030	2.64 ± 0.26	3.95 ± 0.75	7.30 ± 1.06
Month 3	0.0561 ± 0.0161	2.62 ± 0.34	5.57 ± 1.09	7.51 ± 0.97
Month 12	0 ± 0	2.94 ± 0.39	4.39 ± 1.18	3.52 ± 0.40
Month 18	0.0115 ± 0.0115	2.84 ± 0.613	6.30 ± 0.34	6.26 ± 0.93
<i>o</i> -Nitrobenzoic acid/creatinine ratio				
Week 2	0.0301 ± 0.0035	0.430 ± 0.072	0.861 ± 0.088 [▲]	1.55 ± 0.05 [▲]
Month 3	0.00952 ± 0.00255	0.392 ± 0.015 [▲]	0.789 ± 0.065 [▲]	1.26 ± 0.13 [▲]
Month 12	0 ± 0	0.369 ± 0.048 [▲]	0.865 ± 0.080 [▲]	0.868 ± 0.087 ^{*▲}
Month 18	0.00146 ± 0.00146	0.345 ± 0.030 [▲]	0.510 ± 0.045 ^{*▲}	0.748 ± 0.029 [*]
<i>o</i> -Nitrobenzylmercapturic acid (mg/24 hours)				
Week 2	0 ± 0	0.216 ± 0.029	0.472 ± 0.100	0.654 ± 0.102
Month 3	0 ± 0	0.107 ± 0.043	0.361 ± 0.094	0.518 ± 0.068
Month 12	0 ± 0	0.0956 ± 0.0299	0.136 ± 0.067	0.209 ± 0.024
Month 18	0.0679 ± 0.0679	0 ± 0	0.176 ± 0.079	0.370 ± 0.122
<i>o</i> -Nitrobenzylmercapturic acid/creatinine ratio				
Week 2	0 ± 0	0.0347 ± 0.0063 [▲]	0.101 ± 0.010 [▲]	0.138 ± 0.007 [▲]
Month 3	0 ± 0	0.0151 ± 0.0064 ^{*▲}	0.0485 ± 0.0047 ^{*▲}	0.0867 ± 0.0085 ^{*▲}
Month 12	0 ± 0	0.0113 ± 0.0030 ^{*▲}	0.0344 ± 0.0122 ^{*▲}	0.0519 ± 0.0067 ^{*▲}
Month 18	0.0065 ± 0.0064	0 ± 0 ^{*▲}	0.0160 ± 0.0076 ^{*▲}	0.0413 ± 0.0106 ^{*▲}
<i>o</i> -Aminobenzoic acid (mg/24 hours)				
Week 2	0.120 ± 0.038	0.305 ± 0.208	0.331 ± 0.208	0.362 ± 0.137
Month 3	0 ± 0	0.094 ± 0.057	0.0429 ± 0.0429	0 ± 0
Month 12	0.0214 ± 0.0214	0.176 ± 0.042	0.158 ± 0.075	0.0701 ± 0.0345
Month 18	0 ± 0	0 ± 0	0 ± 0	0 ± 0
<i>o</i> -Aminobenzoic acid/creatinine ratio				
Week 2	0.0303 ± 0.0100	0.0338 ± 0.0185	0.0687 ± 0.0335	0.0749 ± 0.0226
Month 3	0 ± 0	0.0108 ± 0.0053	0.00464 ± 0.00464	0 ± 0
Month 12	0.00285 ± 0.00285	0.0213 ± 0.0036	0.0368 ± 0.0158	0.0158 ± 0.0080
Month 18	0 ± 0	0 ± 0	0 ± 0	0 ± 0

* Significantly different (P ≤ 0.05) from corresponding 2-week data by ANOVA by Fisher's least significant difference test

▲ Significantly different (P ≤ 0.05) from corresponding data for males by ANOVA by Fisher's least significant difference test

^a Data are presented as mean ± standard error.

TABLE F2
Urinary Biomarker Data for Mice in the 2-Year Feed Study of o-Nitrotoluene^a

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Male				
n				
Week 2	3	2	0	3
Month 3	4	5	5	5
Month 12	5	5	5	5 _b
Month 18	5	5	5	0 ^b
Volume (mL/24 hours)				
Week 2	0.5 ± 0.3	0.4 ± 0.1	—	0.3 ± 0.1
Month 3	0.6 ± 0.1	0.8 ± 0.2	1.0 ± 0.2	0.9 ± 0.1
Month 12	1.0 ± 0.1	0.7 ± 0.1 ^c	0.8 ± 0.1	1.2 ± 0.1
Month 18	1.2 ± 0.2	1.5 ± 0.1 ^c	0.9 ± 0.2	—
Creatinine (mg/dL)				
Week 2	36.7 ± 8.5	40.5 ± 7.8	—	40.5 ± 5.5
Month 3	39.5 ± 2.0	54.3 ± 12.9	38.1 ± 6.6	38.9 ± 3.0
Month 12	53.2 ± 7.8	58.6 ± 15.5 ^c	50.8 ± 2.9	33.2 ± 2.0
Month 18	48.9 ± 6.7	49.5 ± 2.6 ^c	42.2 ± 4.8	—
o-Nitrobenzoic acid (mg/24 hours)				
Week 2	0.0164 ± 0.0067	0.452 ± 0.068	—	1.61 ± 0.18
Month 3	0 ± 0	0.378 ± 0.077	1.06 ± 0.04	1.50 ± 0.25
Month 12	0 ± 0	0.161 ± 0.043	0.793 ± 0.103	0.777 ± 0.185
Month 18	0.0134 ± 0.0017	0.418 ± 0.077	0.448 ± 0.066	—
o-Nitrobenzoic acid/creatinine ratio				
Week 2	0.0950 ± 0.0159	3.24 ± 0.40	—	13.4 ± 2.5
Month 3	0 ± 0	1.05 ± 0.21	1.15 ± 0.17	4.21 ± 0.50
Month 12	0 ± 0	0.365 ± 0.119	0.824 ± 0.250	2.44 ± 0.67
Month 18	0.0282 ± 0.0073	0.840 ± 0.177	0.954 ± 0.279	—
o-Nitrobenzylmercapturic acid (mg/24 hours)				
Week 2	0 ± 0	0 ± 0	—	0.00132 ± 0.00072
Month 3	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Month 12	0 ± 0	0 ± 0	0.0066 ± 0.0059	0.0230 ± 0.0165
Month 18	0.0605 ± 0.0173	0.0192 ± 0.0148	0 ± 0	—
o-Nitrobenzylmercapturic acid/creatinine ratio				
Week 2	0 ± 0	0 ± 0	—	0.00124 ± 0.00079
Month 3	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Month 12	0 ± 0	0 ± 0	0.0210 ± 0.0187	0.0372 ± 0.0332
Month 18	0.100 ± 0.025	0.0226 ± 0.0175	0 ± 0	—
o-Aminobenzoic acid (mg/24 hours)				
Week 2	0 ± 0	0 ± 0	—	0 ± 0
Month 3	0.0123 ± 0.0031	0.00476 ± 0.00426	0 ± 0	0 ± 0
Month 12	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Month 18	0 ± 0	0 ± 0	0 ± 0	—
o-Aminobenzoic acid/creatinine ratio				
Week 2	0 ± 0	0 ± 0	—	0 ± 0
Month 3	0.0601 ± 0.0140	0.0144 ± 0.0129	0 ± 0	0 ± 0
Month 12	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Month 18	0 ± 0	0 ± 0	0 ± 0	—

TABLE F2
Urinary Biomarker Data for Mice in the 2-Year Feed Study of *o*-Nitrotoluene

	0 ppm	1,250 ppm	2,500 ppm	5,000 ppm
Female				
n				
Week 2	2	4	2	2
Month 3	5	5	5	5
Month 12	5	5	5	5
Month 18	5	5	5	5
Volume (mL/24 hours)				
Week 2	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	1.4 ± 0.7
Month 3	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
Month 12	0.9 ± 0.0	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
Month 18	1.0 ± 0.2	1.3 ± 0.3	0.9 ± 0.2	1.2 ± 0.1
Creatinine (mg/dL)				
Week 2	43.8 ± 3.0	46.5 ± 4.8	40.4 ± 12.1	17.9 ± 8.7
Month 3	88.9 ± 14.3	82.0 ± 9.4	59.1 ± 7.2	54.3 ± 8.0
Month 12	51.4 ± 5.3	48.4 ± 3.6	47.7 ± 7.2	45.7 ± 1.6
Month 18	65.8 ± 8.1	43.4 ± 6.0	58.6 ± 3.1	38.2 ± 3.6
<i>o</i> -Nitrobenzoic acid (mg/24 hours)				
Week 2	0.0173 ± 0.0019	0.501 ± 0.075	0.840 ± 0.243	2.13 ± 0.03
Month 3	0.00534 ± 0.0020	0.207 ± 0.059	0.346 ± 0.057	1.14 ± 0.24
Month 12	0 ± 0	0.190 ± 0.034	0.370 ± 0.038	0.398 ± 0.078
Month 18	0.0181 ± 0.0046	0.372 ± 0.161	0.542 ± 0.076	0.837 ± 0.115
<i>o</i> -Nitrobenzoic acid/creatinine ratio				
Week 2	0.0891 ± 0.018	3.02 ± 0.49	5.73 ± 0.09	11.5 ± 0.4
Month 3	0.0190 ± 0.0086	0.815 ± 0.215	1.77 ± 0.51	3.59 ± 0.53
Month 12	0 ± 0	0.386 ± 0.061	0.858 ± 0.166	0.866 ± 0.170
Month 18	0.0275 ± 0.0095	0.730 ± 0.250	0.968 ± 0.203	2.20 ± 0.32
<i>o</i> -Nitrobenzylmercapturic acid (mg/24 hours)				
Week 2	0 ± 0	0.00324 ± 0.00281	0 ± 0	0 ± 0
Month 3	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Month 12	0 ± 0	0 ± 0	0.0158 ± 0.0142	0.0075 ± 0.0067
Month 18	0 ± 0	0.00284 ± 0.00254	0.0667 ± 0.0364	0 ± 0
<i>o</i> -Nitrobenzylmercapturic acid/creatinine ratio				
Week 2	0 ± 0	0.0193 ± 0.0168	0 ± 0	0 ± 0
Month 3	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Month 12	0 ± 0	0 ± 0	0.0316 ± 0.0283	0.0317 ± 0.0283
Month 18	0 ± 0	0.0252 ± 0.0226	0.102 ± 0.049	0 ± 0
<i>o</i> -Aminobenzoic acid (mg/24 hours)				
Week 2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Month 3	0.00176 ± 0.00157	0.00227 ± 0.00203	0 ± 0	0 ± 0
Month 12	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Month 18	0 ± 0	0 ± 0	0 ± 0	0 ± 0
<i>o</i> -Aminobenzoic acid/creatinine ratio				
Week 2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Month 3	0.00523 ± 0.00470	0.0149 ± 0.0163	0 ± 0	0 ± 0
Month 12	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Month 18	0 ± 0	0 ± 0	0 ± 0	0 ± 0

^a Data are presented as mean ± standard error. Each group consisted of five animals; however, an insufficient sample for complete analysis was obtained from some animals. Therefore, n=number of animals for which metabolite data were obtained.
^b No data were available at this time point due to 100% mortality.
^c n=4

APPENDIX G
ORGAN WEIGHTS
AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE G1	Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male Rats at the 3-Month Interim Evaluation in the 2-Year Feed Study of <i>o</i>-Nitrotoluene	296
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TABLE G1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male Rats
at the 3-Month Interim Evaluation in the 2-Year Feed Study of o-Nitrotoluene^a

	0 ppm	2,000 ppm (Stop-Exposure)	5,000 ppm (Stop-Exposure)
n	10	10	10
Necropsy body wt	360 ± 5	319 ± 7**	264 ± 4**
Heart			
Absolute	0.936 ± 0.023	0.928 ± 0.019	0.928 ± 0.016
Relative	2.595 ± 0.038	2.909 ± 0.026**	3.518 ± 0.057**
R. Kidney			
Absolute	1.162 ± 0.018	1.057 ± 0.026**	1.096 ± 0.022
Relative	3.225 ± 0.034	3.312 ± 0.043	4.153 ± 0.068**
Liver			
Absolute	12.674 ± 0.431	12.860 ± 0.294	15.858 ± 0.248**
Relative	35.100 ± 0.801	40.293 ± 0.354**	60.110 ± 0.822**
Lung			
Absolute	1.379 ± 0.017	1.302 ± 0.044	1.205 ± 0.018**
Relative	3.829 ± 0.036	4.075 ± 0.089**	4.566 ± 0.046**
R. Testis			
Absolute	1.498 ± 0.030	1.348 ± 0.030*	1.111 ± 0.061**
Relative	4.160 ± 0.082	4.232 ± 0.097	4.226 ± 0.257
Thymus			
Absolute	0.266 ± 0.010	0.219 ± 0.015*	0.234 ± 0.012
Relative	0.739 ± 0.029	0.686 ± 0.047	0.886 ± 0.044*

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

** $P \leq 0.01$

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

APPENDIX H

CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

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CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF o-NITROTOLUENE

o-Nitrotoluene was obtained from Aldrich Chemical Company (Milwaukee, WI) in one lot (8056-58-05RTI). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Research Triangle Institute (Research Triangle Park, NC), and the study laboratory. Reports on analyses performed in support of the o-nitrotoluene studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a yellow-green liquid, was identified as o-nitrotoluene by infrared, ultraviolet/visible, proton nuclear magnetic resonance (NMR), and low- and high-resolution mass spectroscopy by the analytical chemistry laboratory and by infrared and NMR spectroscopy by the study laboratory. All spectra were consistent with the literature spectra (*Sadtler*, 1979; *Aldrich*, 1981; *Handbook of Proton NMR Spectra and Data*, 1985; *NIST Standard Reference Database*) and with the structure of o-nitrotoluene. The infrared and NMR spectra are presented in Figures H1 and H2. The observed boiling point of 222° C and density of 1.154 at 22° C were in agreement with literature values (*Merck Index*, 1996).

The purity of lot 8056-58-05RTI was determined by Karl Fischer water analysis and gas chromatography by systems A and B (Table H1). The study laboratory performed gas chromatography using system C with naphthalene added as an internal standard.

Karl Fischer analysis indicated 0.29% water. Gas chromatography using system A indicated one major peak, one impurity peak with an area of 0.11% of the total integrated area, and five minor peaks, each with an area of less than 0.1%. System B indicated one major peak and six minor peaks, each accounting for less than 0.1% of the total integrated area. Gas chromatography by system C indicated one major peak and no impurities. The overall purity of lot 8056-58-05RTI was determined to be greater than 99%.

Accelerated stability studies of the bulk chemical were performed by the analytical chemistry laboratory, using gas chromatography by system D. These studies indicated that o-nitrotoluene was stable as a bulk chemical for at least 2 weeks when stored protected from light at temperatures up to 25° C. To ensure stability, the bulk chemical was stored in amber glass bottles inside metal cans at room temperature. Stability was monitored during the studies with gas chromatography (system C). No degradation of the bulk chemical was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared every 2 weeks by mixing o-nitrotoluene with feed (Table H2). Nonirradiated NTP-2000 feed was used through June 26, 1996; irradiated NTP-2000 feed was used thereafter. A premix was prepared by hand and then blended with additional feed in a Patterson-Kelly twin-shell blender for 15 minutes using an intensifier bar for the initial 5 minutes. Formulations were stored in doubled opaque plastic bags inside plastic buckets at approximately 5° C, protected from light and with minimal headspace, for 35 days.

Homogeneity studies of 625 and 5,000 ppm dose formulations in nonirradiated NTP-2000 feed were performed with gas chromatography by the analytical chemistry laboratory (system E) and by the study laboratory (system F). Stability studies of the 625 ppm dose formulation in nonirradiated NTP-2000 feed were performed by the analytical chemistry laboratory with gas chromatography by system E. Homogeneity was confirmed, and stability was confirmed for 36 days for dose formulations stored in sealed containers, protected from light at temperatures up to 3° C. Samples stored at room temperature under simulated dosing conditions, open to air and light, were not stable. Losses were shown to be due to volatility. Due to the volatile losses during formulation, the study laboratory prepared dose formulations at concentrations up to 115% of the target concentrations. Dose

formulations were replaced in animal room feeders on a 2-day, 2-day, 3-day schedule. After the change to irradiated feed, the study laboratory performed additional homogeneity analyses of the 625, 2,000, and 5,000 ppm dose formulations with gas chromatography (system F) to compare the homogeneity of dose formulations prepared with nonirradiated feed to that of dose formulations prepared with irradiated feed. The homogeneity of all samples was confirmed.

Analyses of the dose formulations of *o*-nitrotoluene were conducted by the study laboratory every 8 to 12 weeks using gas chromatography by system F. Because of the expected loss of *o*-nitrotoluene during mixing, the NTP permitted the use of dose formulations within the range of 90% to 115% of the target concentrations. Of the dose formulations analyzed for rats, 202 of 210 had concentrations that were 90% to 115% of the target concentrations; the concentrations in animal room samples analyzed after 3 days of use in the feeders for rats ranged from 75% to 94% of the target concentrations. Of the dose formulations analyzed for mice, all 63 were within 90% to 115% of the target concentrations; concentrations in animal room samples analyzed after 3 days of use in the feeders for mice ranged from 53% to 81% of the target concentrations. Of the eight dose formulations for rats that were not within 90% to 115% of the target concentration, seven were remixed and were found to be within the specified range; one was discarded without being remixed because a sufficient amount of dose formulation at this concentration was available for dosing.

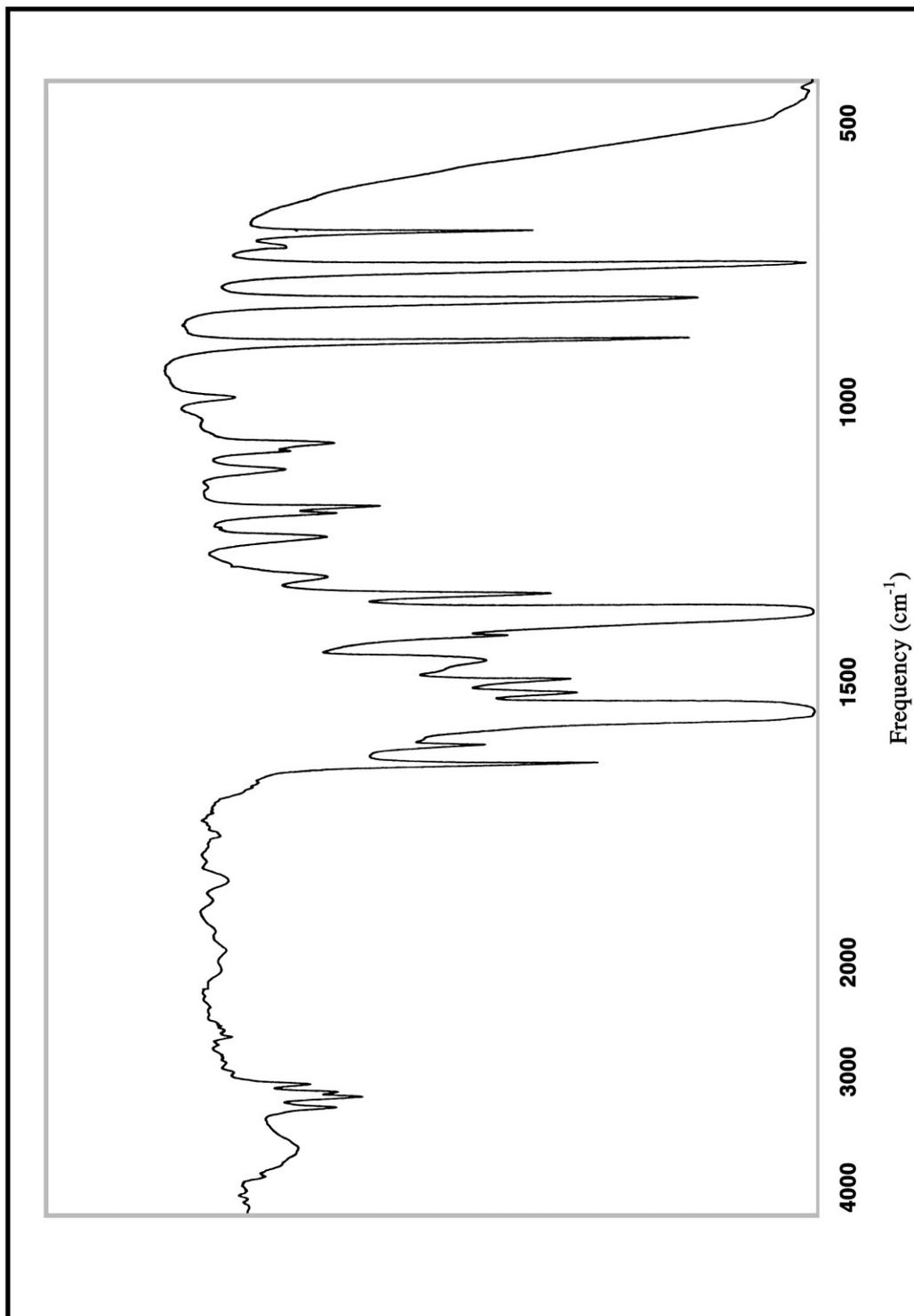


FIGURE H1
Infrared Absorption Spectrum of *o*-Nitrotoluene

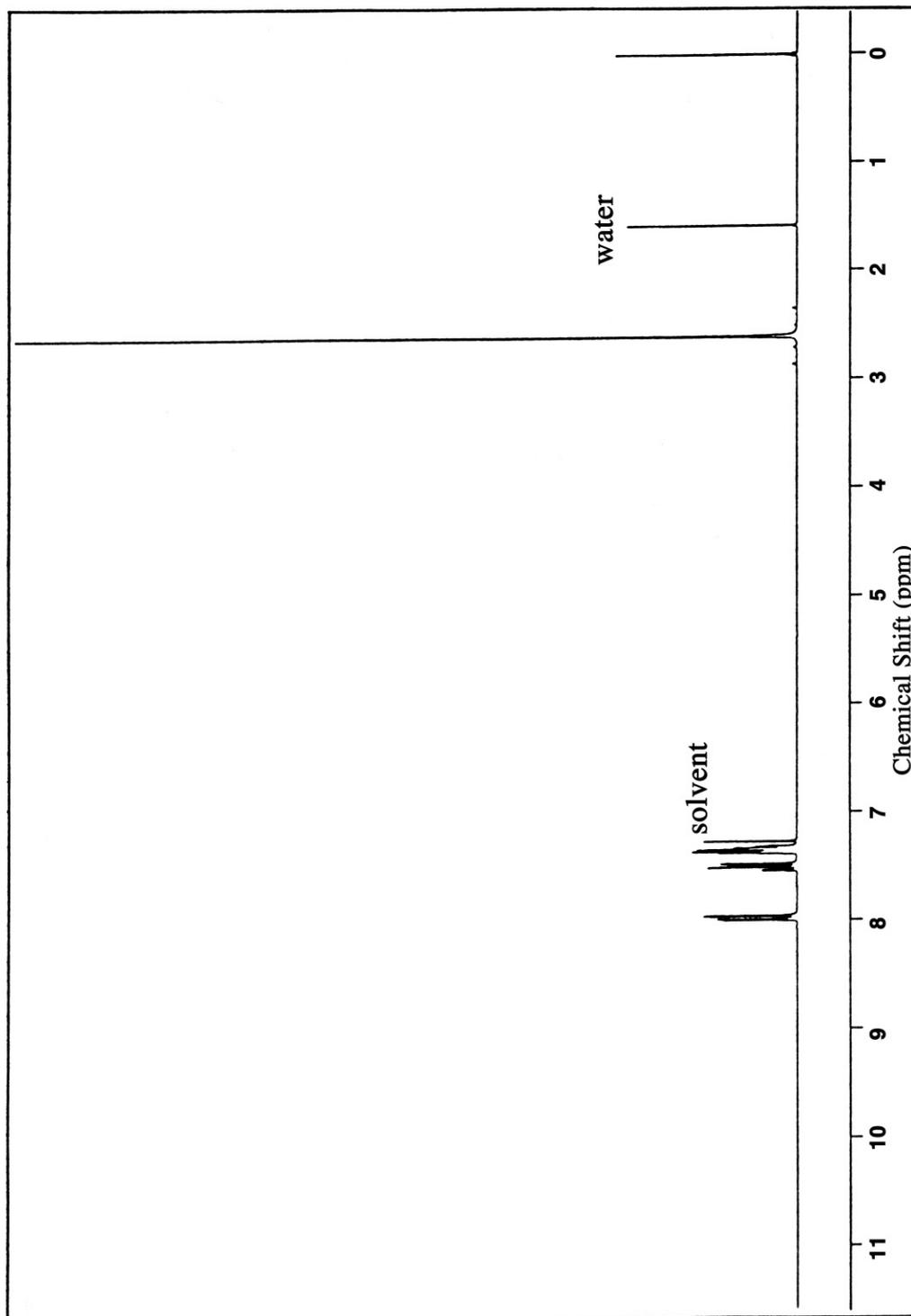


FIGURE H2
Nuclear Magnetic Resonance Spectrum of *o*-Nitrotoluene

TABLE H1
Gas Chromatography Systems Used in the 2-Year Feed Studies of *o*-Nitrotoluene^a

Detection System	Column	Carrier Gas	Oven Temperature Program
System A Flame ionization	SE-30, 30 m × 0.25 mm, 0.25- μ m film (J&W Scientific, Folsom, CA)	Helium at 1 mL/minute	50° C for 5 minutes, then 10° C/minute to 250° C, held for 15 minutes
System B Flame ionization	DB-17, 30 m × 0.25 mm, 0.25- μ m film (J&W Scientific)	Nitrogen at 1 mL/minute	40° C for 5 minutes, then 10° C/minute to 220° C, held for 17 minutes
System C Flame ionization	Rtx5, 30 m × 0.53 mm, 1.0- μ m film (Restek, Bellefonte, PA)	Helium at approximately 12 mL/minute	Isothermal at 130° C
System D Flame ionization	SE-30, 30 m × 0.25 mm, 0.25- μ m film (J&W Scientific)	Helium at 1 mL/minute	Isothermal at 130° C for 7 minutes
System E Flame ionization	SE-30, 30 m × 0.25 mm, 0.25- μ m film (J&W Scientific)	Nitrogen at 1 mL/minute	120° C for 7 minutes, then 30° C/minute to 300° C, held for 7 minutes
System F Flame ionization	Rtx5, 30 m × 0.53 mm, 1.0- μ m film (Restek)	Helium at approximately 12 mL/minute	110° C for 10 minutes, then 70° C/minute to 230° C, held for 10 minutes

^a All gas chromatographs were manufactured by Hewlett Packard (Palo Alto, CA).

TABLE H2
Preparation and Storage of Dose Formulations in the 2-Year Feed Studies of *o*-Nitrotoluene

Preparation

A premix of feed and *o*-nitrotoluene was prepared, then layered into the remaining feed and blended in a Patterson-Kelly twin-shell blender with the intensifier bar on for 5 minutes and off for 10 minutes. The dose formulations were prepared every 2 weeks.

Chemical Lot Number

8056-58-05RTI

Maximum Storage Time

35 days

Storage Conditions

Stored in doubled opaque plastic bags inside plastic buckets at approximately 5° C, protected from light and with minimal headspace

Study Laboratory

Southern Research Institute (Birmingham, AL)

TABLE H3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Feed Studies
of *o*-Nitrotoluene

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration ^a (ppm)	Difference from Target (%)
Rats				
February 7-8, 1996	February 7-9, 1996	625	669	+7
		625	678	+8
		625	677	+8
		625	677	+8
		625	664	+6
		625	718	+15
		1,250	1,330	+6
		1,250	1,340	+7
		1,250	1,310	+5
		1,250	1,320	+6
		1,250	1,310	+5
		1,250	1,260	+1
		2,000	2,100	+5
		2,000	2,110	+6
		2,000	2,090	+5
		2,000	2,140	+7
		2,000	2,110	+6
		2,000	1,950	-2
		2,000	1,980	-1
		2,000	1,970	-1
		2,000	1,970	-1
		2,000	1,990	0
		5,000	5,310	+6
		5,000	5,040	+1
		5,000	4,970	-1
		5,000	4,970	-1
			February 26-27, 1996 ^b	625
		1,250	1,030	-18
		2,000	1,500	-25
		5,000	3,970	-21

TABLE H3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Feed Studies
of o-Nitrotoluene

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)
Rats (continued)				
April 17-18, 1996	April 18-19, 1996	625	627	0
		625	621	-1
		625	620	-1
		625	625	0
		625	620	-1
		625	632	+1
		1,250	1,280	+2
		1,250	1,270	+2
		1,250	1,280	+2
		1,250	1,250	0
		1,250	1,280	+2
		1,250	1,270	+2
		2,000	2,020	+1
		2,000	2,000	0
		2,000	2,010	+1
		2,000	1,980	-1
		2,000	1,950	-2
		2,000	2,000	0
		2,000	2,110	+6
		2,000	2,040	+2
		2,000	2,050	+3
2,000	2,020	+1		
5,000	5,030	+1		
5,000	4,910	-2		
5,000	4,930	-1		
5,000	4,930	-1		
June 26-27, 1996	June 26-29, 1996	625	605	-3
		625	638	+2
		625	616	-1
		625	659	+5
		625	634	+1
		625	664	+6
		625	600	-4
		1,250	1,310	+5
		1,250	1,310	+5
		1,250	1,300	+4
		1,250	1,290	+3
		1,250	1,290	+3
		1,250	1,280	+2
		2,000	2,020	+1
		2,000	1,780 ^c	-11
		2,000	1,990	0
		2,000	1,980	-1
		2,000	1,970	-1
		2,000	1,930	-3
		2,000	1,910	-4
		July 5, 1996	July 9-11, 1996	2,000

TABLE H3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Feed Studies
of *o*-Nitrotoluene

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)
Rats (continued)				
September 4-5, 1996	September 4-6, 1996	625	518 ^c	-17
		625	565	-10
		625	546 ^c	-13
		625	577	-8
		625	584	-7
		625	582	-7
		1,250	1,160	-7
		1,250	1,170	-6
		1,250	1,180	-6
		1,250	1,180	-6
		1,250	1,150	-8
		1,250	1,130	-10
		2,000	1,930	-3
		2,000	1,900	-5
		2,000	1,930	-3
		2,000	1,890	-5
2,000	2,000	0		
2,000	1,990	0		
September 9, 1996	September 9, 1996	625	604 ^d	-3
		625	616 ^d	-1
October 30, 1996	October 30-November 1, 1996	625	614	-2
		625	638	+2
		625	613	-2
		625	616	-1
		625	619	-1
		625	600	-4
		1,250	1,220	-2
		1,250	1,220	-2
		1,250	1,200	-4
		1,250	1,220	-2
		1,250	1,250	0
		1,250	1,230	-2
		2,000	1,910	-4
		2,000	1,880	-6
		2,000	1,990	0
		2,000	1,970	-1
2,000	1,990	0		
2,000	2,030	+2		
November 21, 1996 ^b	November 21, 1996 ^b	625	533	-15
		1,250	976	-22
		2,000	1,510	-24

TABLE H3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Feed Studies
of o-Nitrotoluene

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)		
Rats (continued)						
January 8-9, 1997	January 8-10, 1997	625	622	0		
		625	631	+1		
		625	643	+3		
		625	641	+3		
		625	642	+3		
		625	645	+3		
		1,250	1,250	0		
		1,250	1,260	+1		
		1,250	1,250	0		
		1,250	1,280	+2		
		1,250	1,230	-2		
		1,250	1,250	0		
		2,000	1,940	-3		
		2,000	1,980	-1		
		2,000	1,960	-2		
		2,000	2,000	0		
		2,000	1,960	-2		
		2,000	1,990	0		
		April 1-2, 1997	April 2-4, 1997	625	582	-7
				625	605	-3
625	603			-4		
625	604			-3		
625	580			-7		
625	595			-5		
1,250	1,200			-4		
1,250	1,250			0		
1,250	1,270			+2		
1,250	1,300			+4		
1,250	1,250			0		
1,250	1,280			+2		
2,000	2,050			+3		
2,000	2,050			+3		
2,000	1,960			-2		
2,000	1,970			-1		
2,000	1,950			-2		
2,000	1,920			-4		

TABLE H3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Feed Studies
of *o*-Nitrotoluene

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)
Rats (continued)				
May 28-29, 1997	May 29-30, 1997	625	572	-8
		625	558 ^c	-11
		625	546 ^c	-13
		625	574	-8
		625	566	-9
		625	565	-10
		1,250	1,180	-6
		1,250	1,180	-6
		1,250	1,220	-2
		1,250	1,230	-2
		1,250	1,220	-2
		1,250	1,210	-3
		2,000	1,940	-3
		2,000	1,980	-1
		2,000	1,990	0
		2,000	1,980	-1
		2,000	2,040	+2
2,000	1,980	-1		
June 2, 1997	June 3, 1997	625	709 ^d	+13
		625	716 ^d	+15
	June 23-24, 1997 ^b	625	571	-9
		1,250	1,080	-14
		2,000	1,760	-12
August 20-21, 1997	August 20-22, 1997	625	654	+5
		625	676	+8
		625	668	+7
		625	639	+2
		625	642	+3
		625	642	+3
		1,250	1,330	+6
		1,250	1,410	+13
		1,250	1,400	+12
		1,250	1,410	+13
		1,250	1,440	+15
		1,250	1,340	+7
		2,000	2,010	+1
		2,000	2,150	+8
		2,000	2,120	+6
		2,000	2,230	+12
		2,000	2,160	+8
2,000	2,170	+9		

TABLE H3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Feed Studies
of o-Nitrotoluene

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)		
Rats (continued)						
October 30, 1997	October 30-November 1, 1997	625	650	+4		
		625	647	+4		
		625	634	+1		
		625	652	+4		
		625	645	+3		
		625	651	+4		
		1,250	1,280	+2		
		1,250	1,320	+6		
		1,250	1,290	+3		
		1,250	1,320	+6		
		1,250	1,300	+4		
		2,000	2,750 ^c	+38		
		2,000	2,330 ^c	+17		
		2,000	2,240	+12		
		2,000	2,220	+11		
November 7, 1997	November 7, 1997	2,000	2,080 ^d	+4		
		2,000	2,240 ^d	+12		
January 8, 1998	January 8-10, 1998	625	619	-1		
		625	621	-1		
		625	622	0		
		625	653	+4		
		625	634	+1		
		625	611	-2		
		1,250	623 ^e	-50		
		1,250	1,260	+1		
		1,250	1,220	-2		
		1,250	1,230	-2		
		1,250	1,210	-3		
		2,000	1,870	-6		
		2,000	1,910	-4		
		2,000	1,910	-4		
		2,000	1,930	-3		
		February 3, 1998 ^b	625	539	-14	
			1,250	1,030	-18	
		2,000	1,770	-11		
Mice						
February 7-8, 1996	February 7-9, 1996	1,250	1,340	+7		
		1,250	1,320	+6		
		2,500	2,430	-3		
		2,500	2,470	-1		
		5,000	4,960	-1		
		5,000	4,950	-1		
			March 6, 1996 ^b	1,250	730	-42
				2,500	1,330	-47
				5,000	2,910	-42

TABLE H3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Feed Studies
of o-Nitrotoluene

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)	
Mice (continued)					
April 17-18, 1996	April 18-19, 1996	1,250	1,200	-4	
		1,250	1,210	-3	
		2,500	2,580	+3	
		2,500	2,480	-1	
		5,000	4,860	-3	
		5,000	4,900	-2	
June 26-27, 1996	June 26-29, 1996	1,250	1,240	-1	
		1,250	1,260	+1	
		2,500	2,440	-2	
		2,500	2,430	-3	
		5,000	4,760	-5	
		5,000	4,850	-3	
September 5, 1996	September 5-6, 1996	1,250	1,200	-4	
		1,250	1,170	-6	
		2,500	2,490	0	
		2,500	2,490	0	
		5,000	4,980	0	
		5,000	4,990	0	
	September 30-October 1, 1996 ^b	September 30-October 1, 1996 ^b	1,250	859	-31
			2,500	1,880	-25
			5,000	3,600	-28
			1,250	1,260	+1
			1,250	1,300	+4
			2,500	2,530	+1
October 30, 1996	October 30-November 1, 1996	2,500	2,520	+1	
		5,000	5,040	+1	
		5,000	5,040	+1	
		1,250	1,290	+3	
		1,250	1,280	+2	
		2,500	2,530	+1	
January 8-9, 1997	January 8-10, 1997	2,500	2,480	-1	
		5,000	5,050	+1	
		5,000	5,130	+3	
		1,250	1,280	+2	
		1,250	1,300	+4	
		2,500	2,490	0	
April 2, 1997	April 2-4, 1997	2,500	2,540	+2	
		5,000	4,960	-1	
		5,000	4,900	-2	

TABLE H3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Feed Studies
of o-Nitrotoluene

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration (ppm)	Difference from Target (%)	
Mice (continued)					
May 28-29, 1997	May 29-30, 1997	1,250	1,260	+1	
		1,250	1,250	0	
		2,500	2,570	+3	
		2,500	2,560	+2	
		5,000	5,010	0	
		5,000	4,990	0	
June 23-24, 1997 ^b		1,250	969	-22	
		2,500	1,890	-24	
		5,000	3,770	-25	
August 20-21, 1997	August 20-22, 1997	1,250	1,400	+12	
		1,250	1,400	+12	
		2,500	2,660	+6	
		2,500	2,710	+8	
		5,000	5,130	+3	
		5,000	5,140	+3	
October 30, 1997	October 30-November 1, 1997	1,250	1,290	+3	
		1,250	1,360	+9	
		2,500	2,750	+10	
		5,000	5,500	+10	
January 8, 1998	January 8-10, 1998	1,250	1,180	-6	
		1,250	1,230	-2	
		2,500	2,430	-3	
		5,000	5,160	+3	
	February 3, 1998 ^b		1,250	888	-29
			2,500	1,630	-35
		5,000	4,060	-19	

^a Results of duplicate analyses

^b Animal room samples

^c Remixed; not used in study

^d Results of remix

^e Sample was discarded and not remixed because there was sufficient dose formulation available for dosing at the 1,250 ppm concentration.

APPENDIX I
FEED AND COMPOUND CONSUMPTION
IN THE 2-YEAR FEED STUDIES
OF *o*-NITROTOLUENE

TABLE I1	Feed and Compound Consumption by Male Rats in the 2-Year Feed Study of <i>o</i>-Nitrotoluene	312
TABLE I2	Feed and Compound Consumption by Female Rats in the 2-Year Feed Study of <i>o</i>-Nitrotoluene	314
TABLE I3	Feed and Compound Consumption by Male Mice in the 2-Year Feed Study of <i>o</i>-Nitrotoluene	315
TABLE I4	Feed and Compound Consumption by Female Mice in the 2-Year Feed Study of <i>o</i>-Nitrotoluene	316

TABLE II
Feed and Compound Consumption by Male Rats in the 2-Year Feed Study of *o*-Nitrotoluene

Week	0 ppm		625 ppm			1,250 ppm			2,000 ppm		
	Feed (g/day) ^a	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose (mg/kg) ^b	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
4	18.1	239	18.4	237	48	18.5	235	99	17.9	228	157
8	17.5	303	17.0	299	36	16.8	295	71	16.1	282	114
12	17.3	344	17.0	337	32	17.1	327	65	16.5	315	105
16	17.3	370	16.8	363	29	16.7	350	60	16.3	329	99
20	17.1	391	15.9	378	26	15.9	366	54	16.0	350	91
24	16.3	401	16.8	385	27	16.3	372	55	16.8	357	94
28	17.7	410	18.2	399	28	18.0	384	59	16.9	363	93
32	16.5	422	16.5	406	25	16.6	392	53	16.5	371	89
36	16.4	429	15.9	413	24	16.0	399	50	15.9	377	84
40	16.4	432	16.5	414	25	16.1	400	50	16.4	378	87
44	16.3	434	16.5	416	25	15.9	400	50	16.2	378	86
48	16.6	433	16.4	417	25	16.4	402	51	16.3	378	86
52	16.9	436	16.4	419	24	16.5	404	51	16.3	383	85
56	16.0	428	16.1	415	24	15.3	398	48	15.8	377	84
60	17.6	439	17.1	423	25	15.6	403	48	16.0	380	84
64	17.3	443	16.9	427	25	16.3	409	50	16.7	388	86
68	16.9	447	16.8	432	24	16.3	417	49	15.6	394	79
72	15.9	446	16.2	433	23	16.1	418	48	15.6	396	79
76	14.9	435	15.7	424	23	15.1	409	46	16.0	393	82
80	15.9	433	15.3	419	23	15.6	405	48	16.7	386	87
84	16.7	430	17.1	423	25	16.5	407	51	16.4	374	88
88	16.2	434	15.4	420	23	14.7	411	45			
92	15.1	428	15.7	425	23	14.5	408	45			
96	15.2	425	16.0	424	24	13.4	395	42			
100	15.5	424	14.9	421	22	16.0	408	49			
104	14.6	425	13.5	415	20						
Mean for weeks											
4-13	17.6	295	17.5	291	39	17.5	285	78	16.8	275	125
14-52	16.8	416	16.6	401	26	16.4	387	53	16.4	367	89
53-104	16.0	434	15.9	423	23	15.5	407	47	16.1	386	84

TABLE II
Feed and Compound Consumption by Male Rats in the 2-Year Feed Study of o-Nitrotoluene

Week	2,000 ppm (Stop-Exposure)			5,000 ppm (Stop-Exposure)		
	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
4	18.0	230	156	16.2	211	384
8	16.2	285	114	14.1	247	286
12	16.4	316	104	14.5	263	276
16	16.4	341		15.8	279	
20	16.7	359		16.6	297	
24	16.5	371		16.1	305	
28	17.5	381		17.4	312	
32	16.2	390		16.2	321	
36	16.0	398		15.7	328	
40	16.2	400		15.8	329	
44	16.4	403		15.7	331	
48	16.2	402		16.1	334	
52	16.9	408		16.2	336	
56	16.1	402		15.5	329	
60	17.3	411		16.6	342	
64	17.0	417		16.0	346	
68	16.4	422		15.8	356	
72	15.9	421		16.0	364	
76	15.8	415		16.0	367	
80	15.3	408		15.5	360	
84	15.7	403		16.8	368	
88	16.2	413				
92	15.0	408				
96	14.2	412				
100	14.2	412				
104	15.4	404				
Mean for weeks						
4-13	16.9	277	125	14.9	240	315
14-52	16.5	385		16.2	317	
53-104	15.7	412		16.0	354	

^a Grams of feed consumed per animal per day

^b Milligrams of o-nitrotoluene consumed per kilogram body weight per day

TABLE I2
Feed and Compound Consumption by Female Rats in the 2-Year Feed Study of *o*-Nitrotoluene

Week	0 ppm		625 ppm			1,250 ppm			2,000 ppm		
	Feed (g/day) ^a	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose (mg/kg) ^b	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
4	11.3	145	11.5	144	50	12.0	145	103	11.5	143	161
8	9.9	171	9.9	168	37	10.2	170	75	9.8	168	117
12	10.2	185	10.0	180	35	10.2	182	70	10.1	181	112
16	10.1	195	10.3	193	33	10.5	193	68	10.3	193	106
20	10.2	203	9.6	200	30	10.1	200	63	9.9	196	101
24	10.0	207	10.2	204	31	10.4	204	64	10.3	201	102
28	10.7	212	10.9	210	33	11.1	209	67	10.4	206	101
32	10.3	221	10.4	217	30	10.5	216	61	10.2	213	96
36	9.8	225	10.0	221	28	10.1	219	57	9.8	214	92
40	10.0	227	10.1	225	28	10.3	224	58	10.0	216	93
44	10.2	231	10.2	228	28	10.6	227	58	10.3	220	93
48	10.5	236	10.4	232	28	10.7	231	58	10.2	222	91
52	10.7	239	10.6	235	28	11.0	235	59	10.5	224	94
56	11.3	244	11.4	241	30	11.1	241	58	10.9	227	96
60	11.6	254	11.0	247	28	11.1	247	56	10.8	230	94
64	11.1	258	11.3	256	28	11.5	251	57	11.0	234	94
68	11.7	266	11.8	263	28	12.0	259	58	10.8	241	89
72	11.9	271	12.2	267	29	12.0	267	56	11.5	243	95
76	12.2	276	12.2	271	28	11.4	268	53	11.4	243	94
80	12.0	281	11.8	274	27	11.6	272	53	11.2	247	91
84	12.0	284	12.4	275	28	12.6	272	58	11.3	247	91
88	11.7	284	12.5	278	28	12.0	274	55	11.3	250	91
92	11.9	290	12.2	285	27	11.9	282	53	10.9	257	85
96	11.7	291	11.8	287	26	11.7	287	51	11.3	264	86
100	11.9	294	11.7	291	25	11.8	293	50	11.3	273	83
104	11.5	299	12.1	302	25	12.3	298	52	11.7	275	85
Mean for weeks											
4-13	10.5	167	10.5	164	40	10.8	166	83	10.5	164	130
14-52	10.3	220	10.3	217	30	10.5	216	61	10.2	210	97
53-104	11.7	276	11.9	272	27	11.8	270	55	11.2	248	90

^a Grams of feed consumed per animal per day

^b Milligrams of *o*-nitrotoluene consumed per kilogram body weight per day

TABLE I3
Feed and Compound Consumption by Male Mice in the 2-Year Feed Study of o-Nitrotoluene

Week	0 ppm		1,250 ppm			2,500 ppm			5,000 ppm		
	Feed (g/day) ^a	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose (mg/kg) ^b	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
4	5.1	24.0	4.7	23.4	250	4.5	22.6	493	4.2	21.4	979
8	4.1	27.7	4.0	27.1	184	3.9	26.2	367	3.6	25.1	722
12	4.6	30.6	4.4	29.0	191	4.3	28.3	376	4.2	26.7	794
16	4.7	34.2	4.6	32.3	179	4.5	30.9	367	4.0	28.3	700
20	4.5	37.3	4.4	35.9	154	4.2	33.8	314	3.8	29.7	636
24	4.9	38.7	4.9	36.4	168	4.7	34.3	341	4.0	30.0	664
28	5.1	40.5	5.1	38.4	165	4.8	35.6	337	4.2	30.8	674
32	5.0	41.9	5.3	39.9	165	5.1	36.8	344	4.2	31.1	673
36	4.9	43.6	4.9	41.0	148	4.8	37.7	318	4.0	31.7	632
40	5.2	44.7	5.2	42.1	153	5.1	38.3	333	4.3	31.9	669
44	5.2	44.8	5.1	42.7	149	5.0	39.0	319	4.2	32.2	646
48	5.0	45.8	5.2	43.2	152	5.4	39.4	341	4.6	32.8	701
56	5.3	47.1	5.3	44.4	150	5.4	40.2	335	3.8	32.4	594
60	5.3	47.0	5.3	44.0	150	5.4	40.1	336			
64	5.3	46.7	5.5	43.4	159	5.1	40.4	318			
68	5.4	47.7	5.4	45.0	151	5.3	40.7	326			
72	5.5	47.3	5.5	44.6	154	5.3	41.0	325			
76	5.8	47.6	5.7	44.2	160	5.2	39.5	326			
80	5.6	48.0	5.9	44.8	166	5.0	38.9	322			
84	5.7	46.3	5.9	43.4	170	5.6	37.3	378			
88	5.4	46.5	5.7	43.6	163						
92	5.0	46.5	5.2	42.1	154						
96	5.3	46.6	5.2	43.1	151						
100	5.1	46.8	5.1	43.7	146						
104	5.4	46.5	5.4	42.6	159						
Mean for weeks											
4-13	4.6	27.4	4.4	26.5	208	4.2	25.7	412	4.0	24.4	832
14-52	4.9	41.3	5.0	39.1	159	4.8	36.2	335	4.1	30.9	666
53-104	5.4	47.0	5.5	43.8	156	5.5	38.6	364	3.8	32.4	594

^a Grams of feed consumed per animal per day

^b Milligrams of o-nitrotoluene consumed per kilogram body weight per day

TABLE I4
Feed and Compound Consumption by Female Mice in the 2-Year Feed Study of o-Nitrotoluene

Week	0 ppm		1,250 ppm			2,500 ppm			5,000 ppm		
	Feed (g/day) ^a	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose (mg/kg) ^b	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
4	3.7	20.6	3.8	21.1	226	3.7	20.4	449	3.3	19.4	857
8	3.4	22.4	3.8	23.2	202	3.6	23.3	388	3.2	22.1	731
12	3.8	25.7	3.9	25.5	193	3.9	25.1	386	3.5	23.2	761
16	4.0	27.8	4.1	27.4	188	4.0	26.8	374	3.7	24.3	758
20	3.7	29.4	3.8	29.3	162	3.9	29.0	336	3.5	25.4	697
24	4.3	31.8	4.5	32.2	177	4.2	30.1	353	3.9	26.4	733
28	4.3	33.2	3.9	33.6	145	4.1	32.0	323	3.8	27.1	704
32	4.6	35.4	4.5	35.8	159	4.7	33.6	348	4.1	28.0	727
36	4.2	37.3	4.1	36.7	141	4.4	35.0	313	3.8	29.0	647
40	4.5	38.1	4.6	37.7	152	4.4	35.9	306	3.9	29.5	666
44	4.4	38.1	4.2	38.6	135	4.3	37.0	288	3.8	30.5	616
48	4.3	39.3	4.4	38.8	143	4.3	37.5	290	3.8	30.4	628
52	4.7	40.4	5.0	40.7	154	4.8	38.1	315	4.6	30.6	747
56	4.2	41.8	4.4	42.6	130	4.2	38.8	269	3.7	31.5	592
60	5.0	42.4	4.9	42.5	143	4.8	39.3	304	4.2	31.6	673
64	4.6	43.7	4.8	43.5	138	4.4	39.9	277	4.1	32.2	644
68	4.3	45.2	4.8	44.4	136	4.5	41.3	274	4.4	33.4	662
72	4.6	44.5	4.8	44.4	135	4.5	41.2	270	4.3	33.3	643
76	4.5	44.8	4.7	43.9	134	4.4	40.8	268	4.4	33.8	653
80	4.2	44.8	4.5	44.2	127	4.4	40.9	268	4.1	33.6	613
84	4.8	43.8	4.7	42.2	139	4.5	38.7	293	4.5	32.8	687
88	5.2	44.7	5.3	43.1	153	5.1	39.3	325	5.0	33.4	745
92	4.3	45.1	4.6	43.9	130	4.5	39.9	283	4.8	33.0	722
96	4.0	45.0	4.5	45.3	123	4.1	39.7	260	5.4	32.7	832
100	4.4	46.2	4.8	47.4	126	4.9	41.3	296	6.0	32.3	934
104	5.1	46.6	5.2	46.9	139	5.5	41.2	334			
Mean for weeks											
4-13	3.6	22.9	3.8	23.3	207	3.7	22.9	408	3.4	21.6	783
14-52	4.3	35.1	4.3	35.1	156	4.3	33.5	325	3.9	28.1	692
53-104	4.5	44.5	4.8	44.2	135	4.6	40.2	286	4.6	32.8	700

^a Grams of feed consumed per animal per day

^b Milligrams of o-nitrotoluene consumed per kilogram body weight per day

APPENDIX J
INGREDIENTS, NUTRIENT COMPOSITION,
AND CONTAMINANT LEVELS
IN NTP-2000 RAT AND MOUSE RATION

TABLE J1	Ingredients of NTP-2000 Rat and Mouse Ration	318
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TABLE J1
Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground hard winter wheat	22.26
Ground #2 yellow shelled corn	22.18
Wheat middlings	15.0
Oat hulls	8.5
Alfalfa meal (dehydrated, 17% protein)	7.5
Purified cellulose	5.5
Soybean meal (49% protein)	5.0
Fish meal (60% protein)	4.0
Corn oil (without preservatives)	3.0
Soy oil (without preservatives)	3.0
Dried brewer's yeast	1.0
Calcium carbonate (USP)	0.9
Vitamin premix ^a	0.5
Mineral premix ^b	0.5
Calcium phosphate, dibasic (USP)	0.4
Sodium chloride	0.3
Choline chloride (70% choline)	0.26
Methionine	0.2

^a Wheat middlings as carrier

^b Calcium carbonate as carrier

TABLE J2
Vitamins and Minerals in NTP-2000 Rat and Mouse Ration^a

	Amount	Source
Vitamins		
A	4,000 IU	Stabilized vitamin A palmitate or acetate
D	1,000 IU	D-activated animal sterol
K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl acetate	100 IU	
Niacin	23 mg	
Folic acid	1.1 mg	
d-Pantothenic acid	10 mg	d-Calcium pantothenate
Riboflavin	3.3 mg	
Thiamine	4 mg	Thiamine mononitrate
B ₁₂	52 μg	
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	d-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

^a Per kg of finished product

TABLE J3
Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	13.6 ± 0.55	12.6 – 14.7	24
Crude fat (% by weight)	8.1 ± 0.32	7.5 – 9.0	24
Crude fiber (% by weight)	9.7 ± 0.64	8.4 – 11.1	24
Ash (% by weight)	5.1 ± 0.29	4.6 – 5.9	24
Amino Acids (% of total diet)			
Arginine	0.731 ± 0.050	0.670 – 0.800	8
Cystine	0.224 ± 0.012	0.210 – 0.240	8
Glycine	0.684 ± 0.041	0.620 – 0.740	8
Histidine	0.333 ± 0.018	0.310 – 0.350	8
Isoleucine	0.524 ± 0.046	0.430 – 0.590	8
Leucine	1.061 ± 0.061	0.960 – 1.130	8
Lysine	0.708 ± 0.056	0.620 – 0.790	8
Methionine	0.401 ± 0.035	0.350 – 0.460	8
Phenylalanine	0.598 ± 0.036	0.540 – 0.640	8
Threonine	0.501 ± 0.051	0.430 – 0.590	8
Tryptophan	0.126 ± 0.014	0.110 – 0.150	8
Tyrosine	0.390 ± 0.056	0.280 – 0.460	8
Valine	0.640 ± 0.049	0.550 – 0.690	8
Essential Fatty Acids (% of total diet)			
Linoleic	3.97 ± 0.284	3.59 – 4.54	8
Linolenic	0.30 ± 0.042	0.21 – 0.35	8
Vitamins			
Vitamin A (IU/kg)	4,699 ± 1,320	2,570 – 8,140	24
Vitamin D (IU/kg)	1,000 ^a		
α-Tocopherol (ppm)	82.2 ± 14.08	62.2 – 107.0	8
Thiamine (ppm) ^b	8.7 ± 1.24	6.6 – 11.7	24
Riboflavin (ppm)	5.6 ± 1.12	4.20 – 7.70	8
Niacin (ppm)	74.3 ± 5.94	66.4 – 85.8	8
Pantothenic acid (ppm)	22.5 ± 3.96	17.4 – 29.1	8
Pyridoxine (ppm)	9.04 ± 2.37	6.4 – 12.4	8
Folic acid (ppm)	1.64 ± 0.38	1.26 – 2.32	8
Biotin (ppm)	0.333 ± 0.15	0.225 – 0.704	8
Vitamin B ₁₂ (ppb)	68.7 ± 63.0	18.3 – 174.0	8
Choline (ppm)	3,155 ± 325	2,700 – 3,790	8
Minerals			
Calcium (%)	0.986 ± 0.053	0.884 – 1.080	24
Phosphorus (%)	0.575 ± 0.029	0.487 – 0.616	24
Potassium (%)	0.659 ± 0.022	0.627 – 0.691	8
Chloride (%)	0.357 ± 0.027	0.300 – 0.392	8
Sodium (%)	0.189 ± 0.019	0.160 – 0.212	8
Magnesium (%)	0.199 ± 0.009	0.185 – 0.213	8
Sulfur (%)	0.178 ± 0.021	0.153 – 0.209	8
Iron (ppm)	160 ± 14.7	135 – 177	8
Manganese (ppm)	50.3 ± 4.82	42.1 – 56.0	8
Zinc (ppm)	50.7 ± 6.59	43.3 – 61.1	8
Copper (ppm)	6.29 ± 0.828	5.08 – 7.59	8
Iodine (ppm)	0.461 ± 0.187	0.233 – 0.843	8
Chromium (ppm)	0.724 ± 0.529	0.330 – 2.000	8
Cobalt (ppm)	0.45 ± 0.628	0.20 – 2.0	8

^a From formulation

^b As hydrochloride (thiamine and pyridoxine) or chloride (choline)

TABLE J4
Contaminant Levels in NTP-2000 Rat and Mouse Ration^a

	Mean ± Standard Deviation ^b	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.24 ± 0.096	0.10 – 0.50	24
Cadmium (ppm)	0.05 ± 0.011	0.04 – 0.09	24
Lead (ppm)	0.10 ± 0.052	0.06 – 0.28	24
Mercury (ppm)	<0.02		24
Selenium (ppm)	0.16 ± 0.029	0.12 – 0.24	24
Aflatoxins (ppb)	<5.00		24
Nitrate nitrogen (ppm) ^c	14.7 ± 5.69	9.04 – 33.6	24
Nitrite nitrogen (ppm) ^c	0.72 ± 0.40	0.40 – 2.00	24
BHA (ppm) ^d	1.1 ± 0.54	0.01 – 3.37	24
BHT (ppm) ^d	1.0 ± 0.37	0.01 – 2.29	24
Aerobic plate count (CFU/g) ^e	88,956 ± 100,661	15 – 260,000	8
Coliform (MPN/g) ^f	134 ± 212.8	9 – 510	5
<i>Escherichia coli</i> (MPN/g)	<10		24
<i>Salmonella</i> (MPN/g)	Negative		24
Total nitrosoamines (ppb) ^g	5.1 ± 2.29	2.7 – 12.6	24
<i>N</i> -Nitrosodimethylamine (ppb) ^g	2.3 ± 1.54	0.9 – 5.7	24
<i>N</i> -Nitrosopyrrolidine (ppb) ^g	2.8 ± 1.75	1.0 – 8.7	24
Pesticides (ppm)			
α-BHC	<0.01		24
β-BHC	<0.02		24
γ-BHC	<0.01		24
δ-BHC	<0.01		24
Heptachlor	<0.01		24
Aldrin	<0.01		24
Heptachlor epoxide	<0.01		24
DDE	<0.01		24
DDD	<0.01		24
DDT	<0.01		24
HCB	<0.01		24
Mirex	<0.01		24
Methoxychlor	<0.05		24
Dieldrin	<0.01		24
Endrin	<0.01		24
Telodrin	<0.01		24
Chlordane	<0.05		24
Toxaphene	<0.10		24
Estimated PCBs	<0.20		24

TABLE J4
Contaminant Levels in NTP-2000 Rat and Mouse Ration

	Mean ± Standard Deviation	Range	Number of Samples
Pesticides (ppm) (continued)			
Ronnel	<0.01		24
Ethion	<0.02		24
Triethion	<0.05		24
Diazinon	<0.10		24
Methyl chlorpyrifos	0.093 ± 0.084	0.010 – 0.300	20
Methyl parathion	<0.02		24
Ethyl parathion	<0.02		24
Malathion	0.224 ± 0.482	0.020 – 2.430	24
Endosulfan I	<0.01		24
Endosulfan II	<0.01		24
Endosulfan sulfate	<0.03		24

^a CFU=colony-forming units; MPN=most probable number; BHC=hexachlorocyclohexane or benzene hexachloride

^b For values less than the limit of detection, the detection limit is given as the mean.

^c Sources of contamination: alfalfa, grains, and fish meal

^d Sources of contamination: soy oil and fish meal

^e Includes three irradiated and five nonirradiated samples. Microbial counts for remaining samples were below the detection limit.

^f Nonirradiated samples. Microbial counts for irradiated samples were below the detection limit.

^g All values were corrected for percent recovery.

APPENDIX K
SENTINEL ANIMAL PROGRAM

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SENTINEL ANIMAL PROGRAM

METHODS

Rodents used in the Carcinogenesis Program of the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of chemical compounds. Under this program, the disease state of the rodents is monitored via serology on sera from extra (sentinel) animals in the study rooms. These animals and the study animals are subject to identical environmental conditions. The sentinel animals come from the same production source and weanling groups as the animals used for the studies of chemical compounds.

Serum samples were collected from randomly selected rats and mice during the 2-year studies. Blood from each animal was collected and allowed to clot, and the serum was separated. The samples were processed appropriately and sent to Microbiological Associates, Inc. (Rockville, MD), for determination of antibody titers. The laboratory serology methods and viral agents for which testing was performed are tabulated below; the times at which blood was collected during the studies are also listed. At 18 months, live mice were shipped to MA BioServices (Rockville, MD) for evaluation of bacterial profile and viral serology according to NIEHS Advisory Number 19.

Method and Test

Time of Analysis

RATS

ELISA

Mycoplasma arthritis

Study termination

Mycoplasma pulmonis

Study termination

PVM (pneumonia virus of mice)

6, 12, and 18 months, study termination

RCV/SDA

(rat coronavirus/sialodacryoadenitis virus)

6, 12, and 18 months, study termination

Sendai

6, 12, and 18 months, study termination

Immunofluorescence Assay

M. arthritis

Study termination

Parvovirus

Study termination

RCV/SDA

6 months

Hemagglutination Inhibition

H-1 (Toolan's H-1 virus)

6, 12, and 18 months

KRV (Kilham rat virus)

6, 12, and 18 months

MICE

Bacterial Assays

Oral	18 months
Fecal	18 months

ELISA

Ectromelia virus	6, 12, and 18 months, study termination
EDIM (epizootic diarrhea of infant mice)	6, 12, and 18 months, study termination
GDVII (mouse encephalomyelitis virus)	6, 12, and 18 months, study termination
LCM (lymphocytic choriomeningitis virus)	6, 12, and 18 months, study termination
Mouse adenoma virus-FL	6, 12, and 18 months, study termination
MHV (mouse hepatitis virus)	6, 12, and 18 months, study termination
<i>M. arthritidis</i>	18 months and study termination
<i>M. pulmonis</i>	18 months and study termination
PVM	6, 12, and 18 months, study termination
Reovirus 3	6, 12, and 18 months, study termination
Sendai	6, 12, and 18 months, study termination

Immunofluorescence Assay

<i>Helicobacter hepaticus</i>	18 months
Mouse adenoma virus-FL	18 months
MCMV (mouse cytomegalovirus)	18 months and study termination
<i>M. arthritidis</i>	Study termination
Parvovirus	Study termination
Reovirus 3	18 months

Hemagglutination Inhibition

K (papovavirus)	6, 12, and 18 months
MVM (minute virus of mice)	6, 12, and 18 months
Polyoma virus	6, 12, and 18 months

RESULTS

For the 2-year study in rats, all serology tests were negative. Bacterial profiles of sentinel mice shipped live to the rodent disease diagnostic laboratory at 20 months indicated *Enterococcus faecalis* in five males and five females and *Klebsiella oxytoca* in five males. These had no impact on the study results. These mice were subjected to comprehensive health evaluations, including histologic evaluation of liver sections by special stains for *Helicobacter* infections. *Helicobacter spp.* were not isolated from any of these mice. Two mice had positive titers for *M. arthritidis* at study termination. Further evaluation of the samples positive for *M. arthritidis* by immunoblot and Western blot procedures indicated that the positive titers may have been due to cross reaction with antibodies of nonpathogenic *Mycoplasma* or other agents. Only sporadic samples were positive and there were no clinical findings or histopathologic changes of *M. arthritidis* infection in animals with positive titers. Accordingly, *M. arthritidis*-positive titers were considered false positives.

APPENDIX L

COMPARATIVE METABOLISM STUDIES OF *o*-NITROTOLUENE

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COMPARATIVE METABOLISM STUDIES OF o-NITROTOLUENE

INTRODUCTION

Studies were conducted in male and female F344/N rats and male B6C3F₁ mice to determine and compare the metabolism and excretion of [¹⁴C]-o-nitrotoluene following the administration of single doses by gavage; similar studies were conducted in male F344/N rats following 14 days of repeated gavage dosing. These studies were conducted by Research Triangle Institute (1995).

MATERIALS AND METHODS

Nonradiolabeled o-nitrotoluene was obtained from Aldrich Chemical Company (St. Louis, MO) in two lots (08506MV and 04328DG). Radiolabeled [¹⁴C]-o-nitrotoluene, uniformly ring-labeled, was obtained from Chemsyn Science Laboratories (Lenexa, KS; lot CSL-91-331-50-27; 15 mCi/mmol, 0.50 mCi/mL) and Du Pont NEN Research Products (Boston, MA; lot 3048-101; 8.99 mCi/mmol, 1.03 mCi/mL).

The identity of the nonradiolabeled o-nitrotoluene was confirmed by mass spectrometry and nuclear magnetic resonance spectroscopy. Radiochemical purity of the [¹⁴C]-o-nitrotoluene was determined to be 98% or greater by the study laboratory using high-performance liquid chromatography (HPLC), a radioactivity detector with a scintillator flow cell, and ultraviolet detection at 254 nm.

Male and female F344/N rats and male B6C3F₁ mice were obtained from Charles River Laboratories, Inc. (Raleigh, NC). The animals were quarantined for at least 1 week before the beginning of each study. Animals were approximately 9 to 14 (male rats), 9 to 12 (female rats), or 9 to 11 (male mice) weeks old at dosing. Animals were housed in polycarbonate cages. The day before dosing, animals in single-dose studies were transferred to individual glass metabolism cages, which provided for separate collection of urine and feces. Rats in the repeat dosing study were housed in polycarbonate cages until the day before administration of the radiolabeled dose, after which they were transferred to glass metabolism cages. Animals received certified Purina Rodent Chow #5002 and tap water *ad libitum*.

The four male F344/N rats in the pharmacokinetic study were implanted with indwelling jugular cannulae to allow serial blood sampling. Rats were anesthetized with an intraperitoneal injection of 60 mg ketamine:xylazine (7:1)/kg body weight before cannulation. The indwelling cannula design was similar to that of Harms and Ojeda (1974) as modified by McKenna and Bieri (1984). Following implantation of the cannulae, rats were returned to metabolism cages to recover for one day prior to dosing.

At the end of each study, rats were anesthetized with an intraperitoneal injection of 60 mg ketamine:xylazine (7:1)/kg body weight before final blood collection. Rats not used for the pharmacokinetic study were sacrificed by an intracardiac injection of 300 mg sodium pentobarbital/kg body weight. Pharmacokinetic study rats were sacrificed by asphyxiation with CO₂. Mice were anesthetized with an intraperitoneal injection of 60 mg/kg sodium pentobarbital prior to final blood collection, and they were sacrificed by cervical dislocation.

Gavage dosing formulations used in the initial 200 mg/kg rat and mouse studies contained 10 to 100 μCi radiolabel, an appropriate amount of unlabeled o-nitrotoluene, and Emulphor EL-620 to yield a dose volume of 5 mL/kg. The doses for the hemoglobin binding study in male and female rats dosed with 200 mg/kg [¹⁴C]-o-nitrotoluene contained 275 to 300 μCi radiolabel and an appropriate amount of unlabeled o-nitrotoluene in the same vehicle. Dosing solutions were prepared one day prior to use and stored at -20° C in the dark. Subsequent gavage dose formulations were prepared in Emulphor:ethanol:water (1:1:8). These formulations contained 14 to 21 μCi (rats) or 1.7 to 1.9 μCi (mice) of radiolabel, an appropriate amount of unlabeled o-nitrotoluene, and sufficient vehicle for a single dose volume of 5 mL/kg (rats) or 0.5 mL (mice).

Oral doses of [¹⁴C]-*o*-nitrotoluene were administered to animals by intragastric gavage at 2 or 200 mg/kg. Doses were delivered using a ball-tipped gavage needle that was wiped clean of excess dose formulation before the filled dosing apparatus was weighed. After dosing, the needle was wiped clean with a Kimwipe[®], and the empty dosing apparatus was reweighed; the Kimwipe[®] was placed into a vial containing 10 mL of scintillation cocktail to determine the quantity of residual radiolabel on the gavage needle. Each dose was calculated as the difference between the weights of the filled and empty dosing apparatus, less the amount found on the wipes. To measure the concentration of [¹⁴C]-*o*-nitrotoluene in the dose formulation, two weighed aliquots were taken before, two after, and one in the midst of dosing a series of animals.

Determination of Excretion and Urinary Metabolites of [¹⁴C]-*o*-Nitrotoluene in Rats and Mice

Groups of three or four male or female rats or male mice were administered single gavage doses of 2 or 200 mg [¹⁴C]-*o*-nitrotoluene/kg body weight in Emulphor. For the initial 200 mg/kg studies, radioactivity was measured in urine and feces collected 4, 8, 24, 48, and 72 hours after dosing. In subsequent studies, urine or urine and feces were collected 24, 48, and 72 hours after dosing. Urine and feces were collected separately into round-bottom flasks cooled with dry ice. Samples were stored at -20° C, protected from light, until analysis.

To determine radioactivity, aliquots of urine and plasma were added to vials containing scintillation cocktail (Ultima Gold[®], Packard Instrument Company, Meriden, CT). Samples of feces and blood were digested in 2 mL Soluene[®]-350 (Packard Instrument Company). After digestion, samples requiring bleaching were decolorized with perchloric acid/hydrogen peroxide before the addition of scintillation cocktail.

To isolate, purify, and characterize urinary metabolites, composite samples of urine from animals administered 200 mg/kg [¹⁴C]-*o*-nitrotoluene by gavage were made by mixing 20% of each sample collected. Samples from male and female rats and male mice were pooled separately. The pooled samples were centrifuged, decanted, and filtered through a Millex HV 0.45- μ m filter (Millipore Corporation, Bedford, MA). Concentrations of radiolabel in the pooled samples were determined by liquid scintillation counting. In groups where metabolite profiles were measured, individual animal urine samples were analyzed separately to obtain group mean and standard deviation estimates.

Urinary metabolite profiles were obtained using HPLC with a Supelco LC-18 DB analytical column (Supelco, Inc., Bellefonte, PA). Metabolites were eluted using a linear gradient, changing from 5% to 90% acetonitrile in 5 mM sodium phosphate buffer (pH 4.5) over 30 minutes; the flow rate was 2 mL/minute. Metabolites eluting from the column were detected using both ultraviolet absorption (254 nm) and flow-through radioactivity detectors.

Glucuronide and sulfate metabolite conjugates were determined by incubating aliquots of urine with glucuronidase/sulfatase (Sigma Chemical Co., St. Louis, MO); 1.5 μ L of enzyme solution and 6 μ L of 1 M ammonium acetate were added to 45 μ L of the pooled urine samples. The mixture was incubated for 24 hours at 37° C. Controls were prepared with 1,4-saccharolactone, an inhibitor of β -glucuronidase, and with heat-deactivated enzyme or no enzyme. Aliquots were taken before and after incubation to determine recovery based on radioactivity. Recovery was greater than 90% in all cases. Alternatively, urine samples were incubated for 18 hours in TRIZMA[®] buffer with sulfatase (Sigma Chemical Co.). These profiles were then compared with the HPLC profiles of the same samples prior to incubation with glucuronidase/sulfatase or sulfatase.

The other metabolites were tentatively identified by coelution with authentic standards using HPLC. Upon reevaluation of the metabolite profiles in urine collected from rats dosed with *o*-nitrotoluene having a higher specific activity, some of the original assignments appeared questionable. In these cases, four metabolite peaks, labeled C, D, E, and F, were collected individually as they eluted from the HPLC column, concentrated, and freed of phosphate buffer by use of a C₁₈ Sep Pak (Waters, Milford, MA) prior to analysis by gas chromatography/mass spectrometry (GC/MS). GC/MS was not effective in characterizing metabolite E; accordingly, additional urine

samples were prepared, and metabolite E was subsequently analyzed using thermospray liquid chromatography/mass spectrometry.

In separate studies of male rats administered single gavage doses of [¹⁴C]-o-nitrotoluene, the urinary profile was assessed following pretreatment with buthionine sulfoximine (an inhibitor of glutathione synthesis) or pentachlorophenol (an inhibitor of O-sulfation). Specifically, three male rats were provided drinking water containing 30 mM buthionine sulfoximine for 6 days before and until 72 hours after a single gavage dose of 200 mg/kg [¹⁴C]-o-nitrotoluene. Alternatively, three male rats were injected intraperitoneally with pentachlorophenol 45 minutes before gavage administration of 200 mg/kg [¹⁴C]-o-nitrotoluene. In a third study, the effect of repeat gavage dosing on the metabolism and excretion of [¹⁴C]-o-nitrotoluene was measured in three male rats. The rats were administered nonradiolabeled doses of 200 mg o-nitrotoluene daily for 11 days; on day 12, the dose was radiolabeled, and after dosing, the animals were placed in metabolism cages to facilitate collection of urine and feces. The rats received single gavage doses of nonradiolabeled o-nitrotoluene (200 mg/kg) on days 13 and 14 and were sacrificed on day 15.

Determination of Plasma Concentration of o-Nitrotoluene

Samples were collected from four rats 15 and 30 minutes and 1, 2, 4, 8, and 24 hours after a single gavage dose of 200 mg/kg o-nitrotoluene. Blood was sampled for liquid scintillation analysis and plasma was prepared by centrifugation. Plasma aliquots were analyzed for total radioactivity and the remainder was extracted to analyze for concentration of o-nitrotoluene. For extraction, the samples were placed in 1 mL vials and 200 µL n-butyl chloride was added to each vial. The vials were capped and mixed on an end-over-end rotator for 30 minutes and then centrifuged. The supernatant was transferred to a 200 µL low-volume insert for GC analysis. To construct a standard curve, seven samples of plasma containing 223 to 26,700 ng o-nitrotoluene/g plasma were prepared and duplicate samples were extracted as above. The samples were analyzed using a DB5 column (J&W Scientific, Folsom, CA) in an isothermal oven at 75° C with a flame ionization detector at 300° C. Each sample was analyzed twice, and the reported concentrations are the average of the two determinations.

Determination of the Role of Sulfation in the Conjugation of 2-Nitrobenzyl Alcohol with Glutathione

2-Nitrobenzyl alcohol was incubated with rat liver cytosol in the presence or absence of a 3'-phosphoadenosine-5'-phosphosulfate (PAPS)-generating system. The incubation mixture contained 1 mL rat liver cytosol, 10 mM [³H]-glutathione, 6 µmol magnesium chloride, and phosphate buffer (pH 7.4). Adenosine triphosphate and sodium sulfate (10 µmol each) were added as cofactors for the PAPS-generating system. The mixtures were incubated for 1 hour at 37° C, with 2-nitrobenzyl alcohol (1 mM) added after 30 minutes; incubations were stopped by the addition of 1 mL methanol. The samples were centrifuged and the supernatants were analyzed by HPLC.

Determination of the Binding of o-Nitrotoluene Equivalents to Hemoglobin

Blood was collected via cardiac puncture into heparinized syringes from four anesthetized male rats that had been administered 200 mg/kg o-nitrotoluene. Plasma was prepared by centrifugation and was stored at -20° C. The erythrocytes were washed three times with an equal volume of 0.9% saline, then lysed by the addition of an equal volume of water. Approximately 1 mL of lysate was mixed with 6 mL of 50 mM hydrogen chloride in isopropanol and centrifuged. The supernatant was decanted, 3 mL of ethyl acetate was added, and the mixture was centrifuged to sediment the precipitated protein pellet containing globin. The pellet was washed first with ethyl acetate, then with n-pentane, resedimenting the pellet by centrifugation as before. The pellet was dried under a gentle stream of nitrogen and then *in vacuo* overnight. Protein content was determined by the method of Lowry *et al.* (1951), and radiochemical content was measured as before. The calculated pmol-equivalents/mg protein were monitored before and after continuous (Soxhlet) extraction (ethyl acetate, 2 days), and changed less than 10%.

RESULTS AND DISCUSSION

Excretion and Urinary Metabolites of [¹⁴C]-o-Nitrotoluene in Rats and Mice

Oral doses of 200 mg/kg *o*-nitrotoluene were excreted mainly in urine, with approximately 86% (male rats), 92% (female rats), and 66% (male mice) of the dose excreted in the first 24 hours after dosing (Table L1). Fecal elimination accounted for 3% to 4% of the dose in both male and female rats and approximately 9% in male mice. Total recoveries of radiolabel for male and female rats were approximately 106%, while those of male mice were approximately 87%. In another study using a higher specific activity dose, the values for the excretion of radioactivity were similar to those of the previous investigation (Table L2).

The profiles of metabolites found in urine collected after a single gavage dose of 200 mg/kg *o*-nitrotoluene were determined by HPLC analysis of pooled urine samples (Figures L1 to L3). The relative amounts of these metabolites found upon analysis of individual urine samples are presented in Table L3. At least eight metabolites are present in the HPLC radiochromatograms of urine collections from male and female rats, and only two major metabolites are present in male mice; *o*-nitrotoluene, which elutes at about 25 minutes, was not detected in these samples. The HPLC radiochromatograms indicate the presence of several polar and nonpolar metabolites in rats and two polar metabolites in mice. The HPLC radiochromatograms of urine from F/344N rats after incubation with glucuronidase/sulfatase are shown in Figure L2. The most pronounced change in the profile of metabolites is the loss of the peak B at retention time 5.3 minutes giving rise to peak G; upon incubation with purified sulfatase, peak B remained, confirming it is a glucuronide rather than a sulfate conjugate. The identity of peak G was confirmed by coelution with the authentic standard, 2-nitrobenzyl alcohol. The HPLC radiochromatogram of male B6C3F₁ mouse urine after incubation with glucuronidase/sulfatase is shown in Figure L3. Again, metabolite B was hydrolyzed to yield peak G by incubation with glucuronidase, but not with sulfatase. The major metabolites excreted in the urine of male F/344N rats were 2-nitrobenzoic acid (peak A, 21.0% of the dose), 2-nitrobenzyl glucuronide (B, 16.6%), 2-aminobenzyl alcohol (D, 18.2%), S-(2-nitrobenzyl)-N-acetylcysteine (*o*-nitrobenzylmercapturic acid) (F, 10.4%), 2-nitrobenzyl alcohol (G, 1.8%), and *o*-toluidine (H, 1.3%). Female rats produced a similar profile of major metabolites, except the production of 2-aminobenzyl alcohol and S-(2-nitrobenzyl)-N-acetylcysteine was significantly less than that of male rats. Male mice produced only two major urinary metabolites, 2-nitrobenzoic acid (38.2%) and 2-nitrobenzyl glucuronide (23.9%). Thermospray mass spectral analysis of isolated metabolite E shows that while its retention time was the same as that of the synthetic standard S-(2-nitrobenzyl)glutathione, its mass spectrum does not match that of the synthesized compound.

Excretion of radioactivity following a 2 mg/kg gavage dose of *o*-nitrotoluene was similar to that found after a 200 mg/kg dose, with about 98% and 60% of the dose excreted in the urine of rats and mice, respectively, in the first 24 hours after dosing (Table L4). The metabolite profiles (Table L5) are similar to those seen after the 200 mg/kg dose, except that rats excreted a greater proportion of the dose as 2-nitrobenzoic acid and 2-nitrobenzyl glucuronide after the lower dose. The sex-dependent variance in metabolite profile remains at the 2 mg/kg dose level, with female rats excreting significantly less 2-aminobenzyl alcohol and *o*-nitrobenzylmercapturic acid than male rats, but more nitrobenzoic acid. Male mice again excreted primarily 2-nitrobenzoic acid and 2-nitrobenzyl glucuronide in urine, but a small amount of 2-aminobenzyl alcohol (4%) was also detected.

In rats administered 200 mg/kg *o*-nitrotoluene for 11 days, followed by a 200 mg/kg radiolabeled dose on day 12 and nonradiolabeled doses on days 13 and 14, excretion of radioactivity during the 72 hours following the radiolabeled dose was similar to that found after a single dose of *o*-nitrotoluene (Table L6). There was a 50% decrease in the amount of *o*-nitrobenzylmercapturic acid in urine (Table L7).

Rats treated with buthionine sulfoximine, an inhibitor of glutathione synthesis, before administration of 200 mg/kg *o*-nitrotoluene excreted significantly less of the dose in urine (57% in 24 hours; Table L8) than nonpretreated rats (83% in 24 hours; Table L2). The amount of *o*-nitrobenzylmercapturic acid excreted was about halved, and the amount of 2-nitrobenzyl alcohol excreted tripled relative to rats that were not pretreated. Rats pretreated with pentachlorophenol, an inhibitor of O-sulfation, also excreted significantly less of the dose (52%) in the urine than

nonpretreated rats (Table L9). The amount of *o*-nitrobenzylmercapturic acid excreted decreased from 9.9% of the dose to 1.5%. These data suggest nitrobenzyl alcohol is converted to an alkylating species by O-sulfation, and that glutathione may serve in a protective role by conjugation with that reactive intermediate. The urinary excretion of 2-nitrobenzyl glucuronide was also decreased by pentachlorophenol pretreatment (8% of the dose compared to 15% for nonpretreated animals); 2-aminobenzyl alcohol decreased from 17% of the dose in nonpretreated rats to 4% in the rats receiving pentachlorophenol. The decrease in the glucuronide may have resulted from competition of pentachlorophenol for glucuronidation. The resulting decreases in its biliary excretion, nitro group reduction, and deconjugation by gut microflora may have led to the observed decrease in the urinary excretion of aminobenzyl alcohol.

Roughly twice as much S-(2-nitrobenzyl)-N-acetylcysteine was detected in the urine of male rats than in the urine of female rats. None of this mercapturate was detected in the urine of male mice. In previous studies conducted by the National Institute of Environmental Health Sciences, only male rats showed hepatotoxicity when exposed to *o*-nitrotoluene in feed (NTP, 1992). Rickert *et al.* (1984) found *o*-nitrotoluene binds covalently to hepatic macromolecules to a greater extent than the other nitrotoluene isomers. In addition, *o*-nitrotoluene binds only when its concentration is greater than 40 pmol *o*-nitrotoluene equivalents per mg macromolecule (type and molecular weight of macromolecule not specified). This binding was inhibited by sulfotransferase inhibitors. These findings, along with the results of the present studies, suggest that there may be a threshold production of reactive intermediates, above which the protective nucleophile glutathione may be depleted, and alkylation of DNA and other crucial macromolecules occurs. Rickert *et al.* (1984) suggested 2-aminobenzyl sulfate was the *o*-nitrotoluene metabolite responsible for binding covalently to hepatic macromolecules. The electrophilic 2-nitrobenzyl sulfate, if formed, could react with glutathione to produce S-(2-nitrobenzyl) glutathione and the corresponding mercapturate. The mercapturate present in the urine of male rats is consistent with the production of a reactive intermediate and may rationalize the toxicities peculiar to that species and gender.

Blood Plasma Concentration of *o*-Nitrotoluene

o-Nitrotoluene concentrations in plasma from male rats peaked at nearly 10,000 ng/g plasma 15 to 60 minutes after a single gavage dose of 200 mg/kg (Table L10). The concentration in plasma rapidly decreased through 24 hours after dosing, and concentrations at 24 hours were below the limit of detection. The half-life of *o*-nitrotoluene in plasma was about 1.5 hours.

Role of Sulfation in the Conjugation of 2-Nitrobenzyl Alcohol with Glutathione

No evidence for the formation of glutathione conjugates with 2-nitrobenzyl alcohol was found by HPLC analysis of incubation mixtures of 2-nitrobenzyl alcohol with glutathione in rat liver cytosol with or without a PAPS-generating system (data not presented).

Binding of *o*-Nitrotoluene Equivalents to Hemoglobin

An abbreviated look at excretion rates and routes in the globin binding study indicated this study was consistent with the earlier 200 mg/kg excretion study (data not presented). Of the total radioactivity in blood 72 hours after dosing, 89% was associated with red blood cells (Figure L4), a much higher value than would be predicted from a simple estimation of hematocrit (approximately 42%). Of the radioactivity in the red blood cells, approximately 40% was associated with the isolated, washed protein pellet. This pellet was subjected to continuous (Soxhlet) extraction to ensure that only covalently bound *o*-nitrotoluene equivalents remained. There were 26.0 pmol-equivalents/mg globin for male rats and 29.9 pmol-equivalents/mg globin for female rats. These data do not indicate a marked sex-related difference in globin binding of *o*-nitrotoluene equivalents and, contrary to the metabolite profiles, do not suggest a differential production of alkylating species by male and female rats.

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TABLE L1
Cumulative Excretion of Radioactivity by F344/N Rats and Male B6C3F₁ Mice
after a Single Gavage Dose of 200 mg/kg [¹⁴C]-*o*-Nitrotoluene^a

	Time (hours after dosing)	Urine	Feces	Total
Rats				
Male				
	4	7.3 ± 5.2	— ^b	7.3 ± 5.2
	8	34.6 ± 8.9	0.1 ± 0.0	34.7 ± 8.9
	24	85.9 ± 7.5	1.9 ± 1.0	87.8 ± 7.9
	48	99.4 ± 4.2	3.3 ± 0.6	103 ± 4
	72	102 ± 3	3.5 ± 0.6	106 ± 2
Female				
	4	9.4 ± 7.2	—	9.4 ± 7.2
	8	33.4 ± 9.3	0.0 ± 0.1	33.4 ± 9.3
	24	91.9 ± 7.4	2.0 ± 0.9	93.9 ± 6.6
	48	101 ± 5	3.0 ± 1.3	104 ± 4
	72	103 ± 4	3.2 ± 1.4	106 ± 3
Mice				
Male				
	4	12.1 ± 17.4	—	12.1 ± 17.4
	8	38.8 ± 6.1	0.5 ± 0.6	39.3 ± 5.7
	24	66.3 ± 12.6	1.1 ± 0.8	67.4 ± 12.2
	48	74.1 ± 13.7	6.6 ± 4.6	80.7 ± 12.8
	72	78.0 ± 13.4	8.6 ± 5.1	86.6 ± 14.6

^a n=4 at each time point; data are presented as cumulative percentage of dose recovered (mean ± standard deviation).

^b Not detected

TABLE L2
Cumulative Urinary Excretion of Radioactivity by F344/N Rats after a Single Gavage Dose
of 200 mg/kg [¹⁴C]-*o*-Nitrotoluene^a

	Time (hours after dosing)	Percent of Dose Recovered
Male		
	0 to 24	82.5 ± 5.5
	24 to 48	89.0 ± 3.5
	48 to 72	89.6 ± 3.4
Female		
	0 to 24	83.9 ± 6.9
	24 to 48	90.8 ± 2.3
	48 to 72	91.5 ± 2.0

^a n=3 for each collection period

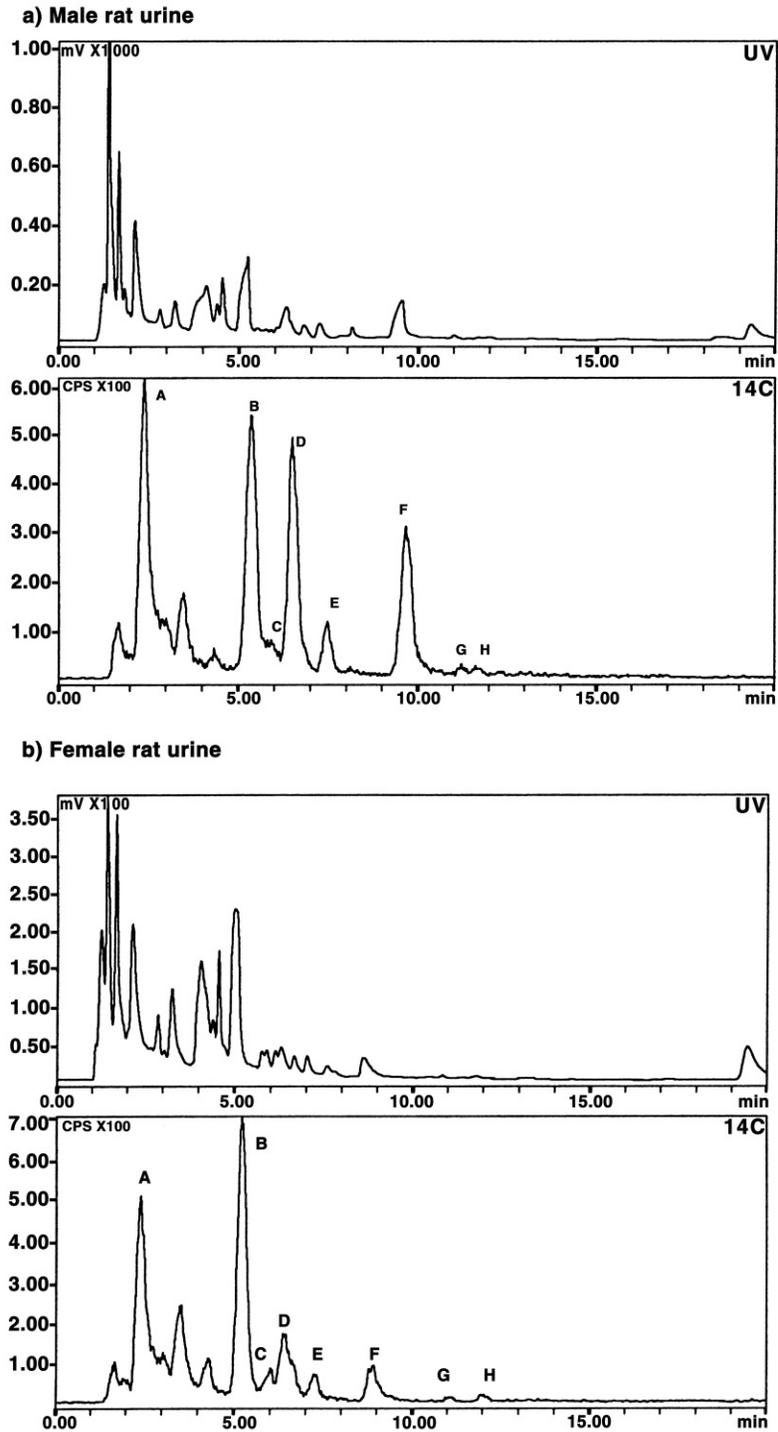


FIGURE L1
HPLC Radiochromatograms of Urine Collected from F344/N Rats 0 to 24 Hours
after a Single Gavage Dose of 200 mg/kg [¹⁴C]-*o*-Nitrotoluene
Metabolites: A=2-nitrobenzoic acid; B=2-nitrobenzyl glucuronide; C=unknown;
D=2-aminobenzyl alcohol; E=unknown; F=S-(2-nitrobenzyl)-N-acetylcysteine;
G=2-nitrobenzyl alcohol; H=*o*-toluidine

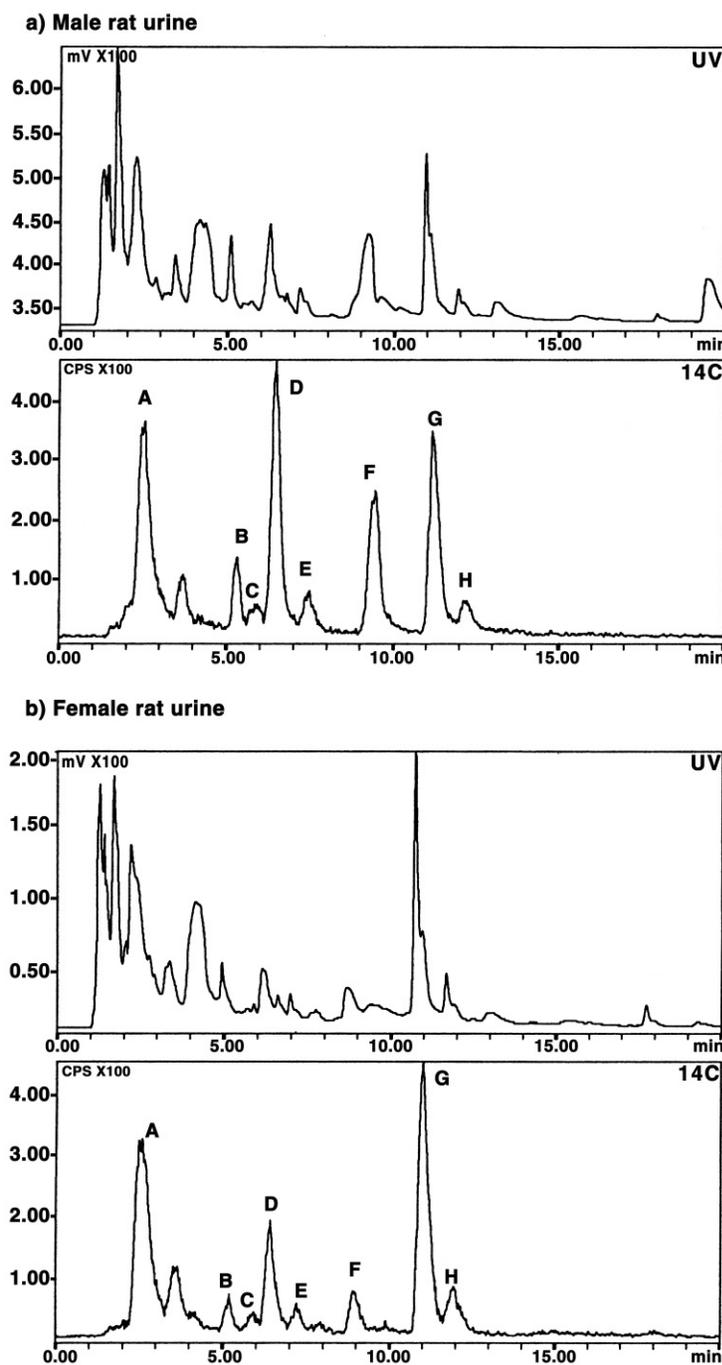


FIGURE L2

HPLC Radiochromatograms of Urine Collected from F344/N Rats 0 to 24 Hours after a Single Gavage Dose of 200 mg/kg [¹⁴C]-*o*-Nitrotoluene and Incubated with β -Glucuronidase/Sulfatase

Metabolites: A=2-nitrobenzoic acid; B=2-nitrobenzyl glucuronide; C=unknown; D=2-aminobenzyl alcohol; E=unknown; F=S-(2-nitrobenzyl)-N-acetylcysteine; G=2-nitrobenzyl alcohol; H=*o*-toluidine

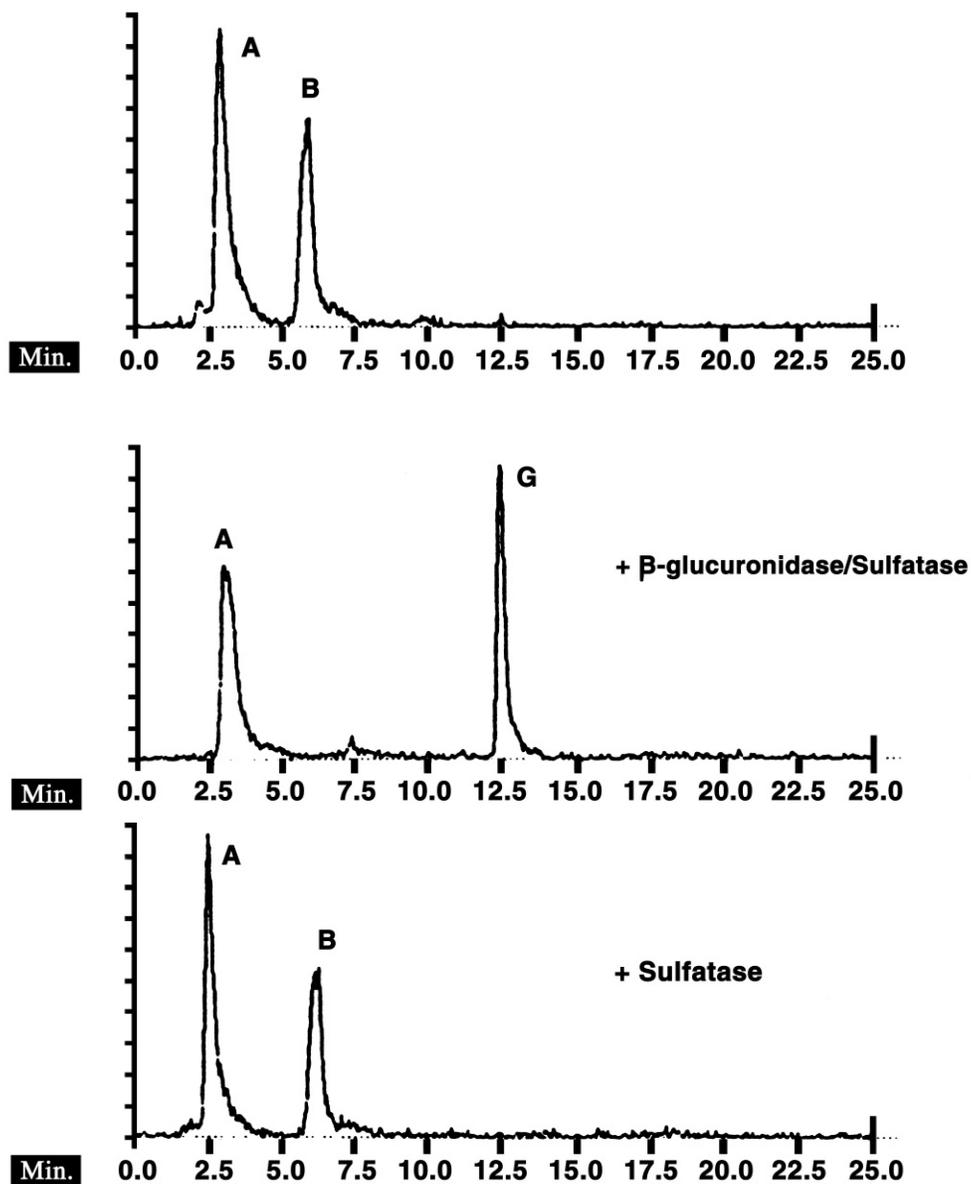


FIGURE L3
HPLC Radiochromatograms of Urine Collected from B6C3F₁ Mice 0 to 24 Hours
after a Single Gavage Dose of 200 mg/kg [¹⁴C]-*o*-Nitrotoluene
Metabolites: A=2-nitrobenzoic acid; B=2-nitrobenzyl glucuronide; G=2-nitrobenzyl alcohol

TABLE L3
Urinary Metabolite Profile for F344/N Rats and Male B6C3F₁ Mice after a Single Gavage Dose of 200 mg/kg [¹⁴C]-o-Nitrotoluene

Time (hours after dosing)	2-Nitrobenzoic acid	2-Nitrobenzyl glucuronide	C ^a	2-Aminobenzyl alcohol	E ^a	S-(2-Nitrobenzyl)-N-acetylcysteine	2-Nitrobenzyl alcohol	o-Toluidine
Rats^b								
Male								
24	19.07 ± 3.20	15.34 ± 1.98		17.01 ± 2.31	3.98 ± 0.36	9.88 ± 1.23	1.77 ± 0.75	1.07 ± 0.71
48	1.90 ± 1.20	1.29 ± 0.47	0.19 ^d	1.16 ± 0.42	0.30 ± 0.07 ^e	0.51 ± 0.12		0.23 ^d
Total ^c	20.97 ± 2.03	16.64 ± 2.16	0.19 ^d	18.17 ± 1.89	4.18 ± 0.48	10.38 ± 1.32	1.77 ± 0.75	1.30 ± 0.79
Female								
24	21.84 ± 2.81	22.09 ± 1.66	2.75 ± 0.27	7.89 ± 0.86	2.43 ± 0.42	3.74 ± 0.35	0.77 ± 0.47	1.38 ± 0.67
48	2.06 ± 1.63	1.65 ± 0.94	0.39 ^d	0.65 ± 0.45	0.22 ± 0.12	0.34 ± 0.21	0.14 ± 0.08	0.06 ± 0.05
Total	23.90 ± 3.43	23.75 ± 1.21	3.14 ± 0.17	8.54 ± 0.61	2.65 ± 0.40	4.08 ± 0.22	0.91 ± 0.41	1.44 ± 0.62
Mice^f								
Male	24	38.2	23.9	— ^g	—	—	—	—

^a Metabolite not identified

^b n=3 at each time point; data are presented as percentage of dose recovered (mean ± standard deviation).

^c Mean of individual animal cumulative urinary excretion from 0 to 48 hours.

^d n=1; standard deviation not calculated because of an insufficient number of samples

^e n=2

^f Urine was pooled from four animals; data are presented as percentage of dose recovered.

^g Metabolite not detected

TABLE L4
Cumulative Excretion of Radioactivity by F344/N Rats and Male B6C3F₁ Mice after a Single Gavage Dose of 2 mg/kg [¹⁴C]-o-Nitrotoluene^a

	Time (hours after dosing)	Urine	Feces	Total
Rats				
Male				
	0 to 24	98.2 ± 5.2	4.93 ± 1.37	103 ± 4
	24 to 48 ^b	104 ± 4	5.31 ± 1.39	110 ± 3
	48 to 72 ^b	106 ± 3	5.54 ± 1.46	112 ± 2
Female				
	0 to 24	97.1 ± 13.5	2.73 ± 0.92	99.9 ± 13.3
	24 to 48 ^b	104 ± 18	3.16 ± 0.78	107 ± 17
	48 to 72 ^b	108 ± 20	3.39 ± 0.84	113 ± 20
Mice				
Male				
	0 to 24	60.4 ± 18.9	15.8 ± 2.4	76.2 ± 19.6
	24 to 48 ^b	69.0 ± 17.6	22.5 ± 2.0	91.5 ± 15.8
	48 to 72 ^b	84.9 ± 8.2	23.2 ± 2.2	108 ± 7

^a n=3 for each collection period; data are presented as cumulative percentage of dose recovered (mean ± standard deviation).

^b Cage rinse included for this collection period

TABLE L5
Urinary Metabolite Profile for F344/N Rats and Male B6C3F₁ Mice after a Single Gavage Dose of 2 mg/kg [¹⁴C]-o-Nitrotoluene

Time (hours after dosing)	2-Nitrobenzoic acid	2-Nitrobenzyl glucuronide	C ^a	2-Aminobenzyl alcohol	E ^a	S-(2-Nitrobenzyl)-N-acetylcysteine	2-Nitrobenzyl alcohol	o-Toluidine
Rats^b								
Male								
24	30.6 ± 4.3	28.0 ± 5.9	2.6 ± 0.4	11.0 ± 4.7	2.4 ± 0.1	12.4 ± 1.2	1.9 ± 1.2	— ^c
Female								
24	43.9 ± 2.6	26.5 ± 6.7	2.5 ± 0.6	4.4 ± 0.7	1.2 ± 0.4	4.9 ± 0.7	1.0 ± 0.4	—
Mice^d								
Male								
24	20.1	27.9	0.0	4.0	0.0	0.0	0.0	—
48	3.5	3.5	0.0	0.0	0.0	0.0	0.0	—

^a Metabolite not identified

^b n=3 at each time point; data are presented as percentage of dose recovered (mean ± standard deviation).

^c Not detected

^d Urine was pooled from three mice at each time point; data are presented as percentage of dose recovered.

TABLE L6
Cumulative Excretion of Radioactivity by Male F344/N Rats Administered 200 mg/kg [¹⁴C]-o-Nitrotoluene in the 14-Day Gavage Study^a

Time (hours after dosing with radiolabel)	Urine	Feces	Total
0 to 24	77.7 ± 12.5	10.2 ± 13.7	87.9 ± 1.3
24 to 48	84.7 ± 16.0	10.7 ± 13.6	95.5 ± 2.2
48 to 72 ^b	87.4 ± 15.9	10.9 ± 13.6	98.4 ± 2.8

^a n=3 for each collection period. Radiolabeled dose was administered on day 12; data are presented as cumulative percentage of dose recovered (mean ± standard deviation).

^b Cage rinse included for this collection period

TABLE L7
Urinary Metabolite Profile for Male F344/N Rats Administered 200 mg/kg [¹⁴C]-o-Nitrotoluene in the 14-Day Gavage Study^a

Time (hours after dosing with radiolabel)	2-Nitrobenzoic acid	2-Nitrobenzyl glucuronide	C ^b	2-Aminobenzyl alcohol	E ^b	S-(2-Nitrobenzyl)-N-acetylcysteine	2-Nitrobenzyl alcohol	o-Toluidine
24	19.7	23.1	0.0	12.0	3.1	4.8	1.3	0.8
48	2.3	1.9	0.0	1.0	0.4	0.5	0.1	0.0
Total	22.1	25.0	0.0	12.9	3.6	5.3	1.4	0.8

^a Radiolabeled dose was administered on day 12. Urine was pooled from three rats at each time point; data are presented as percentage of dose recovered.
^b Metabolite not identified

TABLE L8
Urinary Metabolite Profile for Male F344/N Rats Pretreated with Buthionine Sulfoxamine after a Single Gavage Dose of 200 mg/kg [¹⁴C]-o-Nitrotoluene^a

Time (hours after dosing)	2-Nitrobenzoic acid	2-Nitrobenzyl glucuronide	C ^b	2-Amino-benzyl alcohol	E ^b	S-(2-Nitrobenzyl)-N-acetylcysteine	2-Nitrobenzyl alcohol	o-Toluidine	% Excreted ^c
0 to 24	13.0	12.9	0.0	10.0	3.8	4.7	5.8	0.6	57.2 ± 6.3
24 to 48	2.6	2.5	0.0	1.4	0.5	0.7	0.3	0.0	65.7 ± 4.4
48 to 72	0.4	0.4	0.0	0.2	0.1	0.1	0.0	0.0	67.2 ± 5.0
Total	16.0	15.8	0.0	11.5	4.4	5.5	6.2	0.6	

^a Urine was pooled from three rats for each collection period; data are presented as percentage of dose recovered.
^b Metabolite not identified
^c Cumulative percentage of the dose recovered as all metabolites excreted in the indicated time period (mean ± standard deviation)

TABLE L9
Urinary Metabolite Profile for Male F344/N Rats Pretreated with Pentachlorophenol
after a Single Gavage Dose of 200 mg/kg [¹⁴C]-*o*-Nitrotoluene^a

Time (hours after dosing)	2-Nitrobenzoic acid	2-Nitrobenzyl glucuronide	C ^b	2-Amino-benzyl alcohol	E ^b	S-(2-Nitrobenzyl)-N-acetylcysteine	2-Nitrobenzyl alcohol	<i>o</i> -Toluidine	% Excreted ^c
0 to 4	1.25	1.34	0.00	0.18	0.11	0.19	0.91	0.00	4.2 ± 2.0
4 to 8	4.45	2.73	0.00	1.07	0.60	0.40	0.51	0.21	13.0 ± 1.7
0 to 24	21.52	7.79	2.06	4.08	1.96	1.50	2.43	0.83	51.6 ± 8.5

^a Urine was pooled from three rats for each collection period; data are presented as percentage of dose recovered.

^b Metabolite not identified

^c Cumulative percentage of the dose recovered as all metabolites excreted in the indicated time period (mean ± standard deviation)

TABLE L10
Plasma Concentration of *o*-Nitrotoluene in Male F344/N Rats after a Single Gavage Dose of 200 mg/kg [¹⁴C]-*o*-Nitrotoluene^a

Time (hours after dosing)	Rat 1	Rat 2	Rat 3	Rat 4	Mean ± Standard Deviation
0.25	8,239	10,928	8,738	7,811	8,929 ± 1,385
0.5	9,647	9,600	8,931	8,943	9,280 ± 397
1	9,091	6,942	9,183	10,249	8,866 ± 1,386
2	8,126	5,227	4,179	7,970	5,200 ± 3,610
4	2,807	2,431	1,257	2,765	2,315 ± 725
8	429	365	347	267	352 ± 67
24	<LD ^b	<LD	<LD	<LD	<LD

^a Data are presented as ng *o*-nitrotoluene/g plasma; values are the average of two determinations.

^b LD (limit of detection) was approximately 50 ng/g plasma.

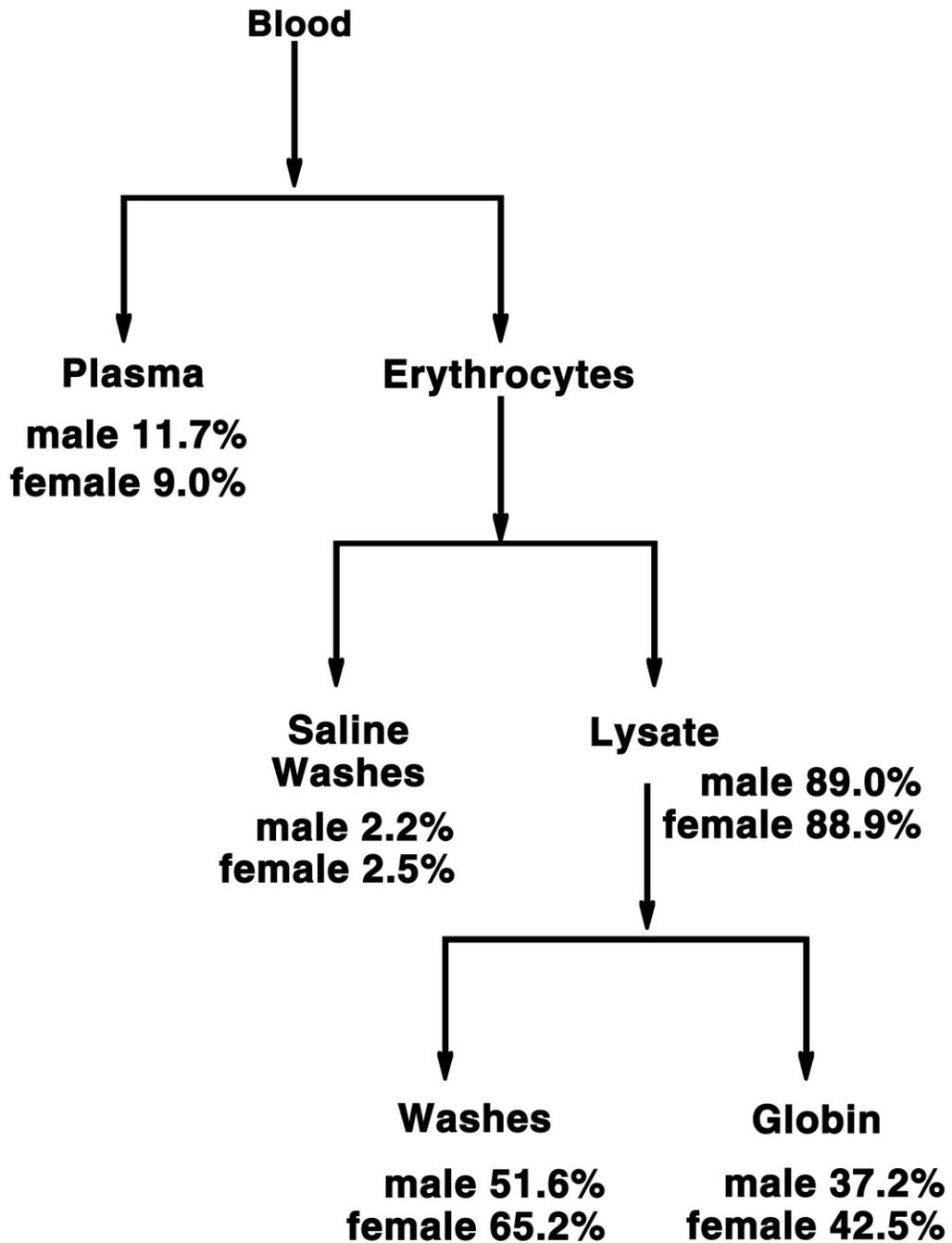


FIGURE L4
Isolation of Globin and Binding of *o*-Nitrotoluene to Globin in F344/N Rats 72 Hours after a Single Gavage Dose of 200 mg/kg [¹⁴C]-*o*-Nitrotoluene
Values are percent of initial blood radioactivity

APPENDIX M
GENETIC ALTERATIONS IN *ras*, *p53*,
AND β -CATENIN GENES
IN HEMANGIOSARCOMAS OF B6C3F₁ MICE
FOLLOWING EXPOSURE TO *o*-NITROTOLUENE
FOR 2 YEARS

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GENETIC ALTERATIONS IN *ras*, *p53*, AND β -CATENIN GENES IN HEMANGIOSARCOMAS OF B6C3F₁ MICE FOLLOWING EXPOSURE TO o-NITROTOLUENE FOR 2 YEARS

INTRODUCTION

In the 2-year o-nitrotoluene mouse study, there were significant increases in the incidences of hemangiosarcomas, cecal carcinomas, and hepatocellular neoplasms. The focus of this study was to evaluate spontaneous and o-nitrotoluene-induced hemangiosarcomas for mutations in cancer genes important in the pathogenesis of human cancer. A representative number of hemangiosarcomas from the 2-year o-nitrotoluene mouse study and spontaneous hemangiosarcomas from the NTP Archives were available for analysis.

In evaluating potential hazards of chemical exposure to humans, it is important to assess how the chemical acts at the molecular level, i.e., through a direct genotoxic mechanism or via indirect mechanisms such as promotion of spontaneous DNA damage. In the past, the patterns of mutations in proto-oncogenes such as *ras* and in tumor suppressor genes such as *p53* have helped in the understanding of tumorigenesis (Harris, 1993; Maronpot *et al.*, 1995). For example, in some neoplasms, the profiles of activating mutations in *ras* genes or inactivating mutations in the *p53* gene are specific for particular chemicals and differ from those detected in spontaneous neoplasms (Trukhanova *et al.*, 1998; Sills *et al.*, 1999). Recently, genetic alterations in the β -catenin gene were identified in human and rodent neoplasms (De La Coste *et al.*, 1998; Mirabelli-Primdahl *et al.*, 1999; Anna *et al.*, 2000). β -catenin is a multifunctional protein involved in two important biological processes: cell-cell adhesion and signal transduction (Behrens, 1999). In this study, hemangiosarcomas from B6C3F₁ mice exposed to o-nitrotoluene in feed were examined for genetic alterations in *ras*, *p53* and β -catenin genes, genes shown to be altered in human neoplasms.

MATERIALS AND METHODS

Hemangiosarcomas: Fifteen subcutaneous, mesentery, and skeletal muscle hemangiosarcomas from mice exposed to 1,250, 2,500, or 5,000 ppm o-nitrotoluene and 13 subcutaneous hemangiosarcomas from control male and female B6C3F₁ mice from previous NTP studies were used. Because this neoplasm is rarely found in control mice, spontaneous hemangiosarcomas were obtained from several studies (Table M1). Hemangiosarcomas from the o-nitrotoluene study were selected based on the samples available in the frozen tissue archives. The frozen samples were not used in this study; however, the paraffin-embedded samples that matched the frozen samples were used for immunohistochemistry and mutation analysis.

Immunohistochemistry, p53 and β -Catenin: o-Nitrotoluene-induced and spontaneous hemangiosarcomas were first screened for *p53* and β -catenin protein expression. Wild-type *p53* protein has a short half-life but, when mutated, can be detected in the nucleus of neoplastic cells. Wild-type β -catenin protein expression is quickly degraded in normal cells, but when a genetic alteration (point mutation, deletion, or splicing intron) occurs in the β -catenin gene, increased expression can be detected in the cell membrane, cytoplasm, or nucleus of neoplastic cells. Hemangiosarcomas were screened for *p53* and β -catenin protein by immunohistochemical analysis using the avidin-biotin-peroxidase detection system (Hsu *et al.*, 1981; Hegi *et al.*, 1993; Rao *et al.*, 1996; Devereux *et al.*, 1999; Vectastain Rabbit Elite Kit, Vector Laboratories, Burlingame, CA). Because mutant *p53* has a much longer half-life than wild-type *p53*, immunohistochemistry primarily detects the mutated protein. Immunohistochemical staining for expression of mutant *p53* protein was performed as previously described (Sills *et al.*, 1995; Trukhanova *et al.*, 1998). A 1:300 dilution of the primary polyclonal rabbit antibody CM-5 (Vector Laboratories), which detects accumulation of the mutant *p53* protein in rodents, was used. Normal rabbit serum (Vector Laboratories) was used as the negative control in place of the primary antibody. Tissue specimens from a

p53 transgenic mouse (mutation in codon 135 of *p53*) served as the positive control. Localization of β -catenin expression was investigated using a polyclonal goat anti- β -catenin antibody at a dilution of 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA). Nonimmune goat IgG (Jackson ImmunoResearch Labs, West Grove, PA) at equivalent conditions in place of the primary antibody was used as the negative control. The positive control for β -catenin protein was a mouse hepatoblastoma where a β -catenin point mutation was confirmed by direct sequencing.

DNA Isolation: The DNA isolation procedure has been described previously (Marmur, 1961; Devereux *et al.*, 1993). DNA was isolated from paraffin-embedded sections containing hemangiosarcomas.

DNA Amplification: DNA was amplified by the polymerase chain reaction (PCR) (Saiki *et al.*, 1988; Sills *et al.*, 1995); details of the use of nested primers for K- and H-*ras* have been described previously (Devereux *et al.*, 1991, 1993). Touch-down PCR was performed for exons 5 through 8 of the *p53* gene; PCR/sequencing primers and annealing temperature profile of the cycles have been described previously (Trukhanova *et al.*, 1998). Positive controls for K-*ras* and H-*ras* or *p53* mutations and no-DNA controls were run with all sets of reactions. DNA amplification by PCR and details of the use of nested primers for β -catenin have been described previously (Devereux *et al.*, 1999; Anna *et al.*, 2000).

Single-Strand Conformational Analysis: For analysis of K-*ras* and *p53* mutations, non-radioactive single-strand conformational polymorphism (SSCP) analysis was performed. A mixture of 5 μ L of PCR product, 0.6 μ L of 1 M methylmercury hydroxide, 1 μ L of 15% (w/v) Ficoll (molecular weight 400,000) loading buffer containing 0.25% bromophenol blue and 0.25% xylene cyanol, and 13.4 μ L of 1X tris-borate EDTA (TBE) buffer (Novex, San Diego, CA) was prepared to yield a total volume of 20 μ L (Hongyo *et al.*, 1993). This mixture was denatured at 85° C for 5 minutes prior to loading on a 20% polyacrylamide TBE gel (Novex). The buffer chamber was filled with 1.5X TBE buffer. The gel was run at 300 volts at 10° and 13° C for K-*ras* exon 1, at 8° C for exon 2, and at 9° C for *p53* until the xylene cyanol light blue dye reached the bottom of the gel. Positive controls for K-*ras* mutations and one undenatured DNA control (without methylmercury hydroxide and no heat) were run with unknown samples. Gels were stained with ethidium bromide. For analysis of β -catenin, SSCP analysis was carried out on PCR products of exon 2 (corresponds to exon 3 in humans) of the mouse β -catenin gene (De La Coste *et al.*, 1998), which contains the glycogen serine-threonine kinase-3 β -targeted phosphorylation sites within residues 33 through 45. The sequences of the intronic PCR primers flanking the borders of exon 2 were: BCAT-1F, 5'-TACAGGTAGCATTTTCAGTTCAC-3' and BCAT-2R, 5'-TAGCTTCCAAACACAAATGC-3' (De La Coste *et al.*, 1998). Inner primers were BCAT-7F, 5'-TAACATACTCTGTTTTTACAGCTG-3', and BCAT-8R, 5'-ACATCTTCTTCCTCAGGGTTG-3'. For DNA from fixed sections, nested PCR was used with primers 1F X 2R for the outer reactions and both 1F X 8R and 7F X 8R for the inner reactions. [³³P]-dATP was incorporated into the inner PCR reactions for SSCP analysis, and two gel conditions were used to detect mutations: 6% acrylamide gels with 10% glycerol were electrophoresed at 40 watts for 6 hours at 5° C, and 0.5X Mutations Detection Enhancement gels (AT Biochem, Malvern, PA) were electrophoresed at 3 watts for 17 hours at room temperature. DNA from normal tissue and no-DNA controls were included with all amplification experiments to confirm no cross-contamination of PCR products.

Cycle Sequencing: To identify mutations, samples were sequenced with a cycle sequencing kit (US Biochemical, Cleveland, OH) that incorporates [α -³³P]-dideoxynucleotide (ddNTP) terminators (A,C,G,T) into the sequencing products. Prior to sequencing, PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). The sequencing primers for K-*ras* were 5'-TCGTACTCATCCACAAAGTG-3' and 5'-ACCTGTCTCTTGGAT-3' for exons 1 and 2 respectively; for H-*ras* exon 2, the sequencing primer was 5'-TGTGCGCATGACTG-3'. The sequencing primers for *p53* have been described previously (Trukhanova *et al.*, 1998). The sequencing reactions were stopped by adding formamide-dye stop solution (Amersham, Cleveland, OH) and were heat denatured at 70° C for 10 minutes prior to electrophoresis on an 8% acrylamide gel containing 8M urea. To identify β -catenin point mutations, small deletions, or splicing introns, samples with altered bands on SSCP gels were reamplified. Other deletions were identified by excision and boiling of the altered band, followed by a fresh amplification. The amplified bands were gel-purified on Qiagen columns

(QIAGEN) before sequencing with a [³³P]-Thermo-Sequenase kit (Amersham). The amplification primers also served as sequencing primers. Mutation identification was confirmed with at least two amplification reactions from original DNA.

RESULTS

Spontaneous hemangiosarcomas from control mice lacked *p53* protein expression (Table M1), and these neoplasms were not screened for *p53* mutations. All of the hemangiosarcomas from B6C3F₁ mice administered *o*-nitrotoluene in feed exhibited positive immunohistochemical staining of *p53* (Table M2). Hemangiosarcomas originating from skeletal muscle, mesentery, and subcutaneous tissues from exposed mice were examined for genetic alterations in the *K-ras*, *H-ras*, *p53* and β -catenin genes. Mutations in at least one of these genes were identified in 13 of 15 (87%) of the *o*-nitrotoluene-induced hemangiosarcomas, and missense mutations in *p53* exons 5 through 8 were detected in 11 of 15 (73%) of these lesions (Table M3). Mutations in *p53* were identified at codons 190, 195, 200, 205, 210, 220, 235, 241, 243, 250, 263 and 264. Of the 15 *p53* mutations identified, six were G-to-A transitions, three were A-to-G transitions, three were C-to-T transitions, and the other three were various base pair alterations. Four hemangiosarcomas from the 5,000 ppm group exhibited double mutations in *p53*; one of these hemangiosarcomas also had a β -catenin mutation, while another had a CTA mutation at codon 61 of *K-ras*.

The lack of *p53* mutations in some of the hemangiosarcomas that were positive by immunohistochemical methods for the *p53* protein may be due to mutations outside the exons 5 to 8 evaluated, or to changes in the expression of other genes that influence the expression of *p53*. Mutations in *ras* genes or the β -catenin gene were not detected in five of the *o*-nitrotoluene-induced hemangiosarcomas that had *p53* mutations.

Seven of 15 (47%) hemangiosarcomas from mice exposed to *o*-nitrotoluene had splice site mutations at the beginning of exon 2 or deletions in the β -catenin gene (Table M3). Two *o*-nitrotoluene-induced hemangiosarcomas had β -catenin genetic alterations but not *p53* or *ras* mutations. There was a generally good correlation (six of seven; 86%) between hemangiosarcomas that had membrane staining for the β -catenin protein and genetic alterations in the β -catenin gene (Tables M2 and M3).

DISCUSSION

This is the first report in which spontaneous subcutaneous hemangiosarcomas have been screened for both *p53* and β -catenin protein expression. The lack of *p53* and β -catenin protein expression in spontaneous hemangiosarcomas provides a basis for comparison with carcinogen-induced hemangiosarcomas. The current findings are consistent with those of Duddy *et al.* (1999a) where *p53* protein expression was not detected in spontaneous hemangiosarcomas in B6C3F₁ mice. Similar to the current study, low frequencies of *ras* mutations were detected in spontaneous or thiazolidinedione-induced hemangiosarcomas in the B6C3F₁ mouse (Duddy *et al.*, 1999b). In contrast, a high frequency of the signature *K-ras* codon 13 G-to-C transversion was detected in 1,3-butadiene-induced hemangiosarcomas (Hong *et al.*, 2000). These studies suggest there may be chemical-specific mutation profiles or pathways in hemangiosarcoma formation.

Similar spectra of *p53* mutations in mice have been identified in hemangiosarcomas following 1,3-butadiene exposure (Hong *et al.*, 2000) and *o*-nitrotoluene exposure (this study). Although most of the *in vitro* genotoxicity tests were negative, the current *in vivo* mutation data suggest *o*-nitrotoluene is metabolized to genotoxic intermediates. *o*-Nitrotoluene has a number of potentially active metabolites that could account for the mutation profile observed in these tumors.

Further evidence that mutation of *p53* plays an important role in *o*-nitrotoluene-induced hemangiosarcoma formation is provided by tumorigenesis findings in *p53* knockout mice. Hemangiosarcomas have been found in six of 56 (11%) untreated *p53* heterozygous (+/-) mice and in 10 of 60 (17%) untreated *p53* homozygous (-/-) knockout mice. Multiple hemangiosarcomas of the liver were found in 14 of 17 (82%) *p53* heterozygous mice

exposed to low concentrations of dimethylnitrosamine (0.0005%) in drinking water (Harvey *et al.*, 1993). The *p53* mutation data from the current study, 1,3-butadiene studies, and studies with *p53* knockout mice collectively provide growing evidence that genetic alterations in the *p53* gene contribute to induction of hemangiosarcomas and that these chemicals target *p53* in tissues in which hemangiosarcomas arise.

β -Catenin protein was detected in 47% of the *o*-nitrotoluene-induced hemangiosarcomas but not in the spontaneous neoplasms, suggesting the Wnt-signaling pathway may be involved in the carcinogenesis process of a subset of hemangiosarcomas. β -Catenin protein accumulation and upregulation of the Wnt-signaling pathway have been shown following mutations in either the adenomatous polyposis coli (APC) or β -catenin gene (Behrens, 1999). The immunohistochemical localization of β -catenin protein at the cell membrane but not in the nucleus of hemangiosarcoma cells was similar to previous findings for chemical-induced mouse hepatocellular neoplasms (Devereux *et al.*, 1999). Because β -catenin plays a pivotal role in cell adhesion through participation in the cadherin/catenin complex of adherence junctions, changes in the expression and structure of β -catenin may lead to loss of adhesiveness and may promote invasiveness and/or metastasis. Consistent with this hypothesis, Gamallo *et al.* (1999) demonstrated that endometrial carcinomas with membrane expression of β -catenin had a poor prognosis. Alternatively, immunohistochemistry may not be the most sensitive measure of signal transduction, and perhaps only a small increase in β -catenin accumulation is necessary for increased nuclear translocation and transcriptional activation. These hypotheses are now being tested.

Activating mutations in the *ras* gene and inactivating mutations in the *p53* gene have been detected in human hemangiosarcomas. For example, in liver hemangiosarcomas from factory workers exposed to vinyl chloride, the *N*⁶-ethenoadduct has been postulated to lead to specific A-to-T transversions detected in the *p53* tumor suppressor gene (Marion *et al.*, 1991; Hollstein *et al.*, 1994). On the other hand, G-to-A transversions at the second base of codon 13 of the *c-kis-ras-2* gene in human hemangiosarcomas associated with vinyl chloride exposure have been shown to be consistent with the known miscoding properties of *N*², 3-ethenoguanine and 3,*N*⁴-ethenocytosine (La and Swenberg, 1996). The finding of chemical-associated mouse hemangiosarcomas with *p53* and β -catenin mutations suggests these neoplasms are good models for the human equivalent. Moreover, the human and mouse neoplasms may have similar carcinogenic pathways with implications for human risk from exposure to this chemical.

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TABLE M1
Summary of Immunohistochemistry for *p53* and β -Catenin Protein Accumulation in Spontaneous Hemangiosarcomas in Control B6C3F₁ Mice in Selected NTP Studies

Study ^a	Technical Report Number	<i>p53</i>	β -Catenin
Sodium fluoride	393	— ^b	—
Chloroprene	467	—	—
Cobalt sulfate	471	—	—
1-Chloro-2-propanol	477	—	—
Oxazepam	468	—	—
<i>t</i> -Butylhydroquinone	459	—	—
Pyridine	470	—	—
Sodium nitrite	495	—	—
Ozone	440	—	—
Primidone	476	—	—
Indium phosphide	499	—	—
Nickel oxide	451	—	—
Glutaraldehyde	490	—	—

^a NTP study from which subcutaneous hemangiosarcoma was obtained from a control mouse; each row represents a single hemangiosarcoma.
^b No positive immunohistochemical response was observed

TABLE M2
Summary of Immunohistochemistry for *p53* and β -Catenin Protein Accumulation in Hemangiosarcomas in B6C3F₁ Mice in the 2-Year Feed Study of *o*-Nitrotoluene

Exposure Concentration ^a (ppm)	<i>p53</i>	β -Catenin
1,250	+ ^b	— ^c
1,250	+	+
2,500	+	—
2,500	+	—
5,000	+	—
5,000	+	+
5,000	+	+
5,000	+	+
5,000	+	—
5,000	+	—
5,000	+	—
5,000	+	—
5,000	+	+
5,000	+	+
5,000	+	+

^a Samples match in sequence with Table M3 samples; each row represents a single hemangiosarcoma.
^b A positive immunohistochemical response was observed
^c No positive immunohistochemical response was observed

TABLE M3
Summary of *ras*, *p53*, and β -Catenin Mutations in Hemangiosarcomas from B6C3F₁ Mice in the 2-Year Feed Study of o-Nitrotoluene^a

Exposure Concentration (ppm)	Tissue	K- <i>ras</i>		H- <i>ras</i>	p53			β -Catenin
		Codon 12	Codon 13	Codon 61	Exon 5	Exon 6	Exon 7	
1,250	Skeletal Muscle	^b —	—	—	—	Codon 190 CAT to CGT (His to Arg)	—	—
1,250	Mesentery	—	—	—	—	—	—	Splicing Intron
2,500	Skeletal Muscle	—	—	—	—	Codon 195 GAA to GGA (Glu to Gly)	—	—
2,500	Skeletal Muscle	—	—	—	—	—	—	—
5,000	Skeletal Muscle	—	—	—	—	Codon 243 ATG to ATA (Met to Ile)	—	Splicing Intron
5,000	Skeletal Muscle	—	—	—	—	Codon 220 CCC to TCC (Pro to Ser)	—	Deletions 25-36
5,000	Skeletal Muscle	—	—	—	—	Codon 200 CCC to CTC (Pro to Leu)	Codon 241 GGG to GAG (Gly to Glu)	Deletions 5-6 Codon 28 CAG to CGG (Gln to Arg)
5,000	Skeletal Muscle	—	—	—	—	—	Codon 235 TGT to TTT (Cys to Phe)	Splicing Intron
5,000	Skeletal Muscle	—	—	—	—	Codon 210 CGC to TGC (Arg to Cys)	—	Codon 263 GGA to AGA (Gly to Arg)
5,000	Subcutaneous Tissues	—	—	—	—	Codon 210 CGC to AGC (Arg to Ser)	—	Codon 263 GGA to AGA (Gly to Arg)

TABLE M3
Summary of *ras*, *p53*, and β -Catenin Mutations in Hemangiosarcomas from B6C3F₁ Mice in the 2-Year Feed Study of *o*-Nitrotoluene

Exposure Concentration (ppm)	Tissue	K- <i>ras</i>		H- <i>ras</i>	<i>p53</i>			β -Catenin
		Codon 12	Codon 13	Codon 61	Exon 5	Exon 6	Exon 7	
5,000	Subcutaneous Tissues	—	—	—	—	—	—	—
5,000	Subcutaneous Tissues	—	CAA to CTA (Gln to Leu)	—	—	Codon 205 GAC to GGC (Asp to Gly)	Codon 250 ACC to TCC (Thr to Ser)	—
5,000	Subcutaneous Tissues	—	—	—	—	—	—	Codon 263 GGA to AGA (Gly to Arg)
5,000	Subcutaneous Tissues	—	—	—	—	—	—	Codon 264 CCG to CAG (Arg to Glu)
5,000	Subcutaneous Tissues	—	—	—	—	—	—	Splicing Intron
Total combined mutations		1/15 (7%)		0/15 (0%)	11/15 (73%)			7/15 (47%)
Hemangiomas containing at least one mutation		13/15 (87%)						

^a His=histidine; Arg=arginine; Glu=glutamic acid; Gly=glycine; Met=methionine; Ile=isoleucine; Pro=proline; Ser=serine; Leu=leucine; Cys=cysteine; Phe=phenylalanine;
 Asp=aspartic acid; Thr=threonine; Gln=glutamine

^b No mutation identified

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Chemical	TR No.	Chemical	TR No.
Acetaminophen	394	Chlorpheniramine Maleate	317
Acetonitrile	447	C.I. Acid Orange 3	335
Acrylonitrile	506	C.I. Acid Orange 10	211
Agar	230	C.I. Acid Red 14	220
Allyl Glycidyl Ether	376	C.I. Acid Red 114	405
Allyl Isothiocyanate	234	C.I. Basic Red 9 Monohydrochloride	285
Allyl Isovalerate	253	C.I. Direct Blue 15	397
1-Amino-2,4-Dibromoanthraquinone	383	C.I. Direct Blue 218	430
2-Amino-4-Nitrophenol	339	C.I. Disperse Blue 1	299
2-Amino-5-Nitrophenol	334	C.I. Disperse Yellow 3	222
11-Aminoundecanoic Acid	216	C.I. Pigment Red 3	407
<i>dl</i> -Amphetamine Sulfate	387	C.I. Pigment Red 23	411
Ampicillin Trihydrate	318	C.I. Solvent Yellow 14	226
Asbestos, Amosite (Hamsters)	249	Cobalt Sulfate Heptahydrate	471
Asbestos, Amosite (Rats)	279	Coconut Oil Acid Diethanolamine Condensate	479
Asbestos, Chrysotile (Hamsters)	246	Codeine	455
Asbestos, Chrysotile (Rats)	295	Comparative Initiation/Promotion Studies (Mouse Skin)	441
Asbestos, Crocidolite	280	Corn Oil, Safflower Oil, and Tricaprylin	426
Asbestos, Tremolite	277	Coumarin	422
L-Ascorbic Acid	247	Cytembena	207
AZT and AZT/ α -Interferon A/D	469	D&C Red No. 9	225
Barium Chloride Dihydrate	432	D&C Yellow No. 11	463
Benzaldehyde	378	Decabromodiphenyl Oxide	309
Benzene	289	Diallyl Phthalate (Mice)	242
Benzethonium Chloride	438	Diallyl Phthalate (Rats)	284
Benzofuran	370	4,4'-Diamino-2,2'-Stilbenedisulfonic Acid, Disodium Salt	412
Benzyl Acetate (Gavage)	250	2,4-Diaminophenol Dihydrochloride	401
Benzyl Acetate (Feed)	431	1,2-Dibromo-3-Chloropropane	206
Benzyl Alcohol	343	1,2-Dibromoethane	210
<i>o</i> -Benzyl- <i>p</i> -Chlorophenol (Gavage)	424	2,3-Dibromo-1-Propanol	400
<i>o</i> -Benzyl- <i>p</i> -Chlorophenol (Mouse Skin)	444	1,2-Dichlorobenzene (<i>o</i> -Dichlorobenzene)	255
2-Biphenylamine Hydrochloride	233	1,4-Dichlorobenzene (<i>p</i> -Dichlorobenzene)	319
2,2-Bis(Bromomethyl)-1,3-Propanediol	452	<i>p,p'</i> -Dichlorodiphenyl sulfone	501
Bis(2-Chloro-1-Methylethyl) Ether	239	2,4-Dichlorophenol	353
Bisphenol A	215	2,6-Dichloro- <i>p</i> -Phenylenediamine	219
Boric Acid	324	1,2-Dichloropropane	263
Bromodichloromethane	321	1,3-Dichloropropene (Telone II)	269
Bromoethane	363	Dichlorvos	342
1,3-Butadiene	288	Dietary Restriction	460
1,3-Butadiene	434	Diethanolamine	478
<i>t</i> -Butyl Alcohol	436	Di(2-Ethylhexyl) Adipate	212
Butyl Benzyl Phthalate	213	Di(2-Ethylhexyl) Phthalate	217
Butyl Benzyl Phthalate	458	Diethyl Phthalate	429
<i>n</i> -Butyl Chloride	312	Diglycidyl Resorcinol Ether	257
<i>t</i> -Butylhydroquinone	459	3,4-Dihydrocoumarin	423
γ -Butyrolactone	406	1,2-Dihydro-2,2,4-Trimethylquinoline (Monomer)	456
Caprolactam	214	Dimethoxane	354
<i>d</i> -Carvone	381	3,3'-Dimethoxybenzidine Dihydrochloride	372
Chloral Hydrate	502	N,N-Dimethylaniline	360
Chlorinated and Chloraminated Water	392	3,3'-Dimethylbenzidine Dihydrochloride	390
Chlorendic Acid	304	Dimethyl Hydrogen Phosphite	287
Chlorinated Paraffins: C ₂₃ , 43% Chlorine	305	Dimethyl Methylphosphonate	323
Chlorinated Paraffins: C ₁₂ , 60% Chlorine	308	Dimethyl Morpholinophosphoramidate	298
Chlorinated Trisodium Phosphate	294	Dimethylvinyl Chloride	316
2-Chloroacetophenone	379	Diphenhydramine Hydrochloride	355
<i>p</i> -Chloroaniline Hydrochloride	351	5,5-Diphenylhydantoin	404
CS ₂	377	Emodin	493
Chlorobenzene	261	Ephedrine Sulfate	307
Chlorodibromomethane	282	Epinephrine Hydrochloride	380
Chloroethane	346	1,2-Epoxybutane	329
2-Chloroethanol	275	Erythromycin Stearate	338
3-Chloro-2-Methylpropene	300	Ethyl Acrylate	259
Chloroprene	467	Ethylbenzene	466
1-Chloro-2-Propanol	477	Ethylene Glycol	413

Chemical	TR No.	Chemical	TR No.
Ethylene Glycol Monobutyl Ether	484	Nitrofurazone	337
Ethylene Oxide	326	Nitromethane	461
Ethylene Thiourea	388	<i>p</i> -Nitrophenol	417
Eugenol	223	<i>o</i> -Nitrotoluene	504
FD&C Yellow No. 6	208	<i>p</i> -Nitrotoluene	498
Fumonisin B ₁	496	Ochratoxin A	358
Furan	402	Oleic Acid Diethanolamine Condensate	481
Furfural	382	Oxazepam (Mice)	443
Furfuryl Alcohol	482	Oxazepam (Rats)	468
Furosemide	356	Oxymetholone	485
Gallium Arsenide	492	Oxytetracycline Hydrochloride	315
Geranyl Acetate	252	Ozone and Ozone/NNK	440
Glutaraldehyde	490	Penicillin VK	336
Glycidol	374	Pentachloroanisole	414
Guar Gum	229	Pentachloroethane	232
Gum Arabic	227	Pentachloronitrobenzene	325
HC Blue 1	271	Pentachlorophenol, Purified	483
HC Blue 2	293	Pentachlorophenol, Technical Grade	349
HC Red 3	281	Pentaerythritol Tetranitrate	365
HC Yellow 4	419	Phenolphthalein	465
Hexachlorocyclopentadiene	437	Phenylbutazone	367
Hexachloroethane	361	Phenylephrine Hydrochloride	322
4-Hexylresorcinol	330	N-Phenyl-2-Naphthylamine	333
Hydrochlorothiazide	357	<i>o</i> -Phenylphenol	301
Hydroquinone	366	Polybrominated Biphenyl Mixture (Firemaster FF-1) (Gavage)	244
8-Hydroxyquinoline	276	Polybrominated Biphenyl Mixture (Firemaster FF-1) (Feed)	398
Indium Phosphide	499	Polysorbate 80 (Glycol)	415
Iodinated Glycerol	340	Polyvinyl Alcohol	474
Isobutene	487	Primidone	476
Isobutyl Nitrite	448	Probenecid	395
Isobutyraldehyde	472	Promethazine Hydrochloride	425
Isophorone	291	Propylene	272
Isoprene	486	1,2-Propylene Oxide	267
Lauric Acid Diethanolamine Condensate	480	Propyl Gallate	240
<i>d</i> -Limonene	347	Pyridine	470
Locust Bean Gum	221	Quercetin	409
60-Hz Magnetic Fields	488	Resorcinol	403
Magnetic Field Promotion	489	Rhodamine 6G	364
Malonaldehyde, Sodium Salt	331	Rotenone	320
Manganese Sulfate Monohydrate	428	Roxarsone	345
D-Mannitol	236	Salicylazosulfapyridine	457
Marine Diesel Fuel and JP-5 Navy Fuel	310	Scopolamine Hydrobromide Trihydrate	445
Melamine	245	Sodium Azide	389
2-Mercaptobenzothiazole	332	Sodium Fluoride	393
Mercuric Chloride	408	Sodium Nitrite	495
Methacrylonitrile	497	Sodium Xylenesulfonate	464
8-Methoxypsoralen	359	Stannous Chloride	231
<i>o</i> -Methylbenzyl Alcohol	369	Succinic Anhydride	373
Methyl Bromide	385	Talc	421
Methyl Carbamate	328	Tara Gum	224
Methyldopa Sesquihydrate	348	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -Dioxin (Dermal)	201
Methylene Chloride	306	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -Dioxin (Gavage)	209
4,4'-Methylenedianiline Dihydrochloride	248	1,1,1,2-Tetrachloroethane	237
Methyleugenol	491	Tetrachloroethylene	311
Methyl Methacrylate	314	Tetracycline Hydrochloride	344
N-Methylolacrylamide	352	Tetrafluoroethylene	450
Methylphenidate Hydrochloride	439	1-Trans-Delta ⁹ -Tetrahydrocannabinol	446
Mirex	313	Tetrahydrofuran	475
Molybdenum Trioxide	462	Tetrakis(Hydroxymethyl)Phosphonium Sulfate	296
Monochloroacetic Acid	396	Tetrakis(Hydroxymethyl)Phosphonium Chloride	296
Monuron	266	Tetranitromethane	386
Nalidixic Acid	368	Theophylline	473
Naphthalene (Mice)	410	4,4-Thiobis(6- <i>t</i> -Butyl- <i>m</i> -Cresol)	435
Naphthalene (Rats)	500	Titanocene Dichloride	399
Nickel (II) Oxide	451	Toluene	371
Nickel Sulfate Hexahydrate	454	2,4- & 2,6-Toluene Diisocyanate	251
Nickel Subsulfide	453	<i>o</i> -Toluidine Hydrochloride	153
<i>p</i> -Nitroaniline	418	Triamterene	420
<i>o</i> -Nitroanisole	416	Tribromomethane	350
<i>p</i> -Nitrobenzoic Acid	442	Trichloroethylene	243
Nitrofurantoin	341	Trichloroethylene	273

Chemical	TR No.	Chemical	TR No.
1,2,3-Trichloropropane	384	4-Vinyl-1-Cyclohexene Diepoxide	362
Tricresyl Phosphate	433	Vinylidene Chloride	228
Triethanolamine	449	Vinyl Toluene	375
Tris(2-Chloroethyl) Phosphate	391	Xylenes (Mixed)	327
Tris(2-Ethylhexyl) Phosphate	274	2,6-Xylidine	278
Turmeric Oleoresin (Curcumin)	427	Zearalenone	235
4-Vinylcyclohexene	303	Ziram	238