Report on Carcinogens
Draft Background Document for

o-Nitrotoluene

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Public Health Services
National Toxicology Program
Research Triangle Park, NC 27709

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FOREWORD

The Report on Carcinogens (RoC) is prepared in response to Section 301 of the Public Health Service Act as amended. The RoC contains a list of identified substances (i) that either are known to be human carcinogens or may reasonably be anticipated to be human carcinogens and (ii) to which a significant number of persons residing in the United States are exposed. The Secretary, Department of Health and Human Services (DHHS), has delegated responsibility for preparation of the RoC to the National Toxicology Program (NTP), which prepares the report with assistance from other Federal health and regulatory agencies and nongovernmental institutions.

Nominations for (1) listing a new substance, (2) reclassifying the listing status for a substance already listed or (3) removing a substance already listed in the RoC are reviewed by a multi-step, scientific review process with multiple opportunities for public comment. The scientific peer-review groups evaluate and make independent recommendations for each substance according to specific RoC listing criteria. This draft Background Document was prepared to assist in the review of *o*-nitrotoluene. The scientific information used to prepare Sections 3 through 5 of this document must come from publicly available, peer-reviewed sources. Information in Sections 1 and 2, including chemical and physical properties, analytical methods, production, use, and occurrence may come from published and/or unpublished sources. The NTP will provide a reference for all published and unpublished sources used in this document. For each study cited in the background document from the peer-reviewed literature, information on funding sources (if available) and the authors' affiliations will be provided in the reference section. Any interpretive conclusions, comments, or statistical calculations made by the authors of this draft document that are not contained in the original citation are identified in brackets [ ]. This draft document will be peer reviewed in a public forum by an *ad hoc* expert panel of scientists from the public and private sectors with relevant expertise and knowledge selected by the NTP in accordance with the Federal Advisory Committee Act and HHS guidelines and regulations. This document will be finalized based on the peer-review recommendations of the expert panel and public comments received for this draft document.

A detailed description of the RoC review process and a list of all substances under consideration for listing in or delisting from the RoC can be obtained by accessing the NTP Home Page at http://ntp.niehs.nih.gov and selecting "Report on Carcinogens." The most recent RoC, the 11th Edition (2004), is available at the above-mentioned website.

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Criteria for Listing Agents, Substances or Mixtures in the Report on Carcinogens

U.S. Department of Health and Human Services
National Toxicology Program

The criteria for listing an agent, substance, mixture, or exposure circumstance in the RoC are as follows:

Known To Be Human Carcinogen:

There is sufficient evidence of carcinogenicity from studies in humans*, which indicates a causal relationship between exposure to the agent, substance, or mixture, and human cancer.

Reasonably Anticipated To Be Human Carcinogen:

There is limited evidence of carcinogenicity from studies in humans*, which indicates that causal interpretation is credible, but that alternative explanations, such as chance, bias, or confounding factors, could not adequately be excluded,

or

there is sufficient evidence of carcinogenicity from studies in experimental animals, which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors (1) in multiple species or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site, or type of tumor, or age at onset,

or

there is less than sufficient evidence of carcinogenicity in humans or laboratory animals; however, the agent, substance, or mixture belongs to a well-defined, structurally related class of substances whose members are listed in a previous Report on Carcinogens as either known to be a human carcinogen or reasonably anticipated to be a human carcinogen, or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to, dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive sub-populations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals, but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans.

* This evidence can include traditional cancer epidemiology studies, data from clinical studies, and/or data derived from the study of tissues or cells from humans exposed to the substance in question that can be useful for evaluating whether a relevant cancer mechanism is operating in people.

Executive Summary

Introduction

o-Nitrotoluene is a nitro aromatic compound consisting of a benzene ring, with a methyl and a nitro group attached ortho to each other. It is one of three isomers of nitrotoluene, with *m*-nitrotoluene and *p*-nitrotoluene being the other two. *o*-Nitrotoluene is an important chemical intermediate used in the synthesis of dyes that are used in the textile, paper, and other industries and also in the synthesis of agricultural, rubber, and other chemicals.

o-Nitrotoluene was nominated for possible listing in the Report on Carcinogens based on the results of a 2002 National Toxicology Program (NTP) two-year feeding bioassay study demonstrating clear evidence of carcinogenicity in rats and mice.

Human Exposure

o-Nitrotoluene is a chemical intermediate used in the synthesis of azo dyes. It is also used (either directly or as an intermediate) in the production of other dyes, agricultural chemicals, rubber chemicals, pesticides, petrochemicals, pharmaceuticals, and explosives. *o*-Nitrotoluene is produced principally by the nitration of toluene with a mixture of nitric acid and either sulfuric, aromatic sulfonic, or phosphoric acid. *o*-Nitrotoluene is a high production volume (HPV) chemical, and its U.S. production was between 10 million and 50 million pounds for every four-year reporting period from 1986 to 2002.

Exposure to *o*-nitrotoluene in the United States is primarily a result of occupational exposure during the production and use of this chemical. Little information is available on environmental occurrence of *o*-nitrotoluene or on human exposure. The compound has been detected in the ambient air at U.S. chemical manufacturing plants where it is used, and in surface water and groundwater in France, the Netherlands, and Germany. *o*-Nitrotoluene, which can be formed as a breakdown product of di- or trinitrotoluenes, has been detected in the effluent or wastewater of plants producing these chemicals. The uses of di- and trinitrotoluenes include the production of commercial and military explosives,

and *o*-nitrotoluene has been found in the groundwater and surface water at munitions production facilities and military training grounds.

Human Cancer Studies

No studies on the relationship between human cancer and specific exposure to *o*-nitrotoluene were identified. *o*-Nitrotoluene may be used to manufacture magenta and thus magenta manufacturing workers may be exposed to *o*-nitrotoluene. IARC reviewed magenta manufacturing in 1987 and 1993 and concluded that there is *sufficient evidence* in humans that the manufacture of magenta entails exposures that are carcinogenic. Their assessment was based on two cohort studies and a case-control study, all of which reported an excess risk of bladder cancer; however, only one study specifically mentioned that the workers were exposed to *o*-nitrotoluene as a part of the manufacturing process. [These studies are limited for the evaluation of the carcinogenicity of *o*-nitrotoluene in humans because the workers were also exposed to other chemicals, such as *o*-toluidine, 2,5-diaminotoluene, and 4,4'-methylenebis(2-methylaniline), and exposure to *o*-nitrotoluene was not specifically assessed.]

Studies in Experimental Animals

The carcinogenicity of *o*-nitrotoluene was evaluated in feeding studies in rats and mice. Mesothelioma was first observed in male rats administered *o*-nitrotoluene for 13 weeks. Tumors at multiple tissues sites were observed in a stop-exposure study in male rats and two-year chronic studies in both sexes of rats and mice. The NTP concluded that there was *clear evidence of carcinogenic activity* of *o*-nitrotoluene in male and female rats, based on increased incidences of subcutaneous skin neoplasia and mammary-gland fibroadenoma in both sexes, and malignant mesothelioma and liver tumors in males. Increased incidences of lung tumors in males and hepatocellular adenoma in female rats also were considered to be exposure related. The NTP also concluded that there was *clear evidence of carcinogenic activity* of *o*-nitrotoluene in male and female mice, based on increased incidences of hemangiosarcoma in both sexes, carcinoma of the large intestine in males, and hepatocellular tumors in females.

Absorption, Distribution, Metabolism, and Excretion

Metabolites of *o*-nitrotoluene have been detected in the urine of factory workers, indicating that absorption occurs in humans from skin contact and inhalation. *o*-Nitrotoluene is absorbed after oral administration to rats and mice. The half-life of *o*-nitrotoluene in plasma of rats is fairly short, approximately 1.5 hours, and the primary route of excretion is urine, occurring mainly in the first 24 hours after exposure. Excretion of *o*-nitrotoluene metabolites also occurs through feces and expired air. A study in rats showed that approximately 29% of an oral dose (male rats) was excreted in the bile in 12 hours; however, the metabolites are subject to reabsorption and further metabolism, most likely leading to urinary excretion.

The major urinary metabolites found in rats and mice are *o*-nitrobenzoic acid and *o*-nitrobenzyl glucuronide; however, other metabolites, such as *S*-(*o*-nitrobenzyl)-*N*-acetylcysteine and *o*-aminobenzyl alcohol, are found only in rats. Female rats excreted less than half as much of the dose as *o*-aminobenzyl alcohol or as *S*-(*o*-nitrobenzyl)-*N*-acetylcysteine as did male rats. The major biliary metabolite of *o*-nitrotoluene was *o*-nitrobenzyl glucuronide, which accounted for 22.1% of the dose in males and 8.3% of the dose in females. The next most abundant metabolite was *S*-(*o*-nitrobenzyl) glutathione, which accounted for 4.9% of the dose in males and 0.4% in females.

Genotoxicity and Mechanistic Data

o-Nitrotoluene did not cause mutations in prokaryotic systems. In mammalian *in vitro* systems, it induced SCE in CHO cells, but it did not induce chromosomal aberrations in CHO cells or DNA repair in rat or human hepatocytes. [The relative lack of genotoxic effects of *o*-nitrotoluene in *in vitro* test systems is likely consistent with the need for metabolism by both mammalian and bacterial enzymes.] In rats exposed to *o*-nitrotoluene *in vivo,* DNA adducts and increased DNA repair were detected in males but not females. *o*-Nitrotoluene induced a slight increase in normochromatic micronuclei in high-dose male mice (equivocal response) but did not induce micronuclei in the bone marrow of male rats (polychromatic), male mice (polychromatic), or female mice (normochromatic).

The genotoxicity of the *o*-nitrotoluene, as measured by the *in vivo–in vitro* DNA repair assay in rats, depends on metabolism (both mammalian and bacterial) and is sex specific. DNA repair was induced only in male rats with an intact intestinal microflora. Incubation of *o*-nitrotoluene *in vitro* with hepatocytes isolated from male rats failed to induce DNA repair. Biliary excretion is an important step in the activation of *o*-nitrotoluene. Interruption of bile flow into the intestine by cannulation of the bile duct decreased the covalent binding of *o*-nitrotoluene-related material at 12 hours post-administration to 7% (in males) or 22% (in females) of that seen in sham-operated animals. Moreover, deleterious effects of *o*-nitrotoluene generally are more severe in male rats than in females and include changes in hepatic, renal, or splenic histopathology, and tumor incidence. The toxicity of *o*-nitrotoluene, particularly in male rats, likely involves its metabolism by oxidation of the methyl group to an alcohol, conjugation of *o*-nitrobenzyl alcohol with glucuronic acid and excretion in bile, deconjugation of *o*-nitrobenzyl glucuronide and reduction of the nitro group by intestinal bacteria, and final activation of *o*-aminobenzyl alcohol by the formation of *o*-aminobenzyl sulfate.

Based on this proposed activation model, female rats should be resistant to a hepatocarcinogenic effect. However, *o*-nitrotoluene caused hepatocellular adenomas in female rats, suggesting that there are other mechanisms of activation for *o*-nitrotoluene. Moreover, *o*-nitrotoluene also caused significantly increased incidences of tumors in tissues other than the liver in both rats and mice, including mammary gland, skin, lung, large intestine, and hemangiosarcomas in various tissues.

Mutations in the *p53* and *β-catenin* genes and production of these proteins were detected in hemangiosarcomas and colon tumors from mice exposed to *o*-nitrotoluene; K-*ras* gene mutations and cyclin D1 protein production also were detected in the colon tumors. Mutations in *p53*, *β-catenin*, and K-*ras* genes may be a result of the genotoxic effects of *o*-nitrotoluene. The pattern of mutations is consistent with targeting of guanine for adduct formation since mutations in the *p53* gene in hemangiosarcomas mainly involved G·C→A·T transitions, and almost all the mutations in the K-*ras* gene in cecal carcinomas were G·C→T·A transversions. Human colorectal cancers also have a high frequency of mutations in the K-*ras* and *p53* genes, and the *β-catenin* and *cyclin D1* genes are

upregulated. As a result of these genetic effects, both human and mouse colon tumors have alterations in pathways that are considered important for the progression of cells from a normal state to cancer; these pathways include the *β-catenin*/*Wnt* signaling pathway, *ras*/*MAP* kinase pathway, and cell-cycle checkpoint genes (e.g., the *cyclin D1* and *p53* genes).

Studies in rats have also provided evidence that cellular and molecular events involved in the induction of mesotheliomas are similar in both experimental animals (rats exposed to *o*-nitrotoluene) and humans. Microarray analysis of peritoneal mesotheliomas from F344 rats treated with *o*-nitrotoluene identified the following carcinogenic pathways: insulin-like growth factor 1 (IGF-1), p38 MAPK, Wnt/β-catenin, and integrin signaling pathways.

The NTP has conducted bioassay studies in experimental animals on another nitrotoluene isomer, *p*-nitrotoluene. Based on the results from this study of *p*-nitrotoluene, the NTP stated that there was *equivocal evidence of carcinogenic activity* in male rats and male mice, *some evidence of carcinogenic activity* in female rats, and *no evidence of carcinogenic activity* in female mice. The NTP concluded that *o*-nitrotoluene had greater carcinogenic potential than *p*-nitrotoluene and that the differences between the two isomers may be due to (1) greater stability of the ortho adduct, (2) higher covalent binding of *o*-nitrotoluene to rat hepatic macromolecules, or (3) greater metabolism of *o*-nitrotoluene to the *o*-nitrobenzyl glucuronide, which gives rise to *o*-aminobenzyl sulfate, the proposed proximal reactive metabolite.

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Abbreviations

ACGIH:	American Conference of Governmental Industrial Hygienists
APC:	adenomatous polyposis coli
b.w.:	body weight
CHO:	Chinese hamster ovary
CIIT	Chemical Industry Institute of Toxicology
CRASF:	Charles River altered Schaedler flora
DOT:	Department of Transportation
EPA:	Environmental Protection Agency
g:	gram
GI:	gastrointestinal
HPV	high production volume
IARC:	International Agency for Research on Cancer
kg:	kilogram
K_{oc}:	soil organic adsorption coefficient
L:	liter
m^3:	cubic meter
mg:	milligram
mL:	milliliter
mol wt:	molecular weight
NADPH:	nicotinamide adenine dinucleotide phosphate
NCEs:	normochromatic erythrocytes
NCI	National Cancer Institute
NIEHS:	National Institute of Environmental Health Sciences
NIOSH:	National Institute for Occupational Safety and Health

ng: nanogram

NTP: National Toxicology Program

OSHA: Occupational Safety and Health Administration

PAPS: 3'-phosphoadenosine 5'-phosphosulfate

PCEs: polychromatic erythrocytes

PGST: placental glutathione *S*-transferase

ppb: parts per billion

ppm: parts per million

RTECS: Registry of Toxic Effects of Chemical Substances

SCE: sister chromatid exchange

TNT: trinitrotoluene

UDS: unscheduled DNA synthesis

μg: microgram

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1 Introduction

o-Nitrotoluene is a nitro aromatic compound used as an important chemical intermediate in the synthesis of azo dyes. It is also used (either directly or as an intermediate) in the production of other dyes, agricultural chemicals, rubber chemicals, pesticides, petrochemicals, pharmaceuticals and explosives. Based on data reported under U.S. EPA's Inventory Update Rule, production of *o*-nitrotoluene in the United States was in the range of 10 million to 50 million pounds per year from the mid 1980s until 2002 (the most recent available data) (EPA 2007).

o-Nitrotoluene was nominated by the National Institute of Environmental Health Sciences for possible listing in the *Report on Carcinogens* based on the results of a National Toxicology Program (NTP) bioassay (NTP 2002b), which reported *clear evidence of carcinogenic activity,* based on the occurrence of malignant tumors at a variety of tissue sites in male and female mice and rats.

1.1 Chemical identification

o-Nitrotoluene (also known as 2-nitrotoluene) is a nitro aromatic compound with the structure illustrated in Figure 1-1. It is one of three isomers of nitrotoluene; the other two are *m*-nitrotoluene (also known as 3-nitrotoluene) and *p*-nitrotoluene (also known as 4-nitrotoluene). The two other nitrotoluene isomers (*m*- and *p*-nitrotoluene) and other nitrotoluene analogues are described in Section 1.3.

Table 1-1 lists chemical identifying information for *o*-nitrotoluene.

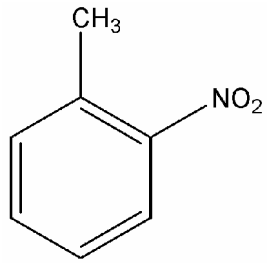

Figure 1-1. Chemical structure of *o*-nitrotoluene

Table 1-1. Chemical identification of *o*-nitrotoluene

Characteristic	Information
CAS Registry number	88-72-2
Molecular formula	$C_7H_7NO_2$
Synonyms	1-methyl-2-nitrobenzene 2-methylnitrobenzene 2-methyl-1-nitrobenzene 2-nitrotoluene 2-nitrotoluol benzene, 1-methyl-2-nitro *o*-nitrotoluol *o*-methylnitrobenzene ONT

Source: ChemIDplus 2007

1.2 Physical-chemical properties

o-Nitrotoluene is a yellow liquid at room temperature with an odor of bitter almonds. It is slightly soluble in water and soluble in acetone, benzene, chloroform, diethyl ether, ethanol, and petroleum ether. It has a flash point of 106°C (closed cup) and an autoignition temperature of 305°C (PTCL 2003). It does not ignite easily; however, it may burn, and containers may explode when heated (HSDB 2007). The physical and chemical properties of *o*-nitrotoluene are summarized in Table 1-2.

Table 1-2. Physical and chemical properties of *o*-nitrotoluene

Property	Information
Molecular weight	137.14
Melting point (°C)	–9.5 (needles); –2.9 (crystals)
Boiling point (°C)	222
Critical temperature (°C)	NA
Specific gravity	1.162 at 19°C/15°C
Solubility in water (at 30°C)	650 mg/L
Octanol-water partition coefficient (log K_{ow})	2.30
Dissociation constant (pK_a)	NA
Vapor pressure (mm Hg)	0.188 at 25°C
Vapor density	4.73
Henry's law constant	1.25 x 10^{-5} atm-cu m/mole @ 25°C

Source: HSDB 2007. NA = not available.

1.3 Identification of metabolites and analogues

Urinary metabolites in workers exposed to *o*-nitrotoluene include *o*-nitrobenzoic acid and *o*-nitrobenzyl alcohol (Jones *et al.* 2005b, Sabbioni *et al.* 2006). The urinary metabolites of *o*-nitrotoluene identified following oral administration to rats and mice include *o*-nitrobenzoic acid, a sulfur-containing conjugate of *o*-acetamidotoluene (tentatively identified as *S*-(*o*-acetamidobenzyl)-*N*-acetylcysteine), *o*-nitrobenzyl glucuronide, *S*-(*o*-nitrobenzyl)-*N*-acetylcysteine (*o*-nitrobenzyl mercapturic acid), a sulfur-containing conjugate of *o*-aminotoluene (tentatively identified as *S*-(*o*-aminobenzyl) glutathione)), *S*-(*o*-nitrobenzyl) glutathione, *o*-aminobenzoic acid, *o*-nitrobenzyl sulfate, *o*-nitrobenzyl alcohol, *o*-aminobenzyl alcohol, and *o*-aminotoluene (*o*-toluidine) (Chism *et al.* 1984, NTP 2002a). See Figure 1-2 for the structures of these metabolites and Section 5.1 for further discussion of metabolism.

The structures of two other nitrotoluene isomers (*m*- and *p*-nitrotoluene), *o*-toluidine (*o*-aminotoluene, prepared by reduction of *o*-nitrotoluene), and the isomers of dinitrotoluene (2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-dinitrotoluene), are shown in Figure 1-3.

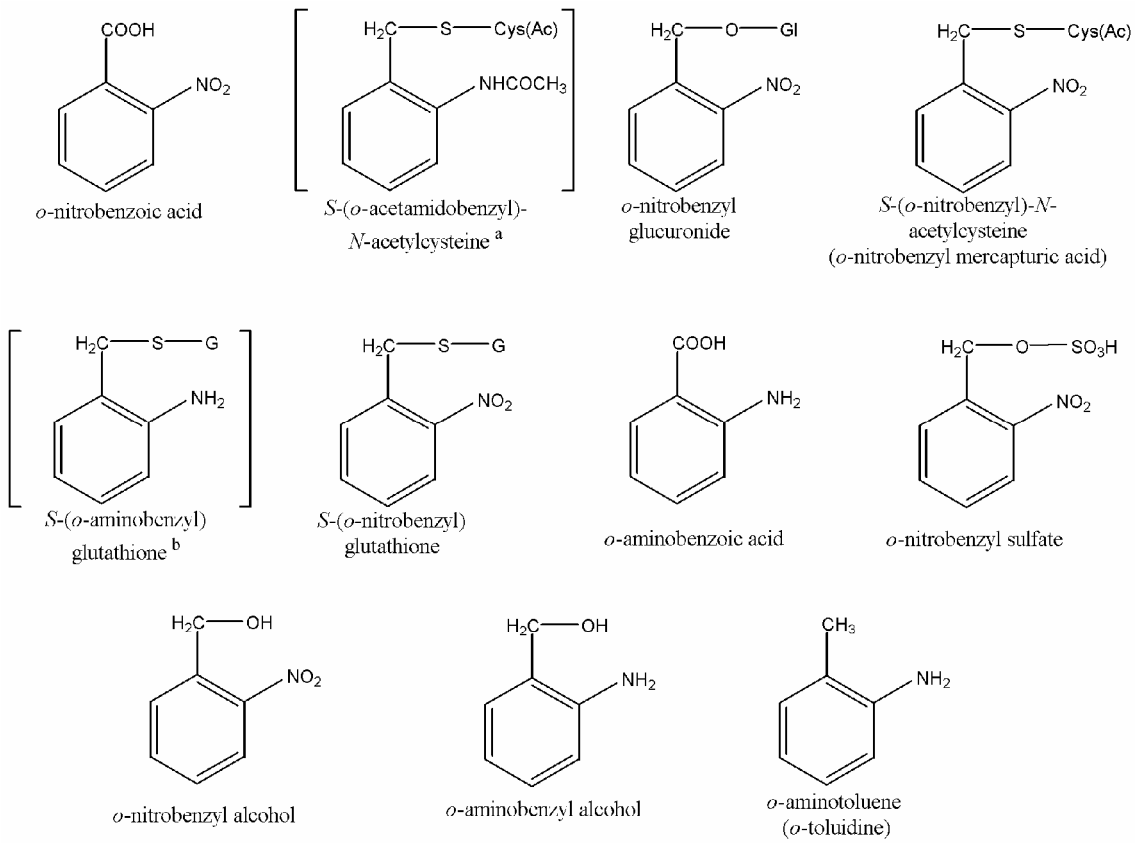

Figure 1-2. Chemical structures of urinary metabolites of *o*-nitrotoluene in rats and mice

Chemical structures of urinary metabolites of *o*-nitrotoluene (see Table 5-3 for data on percent of dose excreted as these metabolites by F344 rats and B6C3F$_1$ mice) are illustrated above. The two bracketed structures indicate structures tentatively identified by Chism and Rickert 1985.

Cys(Ac) = acetylcysteine, G = glutathione, Gl = glucuronide.

[a]Tentatively identified by Chism and Rickert (1985) as the structure of the metabolite listed as a sulfur-containing conjugate of *o*-acetamidotoluene in Table 5-3.

[b]Tentatively identified by Chism and Rickert (1985) as the structure of the metabolite listed as a sulfur-containing conjugate of *o*-aminotoluene in Table 5-3.

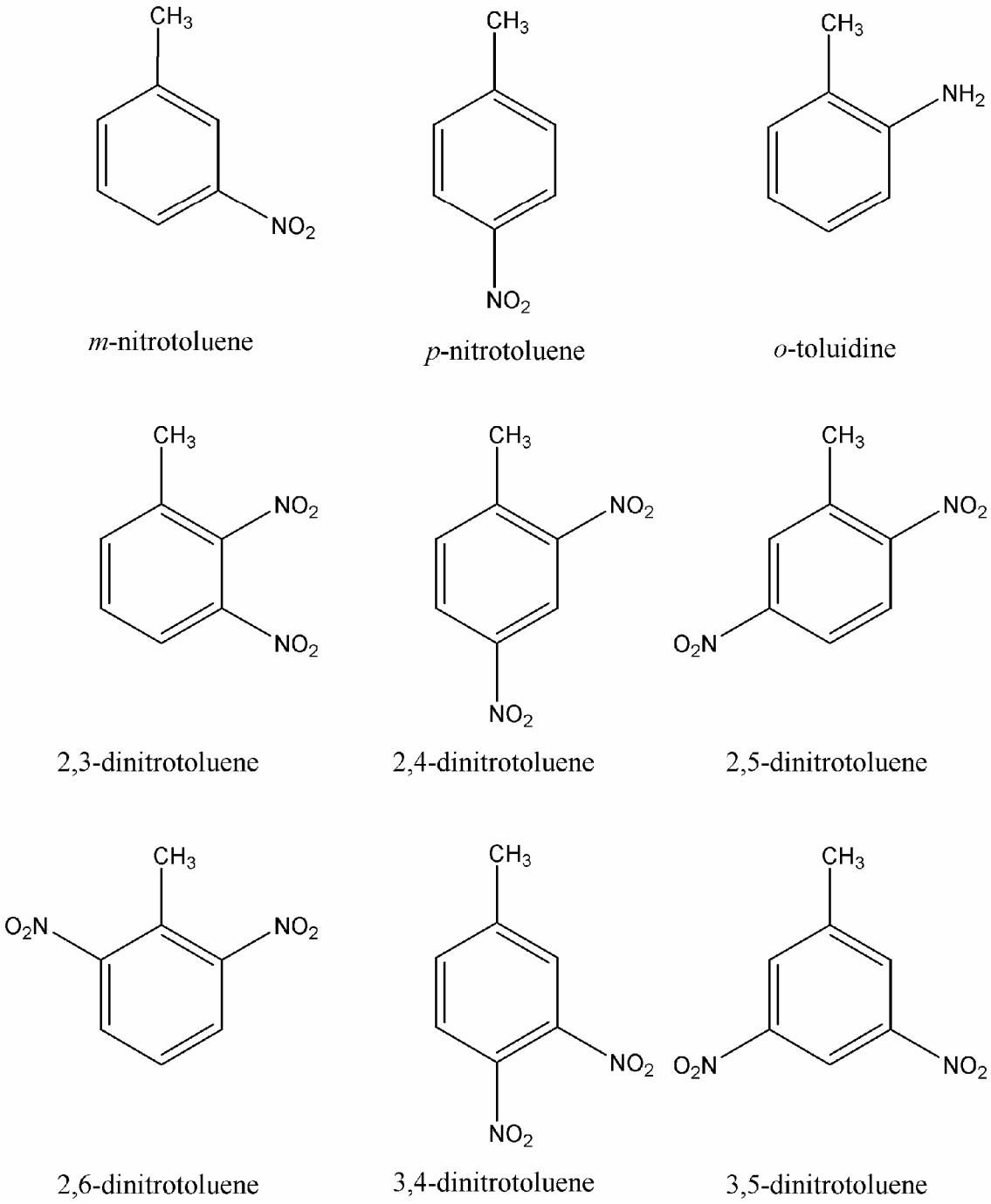

Figure 1-3. Chemical structures of *o*-nitrotoluene analogues

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2 Human Exposure

Exposure to *o*-nitrotoluene in the United States is primarily a result of occupational exposure during the production and use of this chemical. The United States produces large quantities of *o*-nitrotoluene (greater than 10 million pounds annually), which is used primarily in the production of important chemical intermediates used in the synthesis of dyes. Thus, there is the potential for significant exposure to *o*-nitrotoluene. However, specific data on the number of workers exposed was not found for either the production of *o*-nitrotoluene or its use in the production of other chemicals. [The lack of information on the number of workers in *o*-nitrotoluene manufacture may be due largely to the manufacture of this chemical by a single U.S. company in recent years (see Section 2.2).] A potential source of exposure to *o*-nitrotoluene for the general public in some areas is through contamination of soil and groundwater in areas of past munitions manufacture, storage, and use; however, no estimates were found for the number of people potentially exposed. *o*-Nitrotoluene also was measured in ambient air at a manufacturing plant in New Jersey, but no information was found on air exposure levels for the general public.

This section discusses information related to human exposure, including uses, production, concentrations of *o*-nitrotoluene in the environment (environmental occurrence) and in occupational facilities (occupational exposure), numbers of potentially exposed workers, biological indices of exposure, and regulations and guidelines to reduce exposure.

2.1 Use

o-Nitrotoluene is primarily used in the production of derivatives, including *o*-toluidine (*o*-aminotoluene), 2-amino-4-chlorotoluene, 2-amino-6-chlorotoluene, and *o*-toluidine-4-sulfonic acid, which are intermediates in the production of various azo dyes (IARC 1996). It is used in the manufacture (or manufacture of intermediates) for other dyes such as magenta (which is produced by at least two companies in the United States) and various sulfur dyes for cotton, wool, silk, leather, and paper (IARC 1996, HSDB 2007). Other uses include as an intermediate in the synthesis of (or synthesis of intermediates for) explosives and of a variety of organic chemicals, including compounds used in the petrochemical, pesticide, pharmaceutical, and rubber industries (HSDB 2007).

2.2 Production

The nitrotoluenes are produced principally by the nitration of toluene with a mixture of nitric acid and either sulfuric, aromatic sulfonic, or phosphoric acid (Kirk-Othmer 1996). Production can be either a batch or a continuous process. In a batch process, toluene is fed into a nitrator and cooled to about 25°C. The acid is added slowly, and the temperature of the reaction mixture is maintained at 25°C by adjustment of the acid feed rate and the amount of cooling. After the acid is added, the temperature is slowly raised to 35°C to 40°C. The reaction mixture is then put in a separator, where the acid is taken from the bottom, and the product is steam distilled to remove excess toluene and then dried by distillation of the remaining traces of water. The isomers are separated by a combination of fractional distillation and crystallization. The ratio of the isomers depends on the production conditions and the catalyst used, but generally is in the range of 45% to 62% *o*-nitrotoluene, 2% to 5% *m*-nitrotoluene, and 33% to 50% *p*-nitrotoluene. *o*-Nitrotoluene is available commercially at a purity of 99.2% to 99.5% and typically contains the following impurities: *m*- and *p*-nitrotoluenes (0.8%), water (0.2%), and toluene (0.1%) (IARC 1996).

Other processes that have been used to produce nitrotoluenes include (1) reaction of toluene with nitronium salts in the presence of crown ethers or polyethers and (2) reaction of toluene with nitric acid in the gas phase in the presence of solid silica-alumina catalysts (Kirk-Othmer 1996). The main advantage of these processes is that sulfuric acid is not used.

U.S. production of *o*-nitrotoluene was calculated as 13 billion grams (29 million pounds) for 1981 (HSDB 2007). U.S. production of *o*- and *p*-nitrotoluene combined was estimated at 20 billion grams (44 million pounds) in 1983 (HSDB 2007), and production of *o*-nitrotoluene only was estimated at 16,120 metric tons (35.5 million pounds) in 1993 (Kirk-Othmer 1996). *o*-Nitrotoluene is listed as a High Production Volume (HPV) chemical, and according to data submitted by companies under the Inventory Update Rule, U.S. production of *o*-nitrotoluene was between 10 million and 50 million pounds for every four-year reporting period from 1986 to 2002 (EPA 2007). One U.S. facility

was reported to produce *o*-nitrotoluene in 2007 (SRI 2007), and 11 suppliers were identified in the United States in 2007 (ChemSources 2007).

No data specific for U.S. imports or exports of *o*-nitrotoluene were found. U.S. imports of nitrated benzene, nitrated toluene, and nitrated naphthalene were 270 million grams (602,000 pounds) in 1984 (HSDB 2007), and 95,000 kilograms (209,400 pounds) in 2005 (2005 data did not include *p*-nitrotoluene) (ITC 2007a). No imports of this group of compounds were reported in 2006. In 2006, the United States exported approximately 12.9 million kilograms (28 million pounds) of hydrocarbon derivatives containing only nitro or nitroso groups (excluding *p*-nitrotoluene and trinitrotoluene) (ITC 2007b).

2.3 Environmental occurrence and fate

o-Nitrotoluene is expected to exist as a vapor in ambient air and has been observed to be completely degraded in aqueous sewage treatment systems, rivers, and streams. There are limited data on environmental levels of *o*-nitrotoluene. Some data show that *o*-nitrotoluene has been released into the air and water from occupational settings (See Sections 2.3.1 and 2.3.2). *o*-Nitrotoluene may also be formed through the degradation or combustion of military munitions (USACE 2002, NAVFAC 2003). In soil, *o*-nitrotoluene is expected to have moderate mobility and to volatilize slowly. *o*-Nitrotoluene is not listed in EPA's Toxics Release Inventory (TRI), and no data were available from that source for its release into the environment.

2.3.1 Air

Little information is available on concentrations of *o*-nitrotoluene in ambient air. The only ambient-air measurements found were made at a manufacturing plant in New Jersey (IARC 1996) (see Section 2.5, Occupational Exposure).

According to a model of gas-particle partitioning of semivolatile organic compounds in the atmosphere, *o*-nitrotoluene released to air is expected to exist in the vapor phase and to be removed mainly by direct photolysis and reaction with photochemically generated hydroxyl radicals (HSDB 2007). The main photoproducts are expected to be 2-methyl-6-nitrophenol and 2-methyl-4-nitrophenol, and a half-life of 42 days has been estimated for removal by reaction with hydroxyl radicals.

2.3.2 Water

o-Nitrotoluene was detected at concentrations ranging from 320 to 16,000 μg/L in effluent from a U.S. plant manufacturing 2,4,6-trinitrotoluene (IARC 1996, HSDB 2007). Other reports from unspecified locations included concentrations ranging from 20 to 140 μg/L in wastewater from a plant producing and purifying 2,4,6-trinitrotoluene, and at 7,800 μg/L in raw effluent from a plant manufacturing dinitrotoluene. *o*-Nitrotoluene also was detected in a paper-mill waste-treatment lagoon (concentration and location not reported).

o-Nitrotoluene, which can be formed as a breakdown product of di- or trinitrotoluenes, has been detected in the effluent or wastewater of plants producing these chemicals. The uses of di- and trinitrotoluenes include the production of commercial and military explosives. In the United States, *o* nitrotoluene has been found in groundwater and surface water at munitions production facilities, in groundwater at a military training facility, and in groundwater at a nuclear weapons assembly/disassembly facility that previously had been a WWII munitions factory (WDHFS 2002, Pantex 2004, 2006, ATSDR 2007). Maximum onsite groundwater levels seen at three munitions manufacturing facilities were 4,600 μg/L for a plant in Texas, 21,000 μg/L for a plant in Illinois, and 140,000 μg/L for a plant in Tennessee (years of analyses not provided) (ATSDR 2007). At a former munitions production site in Wisconsin between 1999 and 2002, *o*-nitrotoluene was detected in offsite, private well-water at a maximum concentration of 0.095 μg/L. For the same facility and the same time period, *o*- and *p*-nitrotoluene combined was detected in 4 of 17 groundwater samples with levels ranging from 0.16 to 17.0 μg/L (ATSDR 2007, WDHFS 2002).The maximum groundwater level measured at a military training facility in Massachusetts was 25 μg/L, and the maximum surface water concentration measured at a munitions manufacturing facility in Missouri was 0.12 μg/L (years of analyses not provided) (ATSDR 2007).

o-Nitrotoluene has been routinely monitored in groundwater since 1995 at a facility in the Texas panhandle where conventional munitions were produced for World War II and where nuclear-weapons assembly and disassembly activities have been performed since

the mid-1970s. Testing has been performed on groundwater from both the Ogallala aquifer and from a perched aquifer above the Ogallala. Since 1999, *o*-nitrotoluene has been detected sporadically in water from both the Ogallala and the perched aquifer. Concentrations in water from the perched aquifer that exceeded the detection limit ranged from a minimum of 0.14 μg/L in 2003 to a maximum of 5 μg/L in 2004, while concentrations in water from the Ogallala aquifer ranged from a minimum of 0.12 μg/L to a maximum of 2.9 μg/L, both in 2004 (Pantex 2003, 2004). *o*-Nitrotoluene was detected in a small percentage of samples (between 1% and 7%) in 2001 to 2004 in the perched aquifer and in 1999 and 2002 in the Ogallala aquifer (Pantex 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005) but was not detected in 2005, the last year for which data were available (Pantex 2006).

Nitrotoluene (*o*- and p-nitrotoluene combined) was detected in the Netherlands (in the 1970s) in the Rhine River at a concentration of 10 μg/L, in the River Waal at 4.5 μg/L, and in the River Maas at 0.3 μg/L (IARC 1996). In Germany, *o*-nitrotoluene was detected at 0.4 and 7.4 μg/L in surface water near a former munitions plant and at 1.2 μg/L in an adjacent river. Two ponds had concentrations of 0.4 and 22.0 μg/L; these ponds fed into the River Oder, which had a concentration of < 0.01 μg/L. The concentration of *o*-nitrotoluene in three samples from the River Elbe ranged from 0.05 to 0.4 μg/L. Concentrations detected in groundwater in France (in 1987) ranged from 90 to 165 μg/L.

In water, *o*-nitrotoluene may undergo direct or indirect photolysis, volatilization, or aerobic biodegradation (HSDB 2007). It is not expected to adsorb to suspended solids or sediment. Half-lives for volatilization of *o*-nitrotoluene were estimated to be 56 hours from a model river (1 m deep, flowing 1 m/s, wind velocity of 3 m/s) and 30 days from a model lake (1 m deep, flowing 0.05 m/s, wind velocity of 0.5 m/s). The half-life for removal by indirect photolysis from a river with a high concentration of humic substances was calculated to be 45 minutes. *o*-Nitrotoluene is not expected to bioaccumulate in aquatic organisms; a bioconcentration factor of 12 was calculated, and experimentally determined bioconcentration factors in fish were low.

2.3.3 Soil

No information was found on concentrations of *o*-nitrotoluene in soil; however, *o*-nitrotoluene can result from anaerobic reduction of trinitrotoluene (TNT) (ACE 2004) and is a constituent of concern at former munitions sites. In December 2001, Congress passed the National Defense Authorization Act (for fiscal year 2002), which, in part, requires the Department of Defense (DoD) to develop an inventory of DoD sites that are no longer in use but are known or suspected to contain military munitions that will require clean-up. In a 2002 Annual Report, DoD provided a list of the 20 munitions constituents of greatest concern at contaminated military munitions sites due to their widespread use and potential environmental impacts, and *o*-nitrotoluene was included in that list. Development of the inventory of sites for clean-up does not require any analytical measurements for the constituents of concern, and therefore, to-date, there are no data on the frequency or levels of *o*-nitrotoluene at these sites. However, there are over 15 million acres in the United States that are either known or suspected to be contaminated with military munitions and "much of the land on which these sites are located has been or will be converted to nonmilitary uses such as farming, residential or commercial development, and recreation" (GAO 2002, 2003).

o-Nitrotoluene is expected to be moderately mobile in soil, with an estimated soil adsorption coefficient (K_{oc}) of 420, and may volatilize from moist soil surfaces (HSDB 2007). Under aerobic conditions, *o*-nitrotoluene persisted for more than 64 days in a silt loam inoculum. Under anaerobic conditions, *o*-nitrotoluene in soil has been observed to degrade to toluidine.

2.4 General population exposure

The general population may be exposed to *o*-nitrotoluene via inhalation of ambient air in the vicinity of production sites (HSDB 2007). In one experiment in which 115 volatile organic chemicals were measured in expired air samples from 54 healthy individuals from an urban population, 19.1% of 387 samples contained *o*-nitrotoluene at a mean concentration of 0.04 ng/L; however, no exposure to *o*-nitrotoluene was documented (Krotoszynski *et al.* 1979).

2.5 Occupational exposure

Limited information is available on occupational exposure to *o*-nitrotoluene via inhalation. As noted above, the compound was detected in ambient air at a chemical manufacturing plant in New Jersey, where a concentration of 47 ng/m^3 (0.000047 mg/m^3) was reported (IARC 1996). It also was detected in the air at concentrations of up to 2.0 mg/m^3 in the nitrotoluene production area of a chemical plant producing pharmaceuticals and explosives. Jones *et al.* (2005b) reported a mean 8-h TWA exposure level for *o*-nitrotoluene of 0.759 mg/m^3 (ranging from undetected to 4.29 mg/m^3) for a group of 98 workers in a Chinese factory (Lianing Province) manufacturing dinitrotoluene and 2,4,6-trinitrotoluene.

2.6 Biological indices of exposure

o-Nitrobenzoic acid and *o*-nitrobenzyl alcohol have been detected in the urine of workers exposed to *o*-nitrotoluene and were considered to provide a good marker for recently absorbed doses (Jones *et al.* 2005b, Sabbioni *et al.* 2006). Three potential biomarkers of exposure to *o*-nitrotoluene have been assessed in rats and mice: the urinary metabolites *o*-nitrobenzylmercapturic acid (*S*-(*o*-nitrobenzyl)-*N*-acetylcysteine), *o*-aminobenzoic acid, and *o*-nitrobenzoic acid. In a two-year study of dietary exposure of rats and mice to *o*-nitrotoluene (NTP 2002b), the concentrations of *o*-nitrobenzylmercapturic acid and *o*-aminobenzoic acid in the urine of mice were below the limit of quantitation at most time points, and the ratio of *o*-nitrobenzoic acid to creatinine in the urine of rats at 2 weeks and at 3, 12, and 18 months was linearly related to *o*-nitrotoluene exposure levels. The NTP did not consider *o*-aminobenzoic acid to be a good biomarker for exposure to *o*-nitrotoluene because *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).

Jones *et al*. (2005a) measured hemoglobin adducts in Chinese workers exposed to the nitrotoluenes in a trinitrotoluene factory and found that for the mononitrotoluenes, the hemoglobin adduct of *o*-nitrotoluene was present in the highest concentrations. The authors concluded that quantitation of hemoglobin adducts provides an effective biomarker of exposure to the nitrotoluenes.

Jones *et al.* (2005b) measured *o*-nitrobenzoic acid and *o*-nitrobenzyl alcohol in the urine of Chinese workers manufacturing dinitrotoluene and 2,4,6-trinitrotoluene. Mean concentrations of *o*-nitrobenzoic acid increased from 1,070 nmol/g preshift to 2,952 nmol/g postshift, while concentrations of *o*-nitrobenzyl alcohol increased from 55 to 213 nmol/g between pre- and post-shift samples. However, the authors noted that no significant correlation (r = 0.17) was found for air concentrations of *o*-nitrotoluene and the urinary metabolites.

The American Conference of Governmental Industrial Hygienists (ACGIH) considers *o*-nitrotoluene to be an inducer of methemoglobin and recommends that methemoglobin in blood be used as a biological index of exposure to *o*-nitrotoluene (and the other nitrotoluene isomers) (ACGIH 2003). French *et al*. (1995) reported that *o*-nitrotoluene caused methemoglobin in sheep erythrocytes *in vitro* both with and without an NADP bioactivation-system.

2.7 Regulations and guidelines

2.7.1 Regulations

U.S. Department of Homeland Security

Minimum requirements have been established for the safe transport of *o*-nitrotoluene on barges

U.S. Department of Transportation (DOT)

Considered a hazardous material; special requirements have been set for marking, labeling, and transporting

U.S. EPA[1]

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 1,000 lb

[1] EPA has not carried out an Integrated Risk Information System (IRIS) assessment for *o*-nitrotoluene.

Occupational Safety and Health Administration (OSHA)

Permissible exposure limit (PEL) = 5 ppm (30 mg/m^3) [skin][2]

2.7.2 Guidelines

ACGIH

Threshold limit value–time-weighted average (TLV-TWA) limit = 2 ppm

NIOSH

Immediately dangerous to life and health (IDLH) = 200 ppm

Recommended exposure limit (REL) = 2 ppm (11 mg/m^3) [skin]

2.8 Summary

o-Nitrotoluene is used primarily to produce intermediates, such as *o*-toluidine, for the manufacture of azo dyes. It is also used (either directly or as an intermediate) in the production of other dyes, agricultural chemicals, rubber chemicals, pesticides, petrochemicals, pharmaceuticals, and explosives. *o*-Nitrotoluene is a HPV chemical, and production in the United States was reported to be greater than 10 million pounds in 2002. Little information is available on environmental occurrence of *o*-nitrotoluene or on human exposure; however, since *o*-nitrotoluene is produced at high levels and is used in the production of many important chemicals, human exposure is expected to be significant. It has been detected in surface water and groundwater in France, the Netherlands, and Germany. It also can be formed as a breakdown product of di- or trinitrotoluenes, and *o*-nitrotoluene has been detected in the effluent or wastewater of plants producing these chemicals. The uses of di- and trinitrotoluenes include the production of commercial and military explosives, and *o*-nitrotoluene has been detected in groundwater, private well water, and surface water at or near munitions production facilities and military training grounds. Biological indices of exposure to *o*-nitrotoluene include *o*-nitrobenzylmercapturic acid (*S*-(*o*-nitrobenzyl)-*N*-acetylcysteine), *o*-aminobenzoic acid (also a product of catabolism of tryptophan and therefore not very useful as a biomarker for *o*-nitrotoluene), and *o*-nitrobenzoic acid in urine, and

[2] The [skin] designation indicates the potential for dermal absorption; skin exposure should be prevented as necessary through the use of good work practices and gloves, coveralls, goggles, and other appropriate equipment.

methemoglobin in blood. Hemoglobin adducts are another potential biomarker of exposure. The U.S. Department of Homeland Security, DOT, EPA, and OSHA have set regulations, and ACGIH and NIOSH have set guidelines for *o*-nitrotoluene.

3 Human Cancer Studies

No human studies on the relationship between cancer and specific exposure to *o*-nitrotoluene were identified; however, *o*-nitrotoluene may be used to manufacture magenta (see Section 2), and thus magenta manufacturing workers may be exposed to *o*-nitrotoluene. IARC (1987, 1993) reviewed magenta manufacturing and concluded that there *is sufficient evidence* in humans that the manufacture of magenta entails exposures that are carcinogenic (Group 1). As part of their assessment, they reviewed a case-control study and two cohort studies, one of which specifically identified *o*-nitrotoluene as an intermediate in the chemical processes. [The utility of these studies for evaluating the carcinogenicity of *o*-nitrotoluene is limited because the workers were also exposed to other chemicals, such as *o*-toluidine, 2,5-diaminotoluene, and 4,4'-methylenebis(2-methylaniline), and exposure to *o*-nitrotoluene was not specifically assessed.]

3.1 Studies of magenta manufacturing workers

This section describes the cohort study (Rubino *et al.* 1982) that specifically mentions exposure to *o*-nitrotoluene, and briefly reports the findings from the other two studies (Case and Pearson 1954, Vineis and Magnani 1985) that do not mention whether *o*-nitrotoluene was used in the manufacture of magenta. Rubino *et al.* conducted a cohort study of 906 male dyestuff factory workers in northern Italy. Workers were included in the study if they had worked at least 1 month any time between 1922 and 1970, and the numbers of deaths were observed from 1946 to 1976; expected numbers of deaths were calculated using national rates for 1951 to 1976. Exposure was assessed based on knowledge of chemical processes for each job listed in personnel records. Workers ever exposed to benzidine and naphthylamines were excluded from the analysis. A significant excess of mortality due to bladder cancer was observed among the 53 fuchsin (magenta) and safranine T manufacturing workers (standardized mortality ratio [SMR] = 62.5; $P < 0.001$; 5 deaths). The authors stated that the type of fuchsin manufactured at the plant was New Fuchsin or New Magenta. The manufacture of fuchsin and safranine T was carried out in two sections, and workers in both sections were potentially exposed to *o*-nitrotoluene. The first section (intermediates manufacture) involved the synthesis of *o*-toluidine and 4,4'-methylenebis(2-methylaniline); *o*-nitrotoluene was an intermediate in

the manufacture of *o*-toluidine from toluene. In the second section (fuchsin and safranine T manufacture), a mixture of *o*-toluidine, 4,4'-methylenebis(2-methylaniline), and *o*-nitrotoluene was heated to obtain fuchsin, and a mixture of *o*-toluidine and 2,5-diaminotoluene was oxidized in the presence of aniline to obtain safranine T. Decarli *et al*. (1985) and Piolatto *et al*. (1991) reported on additional follow-up of the Italian dyestuff workers, but no additional cases of bladder cancer in workers exposed to fuchsin or safranine T were identified.

Case and Pearson (1954) conducted a cohort study of men who had worked at least 6 months in the manufacture of auramine and magenta in the British chemical industry between 1910 and 1952; workers exposed to benzidine or α- or β-naphthylamine were excluded from the study. A significant excess of mortality was observed among the 85 magenta manufacturing workers that were not involved in auramine manufacturing (SMR = 23.8; $P < 0.005$; 3 observed cases; statistics reported by IARC 1993). No information regarding exposure levels or duration or potential confounders was provided. It is unknown whether the workers were exposed to *o*-nitrotoluene in this study because the authors did not describe the magenta manufacturing process.

The third study on magenta manufacturing was a case-control study conducted in Italy between 1978 and 1983 that included 512 cases of bladder cancer in males and 596 hospital controls (Vineis and Magnani 1985). Exposure to specific chemicals was assessed using job titles, job activities, and knowledge of industrial use of chemicals from the published literature. An increased risk of bladder cancer was found among workers potentially exposed to magenta (relative risk [RR] = 1.8, 95% confidence interval [CI] = 1.1 to 2.9, when calculated using industrial branches and RR = 3.0, 95% CI = 0.4 to 20.0, when calculated from job titles). It is not clear whether the workers exposed to magenta were only involved in the manufacturing of magenta or also included workers using the dye magenta (who probably would not be exposed to *o*-nitrotoluene).

3.2 Discussion and summary

[Two cohort studies and one case-control study have reported excess risk of bladder cancer among magenta manufacturing workers. Efforts were made to exclude workers

exposed to benzidine and α- and β-naphthylamine in the cohort studies, but there were limited exposure data (e.g., levels, durations, etc.), limited information on potential exposure to other agents, and small numbers of exposed workers. However, the risk estimates in the cohorts were very high, and the IARC working group concluded that the manufacture of magenta *entails exposures that are carcinogenic* (Group 1). Suspected substances used in the manufacturing process and thought to cause cancer include *o*-nitrotoluene, magenta, *o*-toluidine, and 4,4'-methylenebis(2-methylaniline) (Siemiatycki *et al.* 2004). These studies are limited for the evaluation of the carcinogenicity of *o*-nitrotoluene in humans. While *o*-nitrotoluene was specifically mentioned in the Italian cohort study, it was not specifically mentioned in the British chemical workers or the case-control study. Even in the Italian cohort study, in which it was known that the workers were exposed to *o*-nitrotoluene, the workers were also exposed to other suspected human carcinogens, such as *o*-toluidine, and 4,4'-methylenebis(2-methylaniline), so it is not possible to evaluate whether specific exposure to *o*-nitrotoluene contributed to the increased risk of bladder cancer in these workers. (*o*-Toluidine is classified by IARC as *probably carcinogenic to humans* [Group 2A] and listed in the Report on Carcinogens as *reasonably anticipated to be a human carcinogen.* 4'-Methylenebis(2-methylaniline) is classified by IARC as *possibly carcinogenic to humans* [Group 2B].)]

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4 Studies of Cancer in Experimental Animals

The results of publicly available, peer-reviewed studies of the carcinogenicity of o-nitrotoluene in experimental animals are summarized in this section. No studies of exposure by routes other than oral administration were found. In 1996 IARC evaluated *o*-nitrotoluene (2-nitrotoluene) and considered it *not classifiable as to its carcinogenicity to humans* (Group 3) based on inadequate evidence in humans and limited evidence in experimental animals (IARC 1996). At the time of the IARC review, no chronic bioassays were available (this was prior to the publication of the NTP two-year bioassay), and IARC's conclusions were based on NTP's sub-chronic study (13 weeks), which reported rare mesotheliomas in male rats receiving 2-nitrotoluene.

Section 4.1 summarizes the studies with rats, and Section 4.2 summarizes the studies with mice. Findings in experimental animals are summarized in Section 4.3. The carcinogenicity of the other nitrotoluene isomers and related compounds is discussed in Section 5.5.

4.1 Rats

The National Toxicology Program (NTP 1992) conducted 13-week studies with F344/N rats exposed to *o*-, *m*-, and *p*-nitrotoluene to compare clinical toxicity, histopathology, and reproductive system toxicity among the isomers. A follow-up subchronic exposure study was conducted (NTP 1996) to confirm carcinogenic effects observed in the 1992 study and to compare the carcinogenicity and toxicity of *o*-nitrotoluene and *o*-toluidine hydrochloride (Section 4.1.1). Finally, NTP conducted a two-year toxicology and carcinogenesis bioassay of *o*-nitrotoluene in the diets of male and female F344/N rats (NTP 2002a) (Section 4.1.2). The findings of these studies related to the carcinogenicity of *o*-nitrotoluene are summarized in this section.

4.1.1 Subchronic exposure

In 13-week studies, groups of 10 male and 10 female F344/N rats per exposure group received diets *ad libitum* containing *o*-nitrotoluene at concentrations of 0, 625, 1,250, 2,500, 5,000, or 10,000 ppm (NTP 1992, Dunnick *et al.* 1994). All animals survived to the end of the studies. Body-weight gains of males and females were reduced in a dose-

related fashion. The number of animals with treatment-related non-neoplastic lesions of the liver, kidney, spleen, testis, and epididymis-mesothelium and the average severity grade for each lesion are summarized in Table 4-1. Males showed more treatment-related toxicity that did females. Most effects in males occurred at concentrations of 2,500 ppm or greater. The most severe lesions were liver vacuolization (grade 3 at 10,000 ppm), kidney nephropathy (grades 2.8 at 5,000 ppm and 2.6 at 10,000 ppm), and testis degeneration (grade 4 at 10,000 ppm). Kidney pigmentation (grade 1.8 at 10,000 ppm) and spleen pigmentation (grade 2 at 5,000 and 10,000 ppm) were the predominant effects in females. No kidney or spleen lesions occurred in females at concentrations less than 2,500 ppm, and no liver lesions were observed in females at any concentration. Two male rats in the high-dose group had mesothelial-cell hyperplasia of the tunica vaginalis on the surface of the epididymis (which was considered to be a preneoplastic lesion), and mesothelioma occurred at the same anatomic location in three male rats in the 5,000-ppm group. At the time of this study, mesothelioma had not previously been identified in exposed or control rats from any of the 13-week toxicity studies conducted by NTP (with any substance). The authors concluded that *o*-nitrotoluene was carcinogenic in male rats, based on the occurrence of mesothelioma and mesothelial hyperplasia.

Table 4-1. Treatment-related lesions in rats[a] dosed with *o*-nitrotoluene for 13 weeks

Lesion	Males/Concentration (ppm)						Females/Concentration (ppm)					
	0	625	1,250	2,500	5,000	10,000	0	625	1,250	2,500	5,000	10,000
Liver												
Inflammation	5 (1.8)[b]	5 (1.0)	5 (1.6)	10 (1.5)	10 (1.8)	8 (1.8)	NF	NF	NF	NF	NF	NF
Vacuolization	0	0	0	6 (1.3)	9 (1.8)	10 (3.0)	NF	NF	NF	NF	NF	NF
Oval cell hyperplasia	0	0	0	2 (1.0)	10 (1.2)	10 (2.2)	NF	NF	NF	NF	NF	NF
Kidney												
Nephropathy	0	0	6 (1.0)	10 (1.6)	10 (2.8)	9 (2.6)	NF	NF	NF	NF	NF	NF
Regeneration	2 (1.0)	6 (1.0)	2 (1.0)	2 (1.0)	5 (1.0)	6 (1.1)	NF	NF	NF	NF	NF	NF
Pigment	0	0	0	0	1 (1.0)	10 (1.0)	0	0	0	3 (1.0)	10 (1.1)	10 (1.8)
Spleen												
Hematopoiesis	0	0	0	6 (1.3)	10 (2.0)	10 (2.0)	0	0	0	0	1 (1.0)	10 (1.0)
Pigment	0	0	0	7 (1.3)	10 (2.0)	10 (2.0)	0	0	0	5 (1.0)	9 (2.0)	10 (2.0)
Capsular hyperplasia	0	0	1 (1.0)	1 (2.0)	1 (1.0)	9 (1.9)	0	0	0	0	1 (1.0)	2 (1.0)
Testis												
Degeneration[c]	0	0	0	0	10 (2.3)	10 (4.0)	NF	NF	NF	NF	NF	NF
Epididymis-Mesothelium												
Hyperplasia[d]	0	0	0	0	0	2	NF	NF	NF	NF	NF	NF
Mesothelioma[e]	0	0	0	0	3	0	NF	NF	NF	NF	NF	NF

Source: NTP 1992, Dunnick *et al.* 1994.
NF = not found.
[a]Ten animals per treatment group/sex/species.
[b]Numbers in parentheses represent severity grade; 1 = minimal, 2 = mild, 3 = moderate, 4 = severe.
[c]Degeneration of seminiferous tubules.
[d]Potential preneoplastic lesions.
[e]Neoplastic lesions.

In a subsequent study (NTP 1996), male rats received *o*-nitrotoluene in feed for either 13 or 26 weeks at a concentration of 5,000 ppm. The dose was based on the results of the 1992 study, in which *o*-nitrotoluene in the diet at 5,000 ppm caused mesothelioma but had little effect on survival or body weight. *o*-Nitrotoluene was administered to 60 rats, and the control group consisted of 20 rats. After 13 weeks of *o*-nitrotoluene exposure, 10 control-group rats and 20 treated rats were killed for a 13-week interim evaluation. Following the 13-week evaluation, administration of *o*-nitrotoluene was discontinued for 20 rats for the remaining 13 weeks (stop-exposure group), and the remaining 20 rats continued to receive *o*-nitrotoluene (26-week exposure). After 26 weeks, all remaining rats were killed, and necropsies and histopathologic exams were performed.

All rats survived until the scheduled evaluations. Mean body-weight gain was lower in exposed rats than in controls. Rats in the stop-exposure group had a slightly greater mean body weight than those in the 26-week-exposure group, but still weighed less than the controls. No other clinical effects of exposure were observed in either group.

Mesothelial-cell hyperplasia (described as a potential preneoplastic lesion) or mesothelioma occurred on the mesothelial surface of the tunica vaginalis of the testis or epididymis of rats in both the 13-week stop-exposure and the 26-week-exposure groups. No tumors were observed after 13 weeks; however, by 26 weeks, mesothelioma was seen in 7 of 20 rats (epididymis) in the continuous-exposure group and in 5 of 20 in the stop-exposure group (epididymis and testis combined). At 26 weeks, focal hyperplasia of the epididymal mesothelium also was seen in 4 rats from these two groups. Significant increases in the number of rats with mesothelioma of the epididymis were noted in the 26-week exposure group. At 26 weeks, 3 cholangiocarcinomas were observed (2 from the stop-exposure group and 1 from the 26-week-exposure group). The authors concluded that these studies confirmed the carcinogenicity of *o*-nitrotoluene, based on the high incidence of mesothelioma and occurrence of cholangiocarcinoma in male rats after short-term exposure. The results are shown in Table 4-2.

The NTP (1996) also conducted 13-week and stop-exposure studies using rats with altered flora to assess the potential role of metabolism by gastrointestinal (GI) flora in *o*-

nitrotoluene-induced toxicity. Rats in the altered-flora groups received a single gavage dose of an antibiotic mixture in water daily for 6 days before the start of the study and daily for 13 weeks thereafter. Exposure conditions were similar to those described above (normal flora) except that there was no 26-week-exposure group. All rats survived until the scheduled evaluations. Similar to the study using rats with normal intestinal flora, mesothelial-cell hyperplasia or mesothelioma occurred on the mesothelial surface of the tunica vaginalis of the testis or epididymis of rats in the 13-week stop-exposure group (Table 4-2). Mesothelioma also occurred in 2 of 20 rats after 13 weeks. In contrast to the study using rats with normal intestinal flora, no cholangiocarcinomas were observed. The NTP was not able to draw any conclusions about the involvement of GI flora in the pathogenesis of *o*-nitrotoluene-induced lesions because of the lower effectiveness of the antibiotic mixture against obligate anaerobic bacteria and the possible development of resistant aerobic bacteria after one week of antibiotic administration.

Table 4-2. Neoplastic lesions identified in F344/N male rats following dietary exposure to *o*-nitrotoluene for 13 or 26 weeks

			Tumor incidence (%)			
			Mesothelioma			Liver
Exposure/GI flora status	Conc. (ppm)	N	Testis	Epididymis	Combined	Cholangio-carcinoma
Normal flora						
13 weeks	0	10	0 (0)	0 (0)	0 (0)	0 (0)
	5,000	20	0 (0)	0 (0)	0 (0)	0 (0)
Stop exposure[a]	0[b]	10	0 (0)	0 (0)	0 (0)	0 (0)
	5,000	20	2 (10)	4 (20)	5 (25)	2 (0)
26 weeks	0[b]	10	0 (0)	0 (0)	0 (0)	0 (0)
	5,000	20	2 (10)	7 (35)*	7 (35)*	1 (5)
Altered flora[c]						
13 weeks	0	10	0 (0)	0 (0)	0 (0)	0 (0)
	5,000	20	0 (0)	2(10)	2(10)	0 (0)
Stop exposure[a]	0	10	0 (0)	0 (0)	0 (0)	0 (0)
	5,000	20	4 (20)	8 (40)*	8 (40)*	0 (0)

Source: NTP 1996.

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

[a]Rats were exposed to *o*-nitrotoluene in feed for 13 weeks and allowed to recover for 13 weeks.

[b]The group of 10 control rats with normal GI flora killed at 26 weeks served as the control group for both the stop-exposure and 26-week groups.

[c]Rats in the altered-flora groups received a single gavage dose of an antibiotic mixture in water daily for 6 days before the start of the study and daily for 13 weeks thereafter.

After 13 weeks, liver weight was greater in rats exposed to *o*-nitrotoluene than in controls, and oval-cell hyperplasia was observed. Liver weight continued to increase and oval-cell hyperplasia persisted in both the stop-exposure and 26-week-exposure groups. Placental glutathione *S*-transferase positive (PGST+) foci (preneoplastic lesions) in the liver were significantly increased in exposed groups. At the end of the recovery period, the numbers of foci observed in the stop-exposure groups (with both normal and altered flora) were less than in the 13-week-exposure groups but significantly greater than in controls (NTP 1996). In a separate analysis of the data on PGST+ foci in the rats with normal flora (Ton *et al.* 1995), the 26-week continuous-exposure group had more and larger foci than the 13-week continuous-exposure group. Although the stop-exposure group at 26 weeks had fewer foci, the mean volume of foci was larger than in the 13-week continuous-exposure group.

4.1.2 Chronic exposure

NTP (2002b) performed a two-year study to investigate the chronic toxicity and carcinogenicity of *o*-nitrotoluene in the diet of rats. *o*-Nitrotoluene concentrations were based on the results of the subchronic toxicity studies (NTP 1992). In the core study, groups of 60 male and 60 female F344/N rats were fed diets containing *o*-nitrotoluene at a concentration of 625, 1,250, or 2,000 ppm for 105 weeks. These dietary concentrations were equivalent to average daily doses of approximately 25, 50, or 90 mg/kg body weight (b.w.) for males and 30, 60, or 100 mg/kg b.w. for females. In a stop-exposure study, groups of 70 male rats were fed diets containing *o*-nitrotoluene at a concentration of 2,000 or 5,000 ppm (equivalent to an average daily dose of roughly 125 or 315 mg/kg b.w.) for 13 weeks, after which *o*-nitrotoluene administration was discontinued for the remainder of the study (two years). Control groups consisted of 70 males and 60 females. After 13 weeks, 10 males from each stop-exposure group and 10 control males were killed for evaluation.

In the stop-exposure study, all rats in the 5,000-ppm group died before the end of the study, and only 11 rats in the 2,000-ppm group survived. Liver weights of the rats in the 5,000-ppm group were significantly greater than those of the controls.

In the two-year chronic-exposure study, survival was reduced in all exposure groups among males and in the highest exposure group among females because of the development of neoplasms. Feed consumption was similar between exposed and control groups; however, mean body weights were lower in all exposed groups (except the 625-ppm group of males). Non-neoplastic lesions included alveolar epithelial hyperplasia in males and females and mammary hyperplasia in females.

As shown in Tables 4-3a and b, similar tumor profiles were observed in the stop-exposure study in males and in the two-year chronic-exposure study. Significantly increased incidences of malignant mesothelioma (mainly large, papillary or solid areas of pleomorphic mesothelial cells) and skin subcutaneous neoplasia (both lipoma and fibroma or fibrosarcoma) were observed in all exposure groups; mammary-gland fibroadenoma also were increased in all exposure groups other than high-dose males in the two-year chronic-exposure study. High incidences of subcutaneous skin fibroma or fibrosarcoma and malignant mesothelioma were observed in the high-dose groups (over 70%, compared with less than 10% in controls). Significantly increased incidences of hepatocellular adenoma or carcinoma (combined) were observed in the high-dose groups in both the chronic and stop-exposure studies, and increased incidences of liver cholangiocarcinoma and alveolar/bronchiolar lung tumors (which were usually papillary and distorted the alveolar architecture) were observed in the high-dose (5,000-ppm) group in the stop-exposure study. The hepatocellular adenomas consisted of nodules of hepatocytes compressing adjacent hepatic parenchyma and lacking the normal lobular and sinusoidal pattern, while hepatocellular carcinomas were solid sheets of hepatocytes or trabeculae three or more cells thick. The incidence of hemangioma or hemangiosarcoma (combined) was significantly increased in the high-dose males in the stop-exposure study, but these tumors were not considered to be exposure related.

In females, a significantly increased incidence of mammary-gland fibroadenoma was observed in all exposure groups, and the incidence of subcutaneous skin fibroma or fibrosarcoma was significantly increased at the two highest dose levels. The incidence of hepatocellular adenoma in females was significantly increased only in the high-dose (2,000-ppm) group. The incidence of mononuclear-cell leukemia was significantly

reduced in all exposure groups of males and females in the two-year chronic exposure study and in the males in the stop-exposure study. In the stop-exposure study, the combined incidence of bilateral or unilateral interstitial-cell adenoma of the testis also was significantly decreased in the high-dose (5,000-ppm) group. The NTP suggested that these decreased tumor incidences were associated with toxicity at the tissue site (spleen or testis).

The NTP concluded that there was *clear evidence of carcinogenic activity* of *o*-nitrotoluene in male and female rats, based on increased incidences of malignant mesothelioma (in males only), subcutaneous skin neoplasia, mammary-gland fibroadenoma, and liver neoplasia (in males only). The increased incidences of lung neoplasia in male rats and hepatocellular adenoma in female rats also were considered to be exposure related.

.

Table 4-3a. Neoplastic lesions identified in F344/N rats following dietary exposure to *o*-nitrotoluene for two years

Sex	N	Conc. (ppm)	Tumor incidence (%)[a]			
			Various tissues	Mammary gland	Skin subcutaneous	
			Malignant mesothelioma	Fibroadenoma	Lipoma	Fibroma/ fibrosarcoma
M	**Chronic exposure**					
	60	0	2 (3.7)	0 (0)	0 (0)	5 (9.3)
	60	625	20 (40.6)**	7 (15.6)**	4 (8.9)*	47 (86.3)**
	60	1,250	29 (62.4)**	10 (26.2)**	13 (33)**	55 (98.7)**
	60	2,000	44 (87.1)**	2 (9.0)	13 (44.8)**	59 (99.8)**
	609	HC[b]	23 (3.8)	26 (4.3)	8 (1.3)	41 (6.7)
	Stop-exposure					
	60	2,000	44 (80.3)**	13 (31.2)**	10 (24.6)**	47 (89)**
	60	5,000	54 (95.1)**	20 (61.1)**	12 (44.7)**	53 (97.8)**
F	**Chronic exposure**					
	60	0	0 (0)	23 (40)	0 (0)	3 (5.3)
	60	625	0 (0)	47 (82.8)**	0 (0)	3 (5.4)
	60	1,250	0 (0)	52 (91.7)**	0 (0)	21 (37.6)**
	60	2,000	0 (0)	56 (96.2)**	0 (0)	22 (40.6)**
	659	HC[b]	NR[c]	284 (43.1)	NR	18 (2.7)

Sources: NTP 2002b, Dunnick *et al.* 2003.

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

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

[a]Adjusted rate by the Poly-3 test except for historical controls.

[b]HC = historical incidence (not adjusted rate) in control male or female rats given NTP-2000 diet.

[c]NR = historical incidence data not reported in NTP 2002b.

Table 4-3b. Neoplastic lesions identified in F344/N rats following dietary exposure to *o*-nitrotoluene for two years (continued)

Sex	N	Conc (ppm)	Tumor incidence (%)[a]				
			Liver				Lung
			Hepatocellular adenoma	Hepatocellular adenoma/carcinoma	Cholangiocarcinoma	Hepatocholangio-carcinoma	Alveolar/ Bronchiolar
M	**Chronic exposure**						
	60	0	2 (3.7)	3 (5.6)	0 (0)	0 (0)	2 (3.7)
	60	625	3 (6.8)	3 (6.8)	0 (0)	1 (2)	5 (11.2)
	60	1,250	3 (8.4)	3 (8.4)	0 (0)	0 (0)	1 (2.9)
	60	2,000	7 (27.1)**	8 (30.2)**	0 (0)	1 (2)	2 (8.7)
	609	HC[b]	5 (0.8)	10 (1.6)	0 (0)	0 (0)	26 (4.3)
	Stop-exposure						
	60	2,000	3 (7.6)	3 (7.6)	0 (0)	0 (0)	3 (7.6)
	60	5,000	4 (18.4)*	6 (25.9)*	3 (13.9)*	0 (0)	11 (42)**
F	**Chronic exposure**						
	60	0	1 (1.8)	1 (2)	0 (0)	0 (0)	1 (1.8)
	60	625	0 (0)	0 (0)	0 (0)	0 (0)	2 (3.6)
	60	1,250	1 (1.9)	1 (2)	0 (0)	0 (0)	0 (0)
	60	2,000	6 (11.2)*	6 (10)*	0 (0)	0 (0)	4 (7.5)
	659	HC[b]	4 (0.6)	NR[c]	NR	NR	NR

Sources: NTP 2002b, Dunnick *et al.* 2003.

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

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

[a]Adjusted rate by the Poly-3 test except for historical controls

[b]HC = historical incidence (not adjusted rate) in control male or female rats given NTP-2000 diet

[c]NR = historical incidence data not reported in NTP 2002b.

4.2 Mice

This section summarizes the subchronic and chronic studies in mice exposed to *o*-nitrotoluene.

4.2.1 Subchronic exposure

NTP (1992) conducted 13-week studies with $B6C3F_1$ mice exposed to *o*-nitrotoluene, to compare clinical toxicity, histopathology, and reproductive-system toxicity among the nitrotoluene isomers (*o*-, *m*-, and *p*-). The compound was administered in feed to groups of 10 males and 10 females per isomer per exposure group at a concentration of 0, 625, 1,250, 2,500, 5,000, or 10,000 ppm. All animals survived to the end of the studies. Body-weight gain was lower in the two highest-exposure groups of males than in controls. The only treatment-related histopathological lesions were olfactory epithelium degeneration/metaplasia in both male and female mice (see Table 4-4). The observed changes included moderate thinning of the olfactory nuclear layer, decreased diameter of the associated nerve fiber bundles in the lamina propria, replacement of the olfactory epithelium by cuboidal respiratory epithelium, and dilation of the submucosa underlying Bowman's glands (Dunnick *et al.* 1994).

Table 4-4. Treatment-related lesions in $B6C3F_1$ mice following dietary exposure to *o*-nitrotoluene for 13 weeks[a]

		Nose
Sex	Concentration (ppm)	Olfactory epithelium degeneration/metaplasia
Male	0	0
	625	0
	1,250	1 (1.0)[b]
	2,500	2 (1.0)
	5,000	10 (2.0)
	10,000	10 (3.0)
Female	0	0
	625	0
	1,250	2 (1.5)
	2,500	9 (1.0)
	5,000	10 (1.9)
	10,000	10 (2.9)

Source: NTP 1992, Dunnick *et al.* 1994.
[a]Ten animals per treatment group/sex/species.
[b]Numbers in parentheses represent average severity grade; 1 = minimal, 2 = mild, 3 = moderate, 4 = severe.

4.2.2 Chronic exposure

In a two-year study to investigate the chronic toxicity and carcinogenicity of *o*-nitrotoluene in B6C3F$_1$ mice (NTP 2002b), groups of 60 male and 60 female mice were fed diets containing *o*-nitrotoluene at a concentration of 0, 1,250, 2,500, or 5,000 ppm for 105 weeks. These concentrations were based on the results of the subchronic exposure study and were equivalent to average daily doses of approximately 165, 360, or 700 mg/kg b.w. for males and 150, 320, or 710 mg/kg b.w. for females.

All males in the two highest-exposure groups died before the end of the study. Survival of males in the 1,250-ppm group and females in the 5,000-ppm group was significantly less than that of controls. Mean body weights were less for all exposed groups of males than for controls, and females in the 5,000-ppm group generally weighed less than controls. Non-neoplastic pathology findings in males and females included significantly increased incidences of eosinophilic and basophilic foci in the liver, renal tubule pigmentation, edema of the subcutaneous tissue, olfactory epithelial degeneration, and, in males only, chronic inflammation of the prostate gland.

The neoplastic pathology findings for both male and female mice are summarized in Table 4-5. All of the males and 50 out of 60 of the females in the high-dose (5,000-ppm) groups developed hemangiosarcoma, and the incidence was significantly increased in all exposure groups of males and in the high-dose group of females (the tumor incidence rate in the females after adjustment for survival by the Poly-3 test was 90%). The 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 incidence of carcinoma of the large intestine (cecum) was significantly increased in the low-dose (1,250-ppm) and mid-dose (2,500-ppm) groups of males, and the incidence of hepatocellular adenoma or carcinoma (combined) was significantly increased in the mid-dose (2,500-ppm) and high-dose (5,000-ppm) groups of females. The incidence of hepatocellular carcinoma was significantly increased in the high-dose females (16 out of 60 animals positive with a survival-adjusted rate of 36.2%). Nonsignificantly increased incidences of carcinoma of the large intestine (cecum) in females were considered by the NTP to be exposure related, because this neoplasm is

extremely rare and had not been seen in female controls. These tumors were characterized by proliferation of glandular structures composed of moderately pleomorphic mucosal epithelial cells that invaded the cell wall.

Based on these results, the NTP concluded that there was *clear evidence of carcinogenic activity* of *o*-nitrotoluene in male and female mice, based on increased incidences of hemangiosarcoma (in males and females), carcinoma of the large intestine (cecum) (in males), and hepatocellular neoplasia (in females).

Table 4-5. Neoplastic lesions identified in B6C3F$_1$ mice following dietary exposure to *o*-nitrotoluene for two years

Sex	N	Conc. (ppm)	Tumor incidence (%)[a]		
			Hemangiosarcoma	Large-intestine carcinoma	Hepatocellular adenoma/carcinoma
Male	60	0	4 (7)	0 (0)	27 (46.1)
	60	1,250	17 (32.7)**	12 (22.7)**	28 (53.7)
	60	2,500	55 (97.9)**	9 (31.6)**	7 (26.7)
	60	5,000	60 (100)**	0 (0)	2 (18.5)
	659	HC[b]	37 (5.6)	1 (0.2)	304 (46.1)
Female	60	0	0 (0)	0 (0)	9 (15.7)
	60	1,250	2 (3.6)	1 (1.9)	9 (16.9)
	60	2,500	3 (5.2)	4 (7)	24 (42.1)**
	60	5,000	50 (90.2)**	3 (7.4)	39 (79.1)**
	659 or 655[c]	HC[b]	15(2.3)	0 (0)	143 (21.8)

Source: NTP 2002b, Dunnick *et al.* 2003.
**Significantly different from the control group ($P < 0.01$) by the Poly-3 test.
[a]Adjusted rate by the Poly-3 test, except for historical controls.
[b]HC = historical incidence (not adjusted rate) in control male or female mice given NTP-2000 diet.
[c]659 for hemangiosarcoma and large-intestine carcinoma, and 655 for hepatocellular tumors.

4.3 Summary

The carcinogenicity of *o*-nitrotoluene was evaluated in feeding studies in rats and mice. Mesothelioma was first observed in male rats administered *o*-nitrotoluene for 13 weeks. Tumors at multiple tissues sites were observed in a stop-exposure study in male rats and two year chronic studies in both sexes of rats and mice. *o*-Nitrotoluene caused tumors of the mammary gland, skin (subcutaneous), liver, lung, and mesothelium of the epididymis

and testis in rats and of the large intestine (cecum), liver, and circulatory system (hemangiosarcoma) in mice. The findings are summarized in Table 4-6.

Table 4-6. Neoplastic lesions identified in rats and mice following dietary exposure to *o*-nitrotoluene

Organ or system	Tumor type	F344/N rats		$B6C3F_1$ mice	
		Male	Female	Male	Female
Mesothelium	mesothelioma	✓			
Skin (subcutaneous)	lipoma	✓			
	fibroma or fibrosarcoma	✓	✓		
Mammary gland	fibroadenoma	✓	✓		
Liver	hepatocellular carcinoma				✓
	hepatocellular adenoma or carcinoma[a]	✓	✓[a]		✓
	chlolangiocarcinoma	✓			
Lung	alveolar/bronchiolar adenoma or carcinoma	✓			
Large intestine (cecum)	carcinoma			✓	+
Circulatory system	hemangiosarcoma			✓	✓

✓ = Significantly greater incidence than in the control group ($P < 0.05$).
+ = Higher incidence than in the control group, but not significantly different.
[a]Only adenomas (no carcinomas) were found in female F344/N rats.

5 Other Relevant Data

This section discusses relevant mechanistic and other information needed to understand the toxicity and potential carcinogenicity of *o*-nitrotoluene. It includes information on absorption, distribution, metabolism, and excretion of *o*-nitrotoluene (Section 5.1); bioactivation of *o*-nitrotoluene (Section 5.2); genetic and related effects (Section 5.3); mechanistic considerations (Section 5.4); carcinogenicity and genotoxicity of *o*-nitrotoluene metabolites and analogues (Section 5.5); and other toxic effects of *o*-nitrotoluene (Section 5.6). Differences in metabolism among the three nitrotoluene isomers offer insight into possible mechanisms of carcinogenicity or mutagenicity. Therefore, the discussions of metabolism and mechanisms include data on *m*- and *p*-nitrotoluene, in addition to *o*-nitrotoluene. The data in this section are summarized in Section 5.7.

5.1 Absorption, distribution, metabolism, and excretion

This section reviews the available information for the absorption, distribution, and/or excretion of *o*-nitrotoluene in humans (Section 5.1.1) and in rodents (Section 5.1.2). This is followed by a description of the *in vitro* metabolism of the three nitrotoluene isomers (Section 5.1.3), the *in vivo* metabolism in rodents of *o*-nitrotoluene (Section 5.1.4) and the other two nitrotoluene isomers (Section 5.1.5), a summary of the comparative metabolism data (Section 5.1.6), and a description of biliary excretion (Section 5.1.7) of the three nitrotoluene isomers. Additional information on the *in vitro* and *in vivo* metabolism of nitrotoluenes can be found in reviews from the mid 1980s by Rickert *et al.* (1986) and Rickert (1987).

5.1.1 Human data

Limited data in humans were available. Information on absorption of *o*-nitrotoluene in humans is based on indirect evidence that hemoglobin adducts in humans were increased after exposure to *o*-nitrotoluene and/or 2,4- and 2,6-dinitrotoluene (Jones *et al.* 2005a). Jones *et al.* (2005b) also reported that the *o*-nitrotoluene metabolites *o*-nitrobenzoic acid and *o*-nitrobenzyl alcohol were detected in the urine of 97% and 99%, respectively, of the workers occupationally exposed to *o*-nitrotoluene in a factory in China (see Section 2.6).

The average concentration of the nitrobenzoic acid metabolite was more than 10-fold higher than the nitrobenzyl alcohol derivative. Post-shift urine samples contained about 3- to 4-fold higher concentrations of the metabolites than measured in pre-shift urine samples. Based on the lack of significant correlation between *o*-nitrotoluene detected in the air and the concentrations of urinary metabolites, the authors suggested that dermal exposure makes up a large proportion of total exposure. Ahlborg *et al.* (1988) also reported that levels of diazo-positive compounds in the urine of workers exposed to aromatic nitroamino compounds, including nitrotoluenes, were significantly higher after a work shift than in unexposed workers, but no data on exposure specifically to *o*-nitrotoluene were reported.

5.1.2 Rodent data

Absorption of *o*-nitrotoluene after oral administration has been studied in rats and mice. In the NTP (2002b) study male and female F344 rats and male B6C3F$_1$ mice received [^{14}C]-*o*-nitrotoluene by gavage at a dose of either 2 or 200 mg/kg b.w. The concentration of *o*-nitrotoluene in plasma peaked at 10,000 ng/g between 15 and 60 minutes post-administration of the 200 mg/kg b.w. dose in male rats but was not measurable at 24 hours post-administration. Male F344 rats excreted approximately 86% of the 200 mg/kg b.w. dose in urine within the first 24 hours while urinary excretion in female rats was slightly higher at 92%. Male B6C3F$_1$ mice excreted approximately 66% of a 200-mg/kg b.w. dose during the first 24 hours. Excretion of radioactivity (parent compound and metabolites) following a 2-mg/kg b.w. gavage dose of [^{14}C]-*o*-nitrotoluene was similar to that with the higher dose, with about 98% of the dose excreted in the urine of rats and 60% of the dose in mice in the first 24 hours after dosing. Fecal elimination accounted for 3% of the dose in male rats, 4% in female rats, and 9% in male mice. Absorption of *o*-nitrotoluene also was reflected in the excretion of metabolites of *o*-nitrotoluene by male F344 rats after oral administration of [^{14}C]-*o*-nitrotoluene at a dose of 200 mg/kg b.w. More than 85% of the total oral dose was recovered in the urine within 24 hours, with lesser amounts in the feces (4.6%) and expired air (0.1%) (Chism *et al.* 1984). These results indicate that most of the dose of *o*-nitrotoluene was absorbed and metabolized in rats and mice.

Another study in male and female F344 rats (Chism and Rickert 1985) demonstrated that 29% of an oral dose (200 mg/kg b.w.) was excreted in the bile in 12 hours; however, these metabolites are subject to reabsorption and further metabolism, most likely leading to urinary excretion (see Section 5.1.7 for discussion of biliary excretion of *o*-nitrotoluene metabolites).

The half-life of *o*-nitrotoluene in plasma of F344 rats receiving a 200-mg/kg b.w. dose was calculated as 1.5 hours (NTP 2002b). *o*-Nitrotoluene or its metabolites are distributed to the liver, as indicated by recovery of radiolabel from *o*-nitrotoluene in the bile after oral administration (Chism and Rickert 1985) and by its binding to hepatic DNA (Rickert *et al.* 1984a). No data documenting the distribution of *o*-nitrotoluene to other tissues was found. However, it has been reported that *o*-nitrotoluene or its metabolites may also form hemoglobin adducts in humans (Jones *et al* 2005a) and rats (NTP 2002b) implying distribution to other tissues.

5.1.3 In vitro *metabolism of* o-, m-, *and* p-*nitrotoluene*

The metabolism of *o*-, *m*-, and *p*-nitrotoluene has been studied in rat hepatic microsomes and in isolated rat hepatocytes (deBethizy and Rickert 1984). In rat hepatic microsomes, the only metabolites formed from the nitrotoluenes were the corresponding nitrobenzyl alcohols. The formation was NADPH dependent, and it was inhibited by carbon monoxide, suggesting that the reaction was catalyzed by cytochrome(s) P450. *o*-Nitrobenzyl alcohol was formed at a rate intermediate between the rates for *m*- and *p*-nitrobenzyl alcohol, but differences in the initial rates of formation were small.

There were no large differences in the rates of disappearance of the nitrotoluenes from incubations with isolated rat hepatocytes. However, there were quantitative and qualitative differences in the metabolites formed. *o*-Nitrotoluene was metabolized to *o*-nitrobenzyl alcohol and the corresponding glucuronide conjugate. These two metabolites accounted for approximately 80% of the metabolism of *o*-nitrotoluene. In addition, small amounts of *o*-nitrobenzoic acid and an unidentified metabolite were formed. *m*-Nitrotoluene also was metabolized to the corresponding nitrobenzyl alcohol and its glucuronide, but those two metabolites accounted for only about 43% of the *m*-

nitrotoluene metabolized. The major metabolite of *m*-nitrotoluene was *m*-nitrobenzoic acid, which accounted for approximately 56% of the *m*-nitrotoluene metabolized. *p*-Nitrotoluene was converted to *p*-nitrobenzyl alcohol and its glucuronide conjugate, but only about 14% of the *p*-nitrotoluene metabolized followed this pathway. About 2% was converted to *p*-nitrobenzoic acid, and the incubation mixture also contained a small amount of *p*-nitrobenzyl sulfate (4% of the metabolized *p*-nitrotoluene). The major metabolite of *p*-nitrotoluene was *S*-(*p*-nitrobenzyl) glutathione. This metabolite apparently arose from a glutathione *S*-transferase–catalyzed reaction between *p*-nitrobenzyl sulfate and glutathione. The reaction did not proceed without glutathione *S*-transferase, suggesting that unlike some arylmethyl sulfates, *p*-nitrobenzyl sulfate is stable (deBethizy and Rickert 1983).

Examination of the metabolism of the nitrobenzyl alcohols by glucuronyl transferase, rat hepatic alcohol dehydrogenase, and sulfotransferase demonstrated that, based on the second-order rate constants for binding and catalysis (V/K) (see Table 5-1), *o*-nitrobenzyl alcohol was the best substrate of the three for glucuronyl transferase (V/K = 11.28), *m*-nitrobenzyl alcohol was the best for alcohol dehydrogenase (V/K = 3.15), and *p*-nitrobenzyl alcohol was the best for sulfotransferase (V/K = 37.21) (Rickert *et al.* 1985), further supporting the predominance of glucuronidation in metabolism of *o*-nitrotoluene.

Table 5-1. Metabolism of nitrobenzyl alcohols by rat hepatic glucuronyltransferase and sulfotransferase

	V/K ($10^3 \cdot$ nmoles/min/mg/μM)		
Substrate	**Glucuronyltransferase**	**Alcohol dehydrogenase**	**Sulfotransferase**
o-nitrobenzyl alcohol	11.28 ± 3.08	NM	4.87 ± 0.10
m-nitrobenzyl alcohol	5.25 ± 0.23	3.15 ± 0.38	4.23 ± 0.13
p-nitrobenzyl alcohol	6.00 ± 0.76	1.87 ± 0.54	37.21 ± 6.09

Source: Rickert *et al*. 1985.
NM = no metabolism observed; V/K = second order rate constant for binding and catalysis.
Note: Values are means (± S.E.) for 4 to 6 experiments.

5.1.4 In vivo *metabolism of* o-*nitrotoluene in rodents*

Male and female F344 rats and male B6C3F$_1$ mice excreted urine containing several metabolites of *o*-nitrotoluene after administration of single oral doses of 2 or 200 mg/kg b.w. (NTP 2002b). Major metabolites in male and female rats and male mice were *o*-nitrobenzoic acid and *o*-nitrobenzyl glucuronide. The male and female rats also excreted *S*-(*o*-nitrobenzyl)-*N*-acetylcysteine and *o*-aminobenzyl alcohol. Similar metabolites in similar percentages of the dose were seen in rats after either dose at 24 hours after administration (see Table 5-2). Although the percentages of the dose excreted in 24 hours as *o*-nitrobenzoic acid (30.6% vs. 19.1%) and *o*-nitrobenzyl glucuronide (28% vs. 15.3%) were a little higher at the low dose than the high dose, there did not seem to be a dose-dependent change in metabolism between 2 and 200 mg/kg b.w. in male rats. The percentage of the dose excreted in urine was similar for male and female rats, as was the metabolite profile at both doses. However, female rats excreted less than half as much of the dose as *o*-aminobenzyl alcohol or as *S*-(*o*-nitrobenzyl)-*N*-acetylcysteine as did male rats.

Male B6C3F$_1$ mice administered a single gavage dose of 200 mg/kg b.w. *o*-nitrotoluene excreted *o*-nitrobenzoic acid and *o*-nitrobenzyl glucuronide within 24 hours after administration (NTP 2002b). The specific metabolites and their percentages were similar after a dose of 2 mg/kg b.w., except that the percentages excreted as *o*-nitrobenzoic acid and *o*-nitrobenzyl glucuronide were somewhat higher than after the 200-mg/kg b.w. dose (Table 5-2).

Table 5-2. Metabolites excreted in urine (% of dose) by male and female F344 rats and male B6C3F$_1$ mice 24 hours after an oral dose of *o*-nitrotoluene

		F344 rats		B6C3F$_1$ mice	
		Dose (mg/kg b.w.)		Dose (mg/kg b.w.)	
Metabolite	Sex	200	2	200	2
o-nitrobenzoic acid	M	19.1	30.6	38.2	20.1
	F	21.8	43.9	NT	NT
o-nitrobenzyl glucuronide	M	15.3	28.0	23.9	27.9
	F	22.1	26.5	NT	NT
S-(*o*-nitrobenzyl)-*N*-acetylcysteine	M	9.9	12.4	–	–
	F	3.7	4.9	NT	NT
o-nitrobenzyl alcohol	M	1.8	1.9	–	–
	F	0.8	1.0	NT	NT
o-aminobenzyl alcohol	M	17.0	11.0	–	–
	F	7.9	4.4	NT	NT
o-aminotoluene (*o*-toluidine)	M	1.1	–	–	–
	F	1.4	–	NT	NT

Source: NTP 2002b.
– = metabolite not found; NT = not tested.

Exposure concentrations were compared to levels of the urinary metabolites *o*-nitrobenzoic acid and *o*-nitrobenzyl mercapturic acid (*S*-(*o*-nitrobenzyl)-*N*-acetylcysteine) at different time points in rats and mice from the NTP bioassay (see Section 2.6 for details related to use of these metabolites as potential biomarkers) (NTP 2002b). The ratios of urinary *o*-nitrobenzoic acid to creatinine determined at 2 weeks and at 3, 12, and 18 months were linearly related to exposure concentration in male and female rats and mice, while the ratio of *o*-nitrobenzyl mercapturic acid to creatinine was related to exposure in male and female rats only; the concentration of *o*-nitrobenzyl mercapturic acid in urine of mice was generally below the limit of quantitation.

The metabolites of *o*-nitrotoluene identified in urine after a single oral dose are summarized in Figure 5-1. There were some quantitative and qualitative differences among rats and mice in the metabolites excreted in urine, however, the relative proportions of the urinary metabolites did not appear to be dose dependent between 2 and 200 mg/kg b.w.

When male F344 rats were given 11 daily doses of unlabeled *o*-nitrotoluene (200 mg/kg b.w.) followed on the 12th day by a dose of [^{14}C]-*o*-nitrotoluene and then by two more daily doses of unlabeled compound, excretion of radioactivity was similar to that found after a single dose of the compound (NTP 2002b). However, the percentage of the radiolabeled dose excreted as *S*-(*o*-nitrobenzyl)-*N*-acetylcysteine (*o*-nitrobenzyl mercapturic acid) in this study was only half that seen after a single radiolabeled dose.

When rats were pretreated with buthionine sulfoxamine, an inhibitor of glutathione synthesis, about half as much of the radioactive dose was excreted in the urine in 24 hours as in non-pretreated rats (NTP 2002b). Excretion of *S*-(*o*-nitrobenzyl)-*N*-acetylcysteine was half that seen in non-pretreated rats, while excretion of *o*-nitrobenzyl alcohol tripled. Excretion of the other identified metabolites was not greatly altered. This study also investigated the urinary excretion of [^{14}C]-*o*-nitrotoluene after pretreatment of male rats with pentachlorophenol, an inhibitor of sulfotransferase. Pretreated rats excreted significantly less of the dose in the urine in 24 hours than did non-pretreated rats. The percentage of the dose excreted as *S*-(*o*-nitrobenzyl)-*N*-acetylcysteine was about 15% of that seen in non-pretreated rats. The authors suggested that nitrobenzyl alcohol was converted by *O*-sulfation to a metabolite that could react with glutathione, resulting in the decreased excretion of *S*-(*o*-nitrobenzyl)-*N*-acetylcysteine after pretreatment with buthionine sulfoxamine or pentachlorophenol. After pentachlorophenol pretreatment, excretion of *o*-nitrobenzyl glucuronide was decreased by about half, as was excretion of *o*-aminobenzyl alcohol; the authors attributed these decreases to competition between *o*-nitrobenzyl alcohol and pentachlorophenol for glucuronyl transferase. Presumably, decreased formation of *o*-nitrobenzyl glucuronide resulted in less of the dose being presented to the gut microflora for deconjugation and reduction, resulting in decreased excretion of *o*-aminobenzyl alcohol.

An earlier report by Chism *et al.* (1984) described similar results for urinary metabolites for male F344 rats given an oral dose of [^{14}C]-*o*-nitrotoluene (200 mg/kg b.w.); however, there were some differences (see Table 5-3). The NTP (2002b) identified *o*-aminobenzyl alcohol as a major metabolite, whereas Chism *et al.* (1984) identified the sulfur-containing conjugates of *o*-acetamidotoluene and aminotoluene as major metabolites.

Section 5.1.5 describes the metabolites of *m*- and *p*-nitrotoluene identified in these studies.

Excretion rates for the metabolites that had not undergone nitro-group reduction peaked within the first 4 hours after administration, while those for metabolites that had undergone nitro-group reduction peaked between 4 and 12 hours after administration (Chism *et al.* 1984). These findings are in contrast to the *in vitro* results discussed in Section 5.1.4, which showed smaller percentages metabolized to *o*-nitrobenzoic acid and higher percentages metabolized to *o*-nitrobenzyl alcohol and *o*-nitrobenzyl glucuronide.

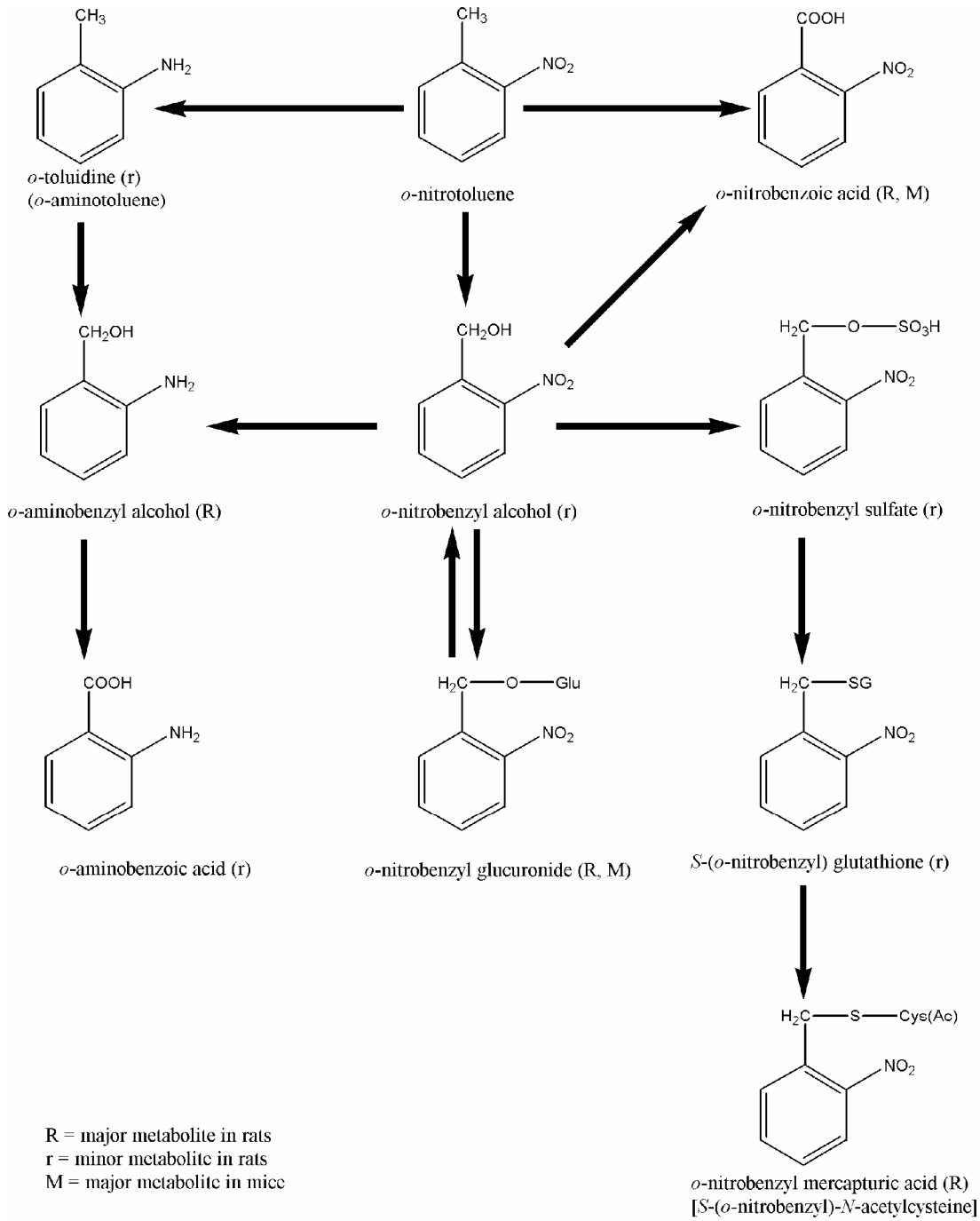


Figure 5-1. Urinary metabolites of *o*-nitrotoluene in rats and mice
Source: NTP 2002b.

The urinary metabolites found in rats and mice are shown above. The letters in parentheses after the name of the molecule denote whether the metabolite is a major or minor metabolite in a particular species. Note that two sulfur-containing metabolites for which quantitative data are included in Table 5-2 are not illustrated above. The sulfur-containing conjugates of *o*-acetamidotoluene and *o*-aminotoluene have been tentatively identified by Chism and Rickert (1985) (see Figure 1-2), but the metabolic pathways giving rise to these urinary products have not been established.

5.1.5 In vivo *metabolism of* m- *and* p-*nitrotoluene in rodents*

The *in vivo* disposition of *m*- and *p*-nitrotoluene after a 200-mg/kg b.w. oral dose also has been studied in male F344 rats (Chism *et al.* 1984). The major urinary metabolites of *m*-nitrotoluene were *m*-nitrohippuric acid, *m*-nitrobenzoic acid, and *m*-acetamidobenzoic acid, and the major urinary metabolites of *p*-nitrotoluene were *p*-nitrobenzoic acid, *p*-acetamidobenzoic acid, *p*-nitrohippuric acid, and *S*-(*p*-nitrobenzyl)-*N*-acetylcysteine (Table 5-3).

The NTP (2002a) studied the *in vivo* metabolism of *p*-nitrotoluene in male and female F344 rats and male and female $B6C3F_1$ mice after oral doses of 2 or 200 mg/kg b.w. At the higher dose, the major metabolites in male rats were *p*-nitrobenzoic acid, *p*-acetamidobenzoic acid, *p*-nitrohippuric acid, and *p*-nitrobenzylmercapturic acid (*S*-(*p*-nitrobenzyl)-*N*-acetylcysteine) along with other unidentified but more polar metabolites that accounted for approximately 5% of the dose; the results reported by NTP and by Chism *et al.* (1984) were generally consistent (Table 5-3). The metabolites excreted by female rats were similar, except that females excreted somewhat more *p*-nitrobenzoic acid and somewhat less *S*-(*p*-nitrobenzyl)-*N*-acetylcysteine. The patterns of excretion in both male and female rats were similar after a 2-mg/kg b.w. dose (data not shown). Male mice given a 200-mg/kg b.w. dose of *p*-nitrotoluene excreted the following major metabolites in urine: *p*-nitrohippuric acid, 2-methyl-5-nitrophenyl sulfate, 2-methyl-5-nitrophenyl glucuronide, *p*-nitrobenzoic acid, and *p*-aetamidobenzoic acid (Table 5-3). Female mice excreted somewhat less of the dose as *p*-nitrohippuric acid or 2-methyl-5-nitrophenyl sulfate and somewhat more of the dose as 2-methyl-5-nitrophenyl glucuronide, *p*-nitrobenzoic acid, or *p*-acetamidobenzoic acid.

The NTP (2002a) also studied the metabolism of *p*-nitrotoluene after multiple 200-mg/kg b.w. doses. After a single radiolabeled dose on day 9, the major urinary metabolite in the urine of male F344 rats was *p*-nitrobenzoic acid; smaller portions of the dose were excreted as *p*-acetamidobenzoic acid, *p*-nitrohippuric acid, and *S*-(*p*-nitrobenzyl)-*N*-acetylcysteine. The half-life of total radioactivity in plasma after an oral dose of radiolabeled *p*-nitrotoluene (200 mg/kg b.w.) to rats was about 1 hour (NTP 2002a), similar to that for *o*-nitrotoluene (NTP 2002b).

5.1.6 Comparison of o-, m-, *and* p-*nitrotoluene metabolism in rodents*

Taken together, the *in vitro* and *in vivo* studies on nitrotoluene metabolism demonstrated some differences in the excretion of *o*-, *m*- and *p*-nitrotoluene metabolites and across species and sex for *o*- and *p*-nitrotoluene metabolites (the results of the *in vivo* studies for all three isomers are summarized in Table 5-3). The first step in nitrotoluene metabolism is conversion of the methyl group to an alcohol. In contrast to what is observed in isolated rat hepatocytes, the main metabolic pathway for each of the isomers *in vivo* is oxidation of the alcohol group to an acid moiety. [Whereas very little of a dose of *m*- or *p*-nitrotoluene is converted to a nitrobenzyl glucuronide, this is an important pathway for *o*-nitrotoluene. Furthermore, the appearance of reduced metabolites in the *in vivo* studies, but not in studies with microsomes or isolated hepatocytes, suggests that nitro-group reduction occurs at a site other than the liver.]

Table 5-3. Metabolites excreted in urine (% of dose) by rats and mice after an oral dose of 200 mg/kg b.w. of *o*-, *m*-, or *p*-nitrotoluene

Metabolite	Sex	*o*-nitrotoluene			*m*-nitrotoluene	*p*-nitrotoluene		
		F344 rats		$B6C3F_1$ mice	F344 rats	F344 rats		$B6C3F_1$ mice
		Ch.	NTP	NTP	Ch.	Ch.	NTP	NTP
x-nitrobenzoic acid[a]	M	28.6	19.1	38.2	21.1	28.0	36.2	5.5
	F	NT	21.8	NT	NT	NT	45.0	10.3
sulfur-containing conjugate of *x*-acetamidotoluene	M	15.9	–	–	–	–	–	–
	F	NT	–	NT	NT	NT	–	–
x-nitrobenzyl glucuronide	M	14.1	15.3	23.9	2.0	1.4	–	–
	F	NT	22.1	NT	NT	NT	–	–
S-(*x*-nitrobenzyl)-*N*-acetylcysteine	M	11.6	9.9	–	–	3.7	7.1	–
	F	NT	3.7	NT	NT	NT	1.2	–
sulfur-containing conjugate of *x*-aminotoluene	M	6.0	–	–	–	–	–	–
	F	NT	–	NT	NT	NT	–	–
S-(*x*-nitrobenzyl) glutathione	M	3.9	–	–	1.3	–	–	–
	F	NT	–	NT	NT	NT	–	–
x-aminobenzoic acid	M	1.8	–	–	1.2	0.8	–	–
	F	NT		NT	NT	NT	–	–
x-nitrobenzyl sulfate	M	0.5	–	–	–	–	–	–
	F	NT	–	NT	NT	NT	–	–
x-nitrobenzyl alcohol	M	0.4	1.8	–	–	–	–	–
	F	NT	0.8	NT	NT	NT	–	–
x-aminobenzyl alcohol	M	–	17.0	–	–	–	–	–
	F	NT	7.9	NT	NT	NT	–	–

Metabolite	Sex	*o*-nitrotoluene			*m*-nitrotoluene	*p*-nitrotoluene		
		F344 rats		$B6C3F_1$ mice	F344 rats	F344 rats		$B6C3F_1$ mice
		Ch.	NTP	NTP	Ch.	Ch.	NTP	NTP
x-nitrohippuric acid	M	–	–	–	23.6	13.0	10.3	20.5
	F	NT	–	NT	NT	NT	8.7	14.7
x-acetamidobenzoic acid	M	–	–	–	11.6	27.1	16.1	5.2
	F	NT	–	NT	NT	NT	19.3	7.0
x-aminotoluene	M	–	1.1	–	–	–	–	–
	F	NT	1.4	NT	NT	NT	NT	–
5-methyl-2-nitrophenyl glucuronide	M	–	–	–	–	0.3	–	–
	F	NT	–	NT	NT	NT	NT	–
5-methyl-2-nitrophenyl sulfate	M	–	–	–	–	0.2	–	–
	F	NT	–	NT	NT	NT	NT	–
2-methyl-5-nitrophenyl glucuronide	M	–	–	–	–	–	–	12.7
	F	NT	–	NT	NT	NT	NT	18.7
2-methyl-5-nitrophenyl sulfate	M	–	–	–	–	–	–	19.0
	F	NT	–	NT	NT	NT	NT	12.0

Sources: Ch. = Chism *et al.* 1984 (72 h data), NTP = NTP 2002b (24 h data) for *o*-nitrotoluene and NTP 2002a (24 h data) for *p*-nitrotoluene.
– = metabolite not found; NT = not tested.
[a] *x* represents *o*-, *m*-, or *p*-nitrotoluene metabolites.

5.1.7 Biliary excretion of o-, m-, *and* p-*nitrotoluene in rats*

In F344 rats given an oral dose (200 mg/kg b.w.) of radiolabeled *o*-nitrotoluene, bile-duct cannulation decreased urinary excretion of radiolabel by about one-quarter in males and by about one-half in females after 12 hours, compared with sham-operated animals (Chism *et al.* 1984). Urinary excretion of radiolabel from *m*- or *p*-nitrotoluene in bile-duct–cannulated F344 rats was also decreased by one-quarter to one-half. In the studies in male rats, 28.6% of an *o*-nitrotoluene dose was eliminated in the bile, while in female rats only 9.6% was eliminated in the bile. Biliary excretion of radiolabel dose due to *m*- or *p*-nitrotoluene accounted for about 10% of the dose in male rats and from 1.3% to 4.3% of the dose in female rats. The major biliary metabolite of *o*-nitrotoluene was *o*-nitrobenzyl glucuronide, which accounted for 22.1% of the dose in males and 8.3% of the dose in females. The next most abundant metabolite was *S*-(*o*-nitrobenzyl) glutathione, which accounted for 4.9% of the dose in males and 0.4% in females. The nitrobenzyl glucuronides formed from *m*- or *p*-nitrotoluene accounted for only 0.1% of the dose in males and 2.8% in females. The major biliary metabolite of *m*-nitrotoluene was *m*-nitrobenzoic acid (3.4% of the dose in males and 1.7% in females). *p*-Nitrotoluene was excreted in the bile primarily as *p*-nitrobenzoic acid and *S*-(*p*-nitrobenzyl) glutathione; each accounted for 2.8% of the dose in male rats.

In another study (NTP 2002a), biliary excretion accounted for 7.7% of a 200-mg/kg b.w. oral dose of [^{14}C]-*p*-nitrotoluene in male rats in 6 hours; the major metabolite was *S*-(*p*-nitrobenzyl) glutathione (4.4% of the dose), followed by *p*-nitrobenzoic acid (2.5%) and *p*-nitrobenzyl glucuronide (0.4%).

These results indicate differences in the biliary excretion of the three nitrotoluene isomers; *o*-nitrotoluene metabolites are excreted via this route in approximately twice the amount as *m*- or *p*-nitrotoluene metabolites. There is also a sex difference in biliary excretion of *o*-nitrotoluene; males excrete about three times as much as *o*-nitrobenzyl glucuronide in the bile as females.

5.2 Bioactivation of *o*-nitrotoluene

Biliary excretion is an important step in the activation of *o*-nitrotoluene (see Figure 5-2). Interruption of bile flow into the intestine by cannulation of the bile duct decreased the covalent binding of *o*-nitrotoluene-related material at 12 hours post-administration to 7% (in males) or 22% (in females) of that seen in sham-operated animals (Chism and Rickert 1985). In intact rats, females tended to excrete *o*-nitrobenzyl glucuronide in the urine to a greater extent than male rats; in bile duct–cannulated animals, similar amounts were excreted in the urine of both sexes.

When covalent binding to hepatic macromolecules was used as an indicator of potential genotoxicity in male F344 rats, 2 to 6 times as much radiolabel from *o*-nitrotoluene was bound to hepatic macromolecules as from *m*- or *p*-nitrotoluene (Rickert *et al.* 1984a). Only *o*-nitrotoluene was bound to hepatic DNA at concentrations above the assay's limit of quantitation. When sulfotransferase activity was inhibited by pretreatment of rats with pentachlorophenol or 2,6-dichloro-4-nitrophenol, the binding of *o*-nitrotoluene-related material to total hepatic macromolecules was decreased to less than half that seen with no pretreatment, and the binding of *o*-nitrotoluene-related material to hepatic DNA was below the limit of quantitation. The binding of *m*- or *p*-nitrotoluene-related material to total hepatic macromolecules was unaffected by pretreatment. These results suggest that sulfotransferase is an important enzyme in the activation of an *o*-nitrotoluene metabolite to a compound capable of reacting with DNA.

[All the above data suggest that, like the activation of 2,6-dinitrotoluene (reviewed in Rickert *et al.* 1984b), the activation of *o*-nitrotoluene to a compound capable of covalently interacting with DNA requires metabolism to a benzyl alcohol, conjugation with glucuronic acid, elimination in bile, deconjugation, reduction of the nitro group, and reabsorption for delivery to the liver. Once in the liver, the final activation of *o*-aminobenzyl alcohol requires the action of sulfotransferase. The involvement of sulfotransferase is suggested by the experiments measuring covalent binding to hepatic DNA in rats pretreated with pentachlorophenol or 2,6-dichloro-4-nitrophenol (Rickert *et al.* 1984a). It is also supported by *in vitro* studies of the binding of *o*-aminobenzyl alcohol to calf thymus DNA (Chism and Rickert 1989). In those experiments, *o*-aminobenzyl

Figure 5-2. Formation and potential bioactivation routes for *o*-aminobenzyl alcohol from *o*-nitrotoluene

Source: Adapted from Chism and Rickert 1985.

o-Nitrobenzyl glucuronide formed from *o*-nitrotoluene in the liver (uppermost box on opposite page) is excreted via the bile into the small intestine (middle box) where intestinal bacteria deconjugate the glucuronide and reduce the nitro group to an amine, forming *o*-aminobenzyl alcohol. *o*-Aminobenzyl alcohol is reabsorbed from the intestine carried by the portal circulation to the liver (lowermost box) where three potential pathways may be followed for further metabolism culminating in the two nitrenium ions and one carbonium ion illustrated. As noted in the text, the pathway through the *o*-aminobenzyl alcohol sulfate (heavier arrows on the right side of the lowermost box) is considered the predominant pathway for bioactivation.

(A) *o*-nitrotoluene

(B) *o*-nitrobenzyl alcohol

(C) *o*-nitrobenzyl alcohol glucuronide

(D) *o*-aminobenzyl alcohol

(E) *o*-(*N*-hydroxylamino)benzyl alcohol

(F) *o*-(*N*-acetylamino)benzyl alcohol

(G) *o*-aminobenzyl alcohol sulfate

(H) *o*-(*N*-hydroxylamino-*N*-sulfoxyl)benzyl alcohol

(I) *o*-(*N*-hydroxy-*N*-acetylamino)benzyl alcohol

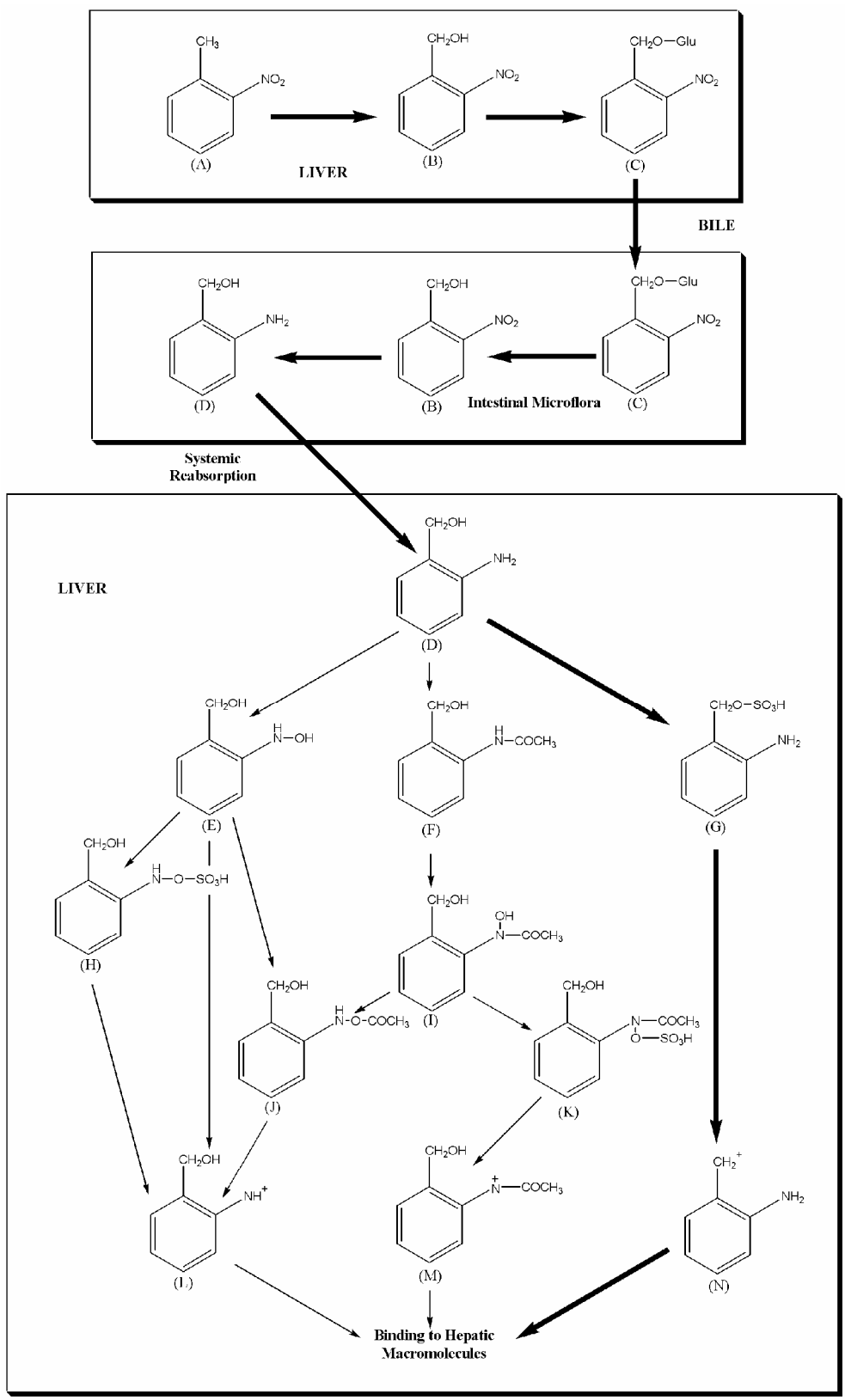
(J) *o*-*N*-acetoxy benzyl alcohol

(K) *o*-acetamido(*N*-sulfoxyl)benzyl alcohol

(L) nitrenium ion

(M) nitrenium ion

(N) carbonium ion


LIVER
BILE
Intestinal Microflora
Systemic Reabsorption
LIVER
Binding to Hepatic Macromolecules

alcohol-related material became bound to calf thymus DNA when incubated with rat hepatic cytosol and 3'-phosphoadenosine 5'-phosphosulfate (PAPS), a source of sulfate for sulfotransferase. No binding was seen when the sulfotransferase inhibitor 2,6-dichloro-4-nitrophenol was added to the incubation mixture. The final reactive intermediate does not appear to be the *N*,*O*-sulfate, as addition of NADPH and microsomes (necessary to produce the precursor hydroxylamine) did not increase covalent binding to calf thymus DNA above that seen in incubations containing cytosol and PAPS. The addition of acetyl coenzyme A did not result in covalent binding, suggesting that the proximal reactive metabolite was not an acetoxyarylamine. Although binding to macromolecules through an *N*-hydroxy intermediate cannot be ruled out, binding in the presence of NADPH and microsomes was much lower than binding in the presence of PAPS and cytosol. This suggests that the proximal reactive metabolite is *o*-aminobenzyl sulfate (see Figure 5-2). This pathway only describes the situation in the liver because other tissues may not have the necessary enzymes to activate aminobenzyl alcohol. Similar structures are proposed to be the reactive intermediates formed from 7,12-dimethylbenz[*a*]anthracene (Watabe *et al.* 1982) and 1'-hydroxysafrole (Boberg *et al.* 1983).]

Although hemoglobin adducts, which release *o*-aminotoluene (*o*-toluidine or *o*-methylaniline) on treatment with mild base, are formed from *o*-nitrotoluene administered to rats, and DNA adducts are formed when *o*-aminotoluene is incubated with calf thymus DNA, no hepatic DNA adducts were detected in rats treated with *o*-aminotoluene (Jones *et al.* 2003, Jones and Sabbioni 2003). Furthermore, the adducts formed when *o*-aminotoluene was incubated with calf thymus DNA were different from those present in hepatic DNA after *o*-nitrotoluene was administered to rats (Jones *et al.* 2003). [These data add support to the possibility that an *o*-nitrotoluene metabolite(s) modifies DNA through the methyl group, rather than an amino group.] Jones *et al.* (2005a) also measured hemoglobin adducts in Chinese workers exposed to the nitrotoluenes in a trinitrotoluene factory and found that for the mononitrotoluenes, the hemoglobin adduct of *o*-nitrotoluene was present in the highest concentrations.

5.3 Genetic damage and related effects

o-Nitrotoluene has been tested for genotoxicity in a number of *in vitro* and *in vivo* test systems. The International Agency for Research on Cancer (IARC 1996) reviewed the available literature and concluded that *o*-nitrotoluene was not genotoxic to bacteria but induced sister chromatid exchange (SCE) in cultured mammalian cells, and formed DNA adducts and induced unscheduled DNA synthesis *in vivo* in rats. This section reviews the available genotoxicity studies of *o*-nitrotoluene in prokaryotic and mammalian *in vitro* and *in vivo* systems, including those cited in the IARC review and studies published subsequently. Studies of mutations and gene expression in oncogenes and tumor suppressor genes are discussed in Section 5.4.2. Genotoxicity studies of nitrotoluene isomers and related compounds are discussed in Section 5.5.

5.3.1 Prokaryotic systems

o-Nitrotoluene has been tested for the ability to induce reverse mutation in several strains of *Salmonella typhimurium* and for differential toxicity in *Bacillus subtilis*. Table 5-4 summarizes the results of tests in prokaryotic systems.

Reverse mutation in Salmonella typhimurium

In studies with *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 (see Table 5-4 for the concentrations tested), *o*-nitrotoluene did not induce reverse mutation, either with or without exogenous metabolic activation (Chiu *et al.* 1978, Tokiwa *et al.* 1981, Spanggord *et al.* 1982a, Spanggord *et al.* 1982b, Haworth *et al.* 1983, Suzuki *et al.* 1983, Shimizu and Yano 1986). In the presence of norharman (200 μg/plate), a co-mutagen found in tobacco tar and in pyrolysate of tryptophan, and with S9 metabolic activation, *o*-nitrotoluene (at concentrations of 100 to 300 μg/plate) induced mutations in *S. typhimurium* strain TA98 but not in TA100 (Suzuki *et al.* 1983). Norharman may alter metabolic activation by the S9 mixture or increase the susceptibility of DNA to damage by intercalation into DNA. The lack of positive results with TA100 may indicate that norharman was effective as a co-mutagen only for the frameshift mutations detectable in strain TA98.

Differential toxicity in Bacillus subtilis

Shimizu and Yano (1986) reported negative results for *o*-nitrotoluene (concentration not reported) in the *rec* assay in *B. subtilis* strains H17 and M45 without metabolic activation.

Table 5-4. Results of genotoxicity testing of *o*-nitrotoluene in prokaryotic systems

Test system	End point (concentration)	Results		Reference
		with S9	without S9	
S. typhimurium TA98, TA100	reverse mutation (0.1–10 μmol)	–	–	Chiu *et al.* 1978
S. typhimurium TA98, TA100	reverse mutation (NR)	–	–	Tokiwa *et al.* 1981
S. typhimurium TA98, TA100	reverse mutation (100–300 μg/plate)	–	–	Suzuki *et al.* 1983
S. typhimurium TA98 with norharman	reverse mutation (100–300 μg/plate)	+	NT	Suzuki *et al.* 1983
S. typhimurium TA100 with norharman	reverse mutation (100–300 μg/plate)	–	NT	Suzuki *et al.* 1983
S. typhimurium TA98, TA1537, TA1538	reverse mutation (0.01–5 μL/plate)	–	–	Shimizu and Yano 1986
S. typhimurium TA98, TA100, TA1535, TA1537	reverse mutation (3–333 μg/plate)	–	–	Haworth *et al.* 1983
S. typhimurium TA98, TA100, TA1535, TA1537, TA1538	reverse mutation (10–5,000 μg/plate)	–	–	Spanggord *et al.* 1982a, 1982b
B. subtilis H17, M45	differential toxicity (NR)	NT	–	Shimizu and Yano 1986

NR = not reported; NT = not tested; + = positive; – = negative.

5.3.2 Mammalian systems

o-Nitrotoluene has been tested for genotoxicity in a number of mammalian *in vitro* systems, including Chinese hamster ovary (CHO) cells, rat and human hepatocytes, and rat pachytene spermatocytes and round spermatids. Mammalian *in vivo* tests of *o*-nitrotoluene included DNA adduct formation in rat livers, the unscheduled DNA synthesis (UDS) assay in rat hepatocytes, and the acute micronucleus test in bone marrow in rats and mice.

In vitro *systems*

Table 5-5 summarizes the results of tests in *in vitro* mammalian systems. Galloway *et al.* (1987) tested the ability of *o*-nitrotoluene to induce SCE and chromosomal aberrations in

cultured CHO cells. Significantly increased incidences of SCE were reported with S9 metabolic activation at *o*-nitrotoluene concentrations from 355 to 423 μg/mL and equivocal results without S9 at concentrations ranging from 117 to 282 μg/mL. The incidence of chromosomal aberrations was not increased, either with S9 at *o*-nitrotoluene concentrations from 375 to 422 μg/mL or without S9 at concentrations from 201 to 394 μg/mL.

Negative results were reported for *o*-nitrotoluene in a number of tests measuring DNA repair as UDS (all of which used concentrations of 10 to 1,000 μM), including tests in male and female F344/N rat hepatocytes (Doolittle *et al.* 1983), human hepatocytes (Butterworth *et al.* 1989), and rat pachytene spermatocytes and round spermatids (Working and Butterworth 1984). Parton *et al.* (1995) reported that culturing rat hepatocytes in serum-free media for 24 hours before exposure increased the assay's sensitivity; these investigators reported that *o*-nitrotoluene induced DNA repair.

Table 5-5. Results of genotoxicity testing of *o*-nitrotoluene in *in vitro* mammalian systems

Test system (tissue or cell type)	End point (conc. Or dose)	Results		Reference
		with S9	without S9	
CHO cells	SCE (355–423 μg/mL)	+	NT	Galloway *et al.* 1987
CHO cells	SCE (117–282 μg/mL)	NT	±	Galloway *et al.* 1987
CHO cells	chromosomal aberrations (375–422 μg/mL)	–	NT	Galloway *et al.* 1987
CHO cells	chromosomal aberrations (201–394 μg/mL)	NT	–	Galloway *et al.* 1987
F344/N rat hepatocytes	DNA repair (10–1,000 μM)	NT	–	Doolittle *et al.* 1983
F344/N rat hepatocytes (serum-free media)	DNA repair (0.1–500 μg/mL)	NT	+	Parton *et al.* 1995
Human hepatocytes	DNA repair (10–1,000 μM)	NT	–	Butterworth *et al.* 1989
Rat spermatocytes and spermatids	DNA repair (10–1,000 μM)	NT	–	Working and Butterworth 1984

NT = not tested; + = positive; – = negative; ± = equivocal.

In vivo *systems*

The results of tests for DNA adduct formation, DNA repair, and micronucleus formation in *in vivo* mammalian systems are summarized below.

DNA adduct formation in rats

Rickert *et al.* (1984a) measured covalent binding of the nitrotoluenes to hepatic macromolecules and DNA in male F344/N rats. A single oral dose (200 mg/kg b.w.) of *o*-nitrotoluene resulted in its covalent binding to hepatic macromolecules and hepatic DNA. Jones *et al.* (2003) reported dose-dependent formation of DNA and hemoglobin adducts in the livers of WELS-Fohm male rats administered *o*-nitrotoluene at a daily oral dose of 40 to 250 mg/kg b.w. for 12 weeks. In another study (Jones and Sabbioni 2003), DNA adduct formation was not observed in the livers of female Wistar rats administered *o*-nitrotoluene (0.1 mL/100 g b.w.) by gavage; however, an exposure marker, hydrolyzable hemoglobin adducts, was detected.

DNA repair in rats

The effects of *o*-nitrotoluene and intestinal bacteria on UDS were assessed in male and female F344 rats, germ-free male F344 rats, and germ-free male F344 rats treated with Charles River Altered Schaedler Flora (CRASF), a mixture of eight bacterial strains used to simulate the autochthonous (native) GI flora (Doolittle *et al.* 1983). *o*-Nitrotoluene was administered via gavage (200 to 500 mg/kg b.w.), and DNA repair was assessed 12 hours later by the *in vivo–in vitro* rat hepatocyte UDS assay in primary cultures of hepatocytes isolated from exposed animals. *o*-Nitrotoluene induced a dose-dependent increase in both the percentage of hepatocytes undergoing DNA repair and the extent of the repair in male rats, but not in female rats. Increases in DNA repair were observed in CRASF rats (albeit to a lesser extent than in rats with conventional flora) but not in the germ-free rats. [The inability of *o*-nitrotoluene to induce DNA repair in germ-free rats and the results of previous studies showing that it did not induce DNA repair in rat hepatocytes *in vitro* led the authors to suggest that intestinal bacteria are needed for the metabolic activation of *o*-nitrotoluene to a hepatic genotoxicant.] Sex differences in the DNA repair responses did not appear to be due to intestinal bacteria, because males and females had nearly

identical types of intestinal flora. The authors suggested that differences between sexes were probably in the hepatic metabolism and/or disposition of *o*-nitrotoluene.

Micronucleus formation in rats and mice

The acute micronucleus test, measuring the frequency of micronucleated polychromatic erythrocytes (PCEs) in bone marrow following a single intraperitoneal (i.p.) dose of *o*-nitrotoluene dissolved in corn oil, was carried out in male F344/N rats according to two protocols: (1) a dose of 625, 1,250, or 2,500 mg/kg b.w. and bone marrow analysis 24 hours post-injection and (2) a dose of 625 or 2,500 mg/kg b.w. and bone marrow analysis 48 hours post-injection. Negative results were reported for both protocols (NTP 2002b).

Male B6C3F_1 mice injected i.p. with *o*-nitrotoluene dissolved in corn oil (100 to 400 mg/kg b.w.) three times at 24-hour intervals showed no significant increase in the frequency of micronucleated PCEs in bone marrow, although small increases were observed at all exposure levels. In a study of mice administered *o*-nitrotoluene in the feed for 13 weeks at concentrations of 625 to 10,000 ppm, no increase in the frequency of micronucleated normochromatic erythrocytes (NCEs) was seen in the peripheral blood of females. In males, the frequency of micronucleated NCEs was slightly increased at the highest exposure level (10,000 ppm); this result was judged to be equivocal (NTP 2002b).

The results of genotoxicity testing of *o*-nitrotoluene in *in vivo* mammalian systems are summarized in Table 5-6.

Table 5-6. Results of genotoxicity testing of *o*-nitrotoluene in *in vivo* mammalian systems

Test system (tissue or cell type)	End point (conc. or dose)	Results	Reference
Male F344 rats (liver, cell type not specified)	Covalent binding to hepatic macromolecules (200 mg/kg b.w.)	+	Rickert *et al.* 1984a
Male F344 rats (liver, cell type not specified)	Covalent binding to DNA (200 mg/kg b.w.)	+	Rickert *et al.* 1984a
Male WELS-Fohm rats (whole liver, cell type not specified)	DNA adducts (40–250 mg/kg b.w.)	+	Jones *et al.* 2003
Female Wistar rats (whole liver, cell type not specified)	DNA adducts (0.1 mL/100 g b.w.)	–	Jones and Sabbioni 2003
Male F344/N rats (hepatocytes)	DNA repair (200–500 mg/kg b.w.)	+	Doolittle *et al.* 1983
Female F344/N rats (hepatocytes)	DNA repair (200–500 mg/kg b.w.)	–	Doolittle *et al.* 1983
Germ-free male F344/N rats (hepatocytes)	DNA repair (200–500 mg/kg b.w.)	–	Doolittle *et al.* 1983
Male F344/N rats raised germ-free but inoculated with CRASF (a mixture of 8 bacteria) 2 weeks pre-exposure (hepatocytes)	DNA repair (200–500 mg/kg b.w.)	+	Doolittle *et al.* 1983
Male F344/N rats (bone marrow)	micronucleated PCEs (625–2,500 mg/kg b.w.)	–	NTP 2002b
Male $B6C3F_1$ mice (bone marrow)	micronucleated PCEs (100–400 mg/kg b.w.)	–	NTP 2002b
Male $B6C3F_1$ mice (bone marrow)	micronucleated NCEs (625–10,000 ppm)	±	NTP 2002b
Female $B6C3F_1$ mice (bone marrow)	micronucleated NCEs (625–10,000 ppm)	–	NTP 2002b

+ = positive; – = negative; ± = equivocal.

5.3.3 Gene expression studies

This section discusses gene expression studies in cultured mouse cells or non-tumor tissue from mice exposed to *o*-nitrotoluene (Table 5-7). Gene expression and mutation studies using *o*-nitrotoluene-induced tumors are discussed in Section 5.4.2 and Table 5-10.

Kim *et al.* (2005) used cDNA microarray analysis to evaluate gene expression profiles for four classes of chemicals differing in their mutagenic and carcinogenic properties: genotoxic carcinogens, genotoxic noncarcinogens, nongenotoxic carcinogens, and nongenotoxic noncarcinogens. *o*-Nitrotoluene was one of the compounds selected to represent the nongenotoxic carcinogens. L5178Y $Tk^{+/-}$ mouse lymphoma cells were used for cDNA microarray analysis. Test chemicals were dissolved in DMSO and diluted in culture media. Cultured cells were exposed for 24 hours. Duplicate RNA samples were obtained for each test substance, and cDNA microarray analysis was performed twice for each RNA sample. Genes were considered differentially expressed when logarithmic gene expression ratios in four independent hybridizations showed a twofold difference in expression. Exposure to *o*-nitrotoluene consistently affected four genes. *Cyp2j6* (an unstable cytochrome P450 isoform), *S100a4* (S100 calcium binding protein A4), which is linked to the invasive and metastatic phenotype of cancer cells, and *IL7*, which has antitumor properties, were upregulated. *Akap10*, a protein kinase A anchoring protein, was downregulated.

Iida *et al.* (2005) investigated early gene expression involved in mouse liver carcinogenesis. Mice were treated for 2 weeks with several animal carcinogens (including *o*-nitrotoluene and oxazepam) and non-carcinogens (including *p*-nitrotoluene). Female mice were treated with 1,250- or 5,000-ppm *o*-nitrotoluene, and quantitative real-time PCR and oligonucleotide microarray analysis were used to identify the gene-expression changes in the liver. Gene-expression changes from *o*-nitrotoluene occurred in only 26 genes from the 1,250-ppm dose and 33 genes from the 5,000-ppm dose; in comparison, expression of 221 genes was noted in male mice and 183 genes in female mice from oxazepam treatment. A dose-dependent loss of expression of the fragile histidine triad gene (*Fhit*) and the WW domain-containing oxidoreductase (*Wwox*) gene was seen from

o-nitrotoluene exposure. The authors noted that these genes are human tumor suppressor genes and are often lost together in many human cancers. *o*-Nitrotoluene treatment also resulted in strong up-regulation of the cell cycle genes, cyclin G1 *(Ccng1)* and p21 (*Cdkn1a*), down-regulation of the epidermal growth factor (*Egfr*) gene, down-regulation of the transcription factor early growth response 1 (*Egr1*) gene at 5,000 ppm in males only, and down-regulation of inhibin β-A (*Inhba*), a member of the TGF-β superfamily, and Jun-B oncogene (*Junb*), a negative regulator of proliferation genes. A comparison of gene expression changes between *o*-nitrotoluene and *p*-nitrotoluene revealed that after 2 weeks of treatment, there were 76 gene-expression changes in the liver of female mice treated with *o*-nitrotoluene and only 33 changes in the *p*-nitrotoluene-treated mice; only 17 of the changes were in common. The difference in gene-expression changes between male and female mice treated with 5,000-ppm *o*-nitrotoluene was investigated, with 20 gene changes reported in common among the 74 to 76 changes detected in males and females. Among these common genes were *Fhit*, *Wwox*, deoxyribonuclease IIα (*Dnase2a*), and cytokine inducible SH2-containing protein (*Cish*), all of which were down-regulated and have a role in promoting apoptosis.

Table 5-7. Gene expression in cultured cells or tissues from mice exposed to *o*-nitrotoluene

Test system (tissue)	*End point (conc. or dose)*	Results	Reference
L5178Y mouse lymphoma cells	*Cyp2j6 gene*	↑ expression	Kim *et al.* 2005
	S100a4 gene	↑ expression	
	IL7 gene	↑ expression	
	Akap10 gene	↓ expression	
$B6C3F_1$ mice – *in vivo* (liver)	fragile histidine triad gene (*Fhit*)	↓ expression	Iida *et al*. 2005
	WW domain-containing oxidoreductase gene (*Wwox*)	↓ expression	
	cyclin G1 (*Ccng1*)	↑ expression	
	p21 (Cdkn1a)	↑ expression	
	epidermal growth factor gene (Egr1)	↓ expression	
	early growth response 1 gene (Egfr)	↓ expression[a]	
	inhibin β-A gene (Inhba)	↓ expression	
	Jun-B gene (Junb)	↓ expression	

[a]In 5,000-ppm males only.

5.4 Mechanistic studies and considerations

This section discusses potential mechanisms of carcinogenicity and genotoxicity of *o*-nitrotoluene including mechanisms related to the bioactivation of *o*-nitrotoluene (discussed in Section 5.2) and studies in rodents evaluating mutations and gene expression from *o*-nitrotoluene–induced tumors.

5.4.1 Potential mechanisms of carcinogenicity and genotoxicity related to the bioactivation of o*-nitrotoluene*

The genotoxicity of the nitrotoluenes as measured by the *in vivo–in vitro* DNA repair assay in rats depends on isomer, sex, and intestinal microflora (Doolittle *et al.* 1983) (see Section 5.3.2). Of the three nitrotoluene isomers, DNA repair was induced only by *o*-nitrotoluene and only in male rats with an intact intestinal microflora. Incubation of *o*-nitrotoluene *in vitro* with hepatocytes isolated from male rats failed to induce DNA repair. [These results suggest that DNA damage and subsequent repair is dependent on metabolism, both mammalian and bacterial.]

[The activation scheme depicted in Figure 5-2 and discussed in Section 5.2 explains the observations on sex and isomer differences in the covalent binding of nitrotoluene-related material in hepatic DNA, and it also explains the sex, isomer, and intestinal flora dependency of the *in vivo–in vitro* DNA repair assay. However, there appear to be other mechanisms of activation of *o*-nitrotoluene. For example, female rats should be resistant to the hepatocarcinogenic effect of *o*-nitrotoluene if only the activation scheme in Figure 5-2 were present. In fact, while evidence for hepatocarcinogenicity in female rats was not as strong as for male rats, females did develop hepatocellular adenomas that were considered to be related to *o*-nitrotoluene administration. In addition, the significantly increased incidences of tumors in other tissues of rats or mice, including mammary gland, skin, lung, large intestine, and hemangiosarcomas in various tissues, support the concept that other activation pathways may exist.]

5.4.2 Gene expression and mutations in oncogenes, tumor suppressor genes, and other critical genes in rodents

Several studies have examined the potential molecular mechanisms underlying the development of tumors in rodents exposed to *o*-nitrotoluene. Hong *et al.* (2003) analyzed gene mutations and gene expression in hemangiosarcomas from mice exposed to *o*-nitrotoluene in the NTP 2-year bioassay, and Sills *et al.* (2004) analyzed gene mutations in colon tumors from the same chronic study. Kim *et al.* (2006) also used quantitative real-time PCR and microarray analysis to characterize the gene expression profile in mesotheliomas collected from rats treated with *o*-nitrotoluene. The major findings in these studies are summarized below. (See Section 5.3 for gene expression studies *in vitro* or from normal tissue of mice exposed to *o*-nitrotoluene).

In an attempt to evaluate the underlying molecular mechanisms for development of *o*-nitrotoluene-induced tumors, Hong *et al*. (2003) analyzed mutations in the K-*ras*, p53, and β-catenin (*Catnb)* genes and production of p53 and β-catenin protein in 15 hemangiosarcomas (from skeletal muscle, subcutaneous tissue, and mesentery) from $B6C3F_1$ mice exposed to *o*-nitrotoluene in the NTP two-year bioassay (see Section 4.2.2) and in 15 spontaneously occurring hemangiosarcomas. Protein production (indicating gene expression) was assessed by immunohistochemical staining, and mutations were

detected by sequencing. None of the spontaneous subcutaneous hemangiosarcomas tested positive for p53 or β-catenin protein expression or had mutations in the p53, β-catenin, or K-*ras* genes. In contrast, all of the *o*-nitrotoluene-induced tumors tested positive for p53 protein, and 73% (11 of 15) of the tumors had missense mutations in the p53 gene. Four tumors had double mutations in the p53 gene, for a total of 15 identified mutations, the majority of which involved G·A base pairs. Deletions in the β-catenin gene (most in exon 2 splice sites) were identified in 47% (7 of 15) of the *o*-nitrotoluene-induced hemangiosarcomas, and a point mutation in the K-*ras* gene (in codon 61) was identified in 1 tumor. Production of β-catenin protein also was detected in 47% of the *o*-nitrotoluene-induced tumors (in 6 of the 7 tumors with a deletion and 1 tumor without a deletion) (see Table 5-8). The authors concluded that the p53 and β-catenin gene mutations were likely a result of the genotoxic effect of *o*-nitrotoluene.

Table 5-8. p53, β-catenin, and K-*ras* mutations in hemangiosarcomas (subcutaneous tissue, skeletal muscle, and mesentery) from B6C3F$_1$ mice orally exposed to *o*-nitrotoluene

Treatment group, mutations	p53 (exon 6-8), number (%)[b]	Catnb[a], number (%)[b]	K-ras (codon 61), number (%)[b]
Controls	0	0	0
o-Nitrotoluene	15	8	1
Total mutations according to dose (ppm)			
1,250	1 (6.7)	1 (12.5)	–
2,500	1 (6.7)	0	–
5,000	13 (86.7)	7 (87.5)	–
Type of mutations identified			
Transitions (all)	12 (80)	1 (12.5)	0
G·C→A·T	9 (60)[c]	0	0
A·T→G·C	3 (20)	1 (12.5)	0
Transversions (all)	3 (20)	0	1 (100)
G·C→T·A	2 (13.3)	0	0
G·C→C·G	0	0	0
A·T→C·G	0	0	0
A·T→T·A	1 (6.7)	0	1 (100)
Deletions	0	7 (87.5)	0

Source: Hong *et al.* 2003.

– = Dose not given.

[a]5 deletions in exon 2 splice sites and 2 deletions in other sites, 1 base substitution in codon 28.

[b]Percent of total mutations identified, not percent of hemangiosarcomas with mutations. Fifteen mutations in *p53* were identified in 11 hemangiosarcomas (4 hemangiosarcomas had double mutations), 8 mutations in C*atnb* were identified in 7 hemangiosarcomas (1 hemangiosarcoma had a double mutation), 1 mutation in K-*ras* was identified in 1 hemangiosarcoma.

[c]6 mutations were G→A transitions and 3 mutations were C→T transitions.

Sills *et al.* (2004) characterized the molecular profile of oncogenes and tumor suppressor genes in carcinomas of the large intestine (cecum) from the same NTP two-year bioassay of *o*-nitrotoluene. In this study, eleven colon tumors from $B6C3F_1$ mice exposed to *o*-nitrotoluene were analyzed for mutations in the p53, K-*ras*, and β-catenin genes by sequencing and by detection of p53, β-catenin, cyclin D1, and adenomatous polyposis coli (APC) protein by immunohistochemical staining. Normal colon tissue was used as a control. Most of the colon tumors had increased protein levels of p53 (8 of 11), β-catenin (8 of 10), and cyclin D1 (8 of 11), but no difference in protein expression was found for APC between the colon tumors and normal colon tissue. Mutations in all three genes were identified in most of the *o*-nitrotoluene-induced tumors; mutations in the β-catenin gene (mainly in exon 2) were detected in all 11 tumors, mutations in the *p53* gene (all in exon 7) were detected in 9 of 11 tumors, and mutations in the K-*ras* gene (in codon 10 to 13) were detected in 9 of 11 tumors. All but one of the K-*ras* gene mutations (all the mutations in exons 10 and 12) were G→T transversions (Gly to Val) (see Table 5-9).

The region of the β-catenin gene where the mutations were identified in both of these studies corresponds to the region of the gene coding for critical sites for phosphorylation and ubiquitination of the β-catenin protein. Since β-catenin is important for cell adhesion, changes in β-catenin expression may lead to a loss of cell adhesion and may promote invasiveness. [The pattern of mutations in both of these studies is consistent with targeting of guanine for adduct formation since mutations in the p53 gene in hemangiosarcomas (see Table 5-7) mainly involved G·C→A·T transitions and almost all the mutations in the K-*ras* gene in cecal carcinomas (see Table 5-8) were G·C→T·A transversions.]

Table 5-9. p53, β-catenin, and K-*ras* mutations in cecal carcinomas from B6C3F$_1$ mice orally exposed to *o*-nitrotoluene

Treatment group, Mutations	p53 (exon 7), number (%)[b]	Catnb[a], number (%)[b]	K-ras (codon 10-13), number (%)[b]
Controls[c]	0	0	0
o-nitrotoluene	12	14	10
Total mutations according to dose (ppm)			
1,250	4	6	5
2,500	8	8	5
Type of mutation identified			
Transitions (all)	4 (33.3)	1 (7.1)	1 (10)
G·C→A·T	3 (25.0)	1 (7.1)	1[d] (10)
A·T→G·C	1 (8.3)	0	0
Transversions (all)	8 (66.7)	3 (21.4)	9 (90)
G·C→T·A	2 (16.7)	0	9[e] (90)
G·C→C·G	0	1 (7.1)	0
A·T→C·G	0	0	0
A·T→T·A	6 (50)	2 (14.3)	0
Deletions	0	10 (71.4)	0

Source: Sills *et al.* 2004.

[a] 10 deletions occurred in exon 2 and/or 5, and 4 base-pair substitutions occurred in codons 15, 25, 37, and 41.

[b] Percent of total mutations identified, not percent of cecal carcinomas with mutations. Twelve mutations in *p53* were identified in 9 carcinomas (3 carcinomas had double mutations), 14 mutations in *Catnb* were identified in 11 carcinomas (3 carcinomas had double mutations), 10 mutation in K-*ras* were identified in 9 carcinomas (1 carcinomas had a double mutation).

[c] No cecal carcinomas occurred in control animals, so normal colon tissue from non-treated mice or non-tumor regions from mice exposed to *o*-nitrotoluene was used as controls.

[d] Codon 13.

[e] 1 mutation in codon 10 and 8 mutations in codon 12.

Table 5-10. Gene mutations and gene expression in *o*-nitrotoluene-induced tumors in rodents.

Test system (species and tumor type)	End point (conc. or dose)	Results	Reference
$B6C3F_1$ mice (hemangiosarcomas)	p53 gene mutation	+	Hong *et al.* 2003
	p53 protein	+	
	β-catenin gene mutation	+	
	β-catenin protein	+	
	K-*ras* gene mutation	–[a]	
$B6C3F_1$ mice (colon tumors)	p53 gene mutation	+	Sills *et al.* 2004
	p53 protein	+	
	β-catenin gene mutation	+	
	β-catenin protein	+	
	K-*ras* gene mutation	+	
	cyclin D1 protein	+	
	APC protein	–	
F344 rats (peritoneal mesotheliomas)	*IGF-1 signaling pathway* akt2, igf-1, igfb2, igfbp3, igfbp6, prkcz	↑ expression	Kim *et al.* 2006
	fos	↓ expression	
	P38 MAPK pathway mapkapk2, stat1, tgfbr2	↑ expression	
	tgfb2, tgfb3, tnfrsf6	↓ expression	
	Wnt/β-catenin pathway akt2, ppp2r2, tgfbr2, wnt4	↑ expression	
	fzd2, gnaq, ilk, tgfb2, tgfb3	↓ expression	
	Integrin pathway itgb2, akt2	↑ expression	
	actn1, actn4, itga8, itgb1, actg2, arf4, colla2	↓ expression	

+ = positive; – = negative
[a] one mutation was detected

Sills *et al.* (2004) discussed the similarity between the pathways affected by these genes and protein products and the pathways that have been described in the development of human colon cancer. Human colorectal cancers also have a high frequency of mutations

in the K-*ras* and p53 genes, and the β-catenin and cyclin D1 genes are upregulated. As a result of these genetic effects, both human and mouse colon tumors have alterations in pathways that are considered important for the progression of cells from a normal state to cancer; these pathways include the β-catenin/Wnt signaling pathway, *ras*/MAP kinase pathway, and cell-cycle checkpoint genes (e.g., the cyclin D1 and p53 genes). Specifically, these pathways may interact in the formation of large-intestine tumors to provide self-sufficiency in growth signaling through the K-*ras* oncogene, upregulation of growth and avoidance of apoptosis through the p53 gene, and increased cyclin D1 production through the combination of β-catenin and *ras* activation. Thus, the authors concluded that the acquisition of similar genetic alterations in both human and mouse large-intestinal cells suggests that the chemically induced tumors in mice model the human cancer quite well, and the results are likely to be relevant to humans.

Kim *et al.* (2006) investigated the genes involved in peritoneal mesotheliomas induced in male F344 rats by exposure to *o*-nitrotoluene or bromochloroacetic acid. Mesotheliomas were collected from four rats, RNA was isolated, and gene expression analysis was conducted using Oligo arrays, with over 20,000 target genes, and real-time PCR. Gene expression was also analyzed in non-transformed mesothelial cell lines that were prepared from the peritoneal (Fred-PE) and pleural (Fred-PL) cavities of normal male F344 rats. Fred-PE of passage 8 was used as a source of reference RNA. Signature genes were defined as those with > 1.5-fold change in expression (tumor/Fred-PE) and a significance level of $P = 0.001$ throughout all replicates and were analyzed further. There were 1,298 transcripts that were differentially expressed in tumor tissue. About 39% (507) of these were increased and 61% (791) were decreased compared to the Fred-PE. Further analysis identified 169 cancer-related genes that were categorized according to cancer-related function (i.e., binding activity, growth and proliferation, cell-cycle progression, apoptosis, and invasion and metastasis). The major carcinogenic pathways involved in peritoneal mesothelioma formation were identified as insulin-like growth factor 1 (IGF-1), p38 MAP kinase, Wnt/β-catenin, and integrin signaling pathways. The authors noted that similar signaling pathways were activated in studies of human mesotheliomas and mesothelioma cell lines.

The results of the studies by Hong *et al.* (2003), Sills *et al.* (2004) and Kim *et al*. (2006) are summarized in Table 5-10.

5.5 Carcinogenicity and genotoxicity of *o*-nitrotoluene analogues and metabolites

A (1992) NTP study included comparative toxicity evaluations of the *o*-, *m*-, and *p*-nitrotoluene isomers in rats and mice. The NTP conducted a two-year carcinogenicity study of rats and mice exposed to *p*-nitrotoluene; however, no studies describing chronic exposure to *m*-nitrotoluene were found. A great deal of research also has been done on the metabolism, mutagenicity, and carcinogenicity of the dinitrotoluenes. The NTP has studied one metabolite of *o*-nitrotoluene, *o*-toluidine (as its hydrochloride), in a subchronic exposure study in male rats. This section briefly discusses the findings of these studies of *o*-nitrotoluene analogues and metabolites.

5.5.1 p-*Nitrotoluene*

Rats and mice exposed to *p*-nitrotoluene in the diet for two years had increased incidences of tumors in a number of organs and systems (e.g., clitoral gland, skin, hematopoietic system or spleen, testis, and lung) (NTP 2002a). However, most of the increased incidences either were not significantly higher than the incidences in concurrent or historical controls or were inconsistent among exposure groups. The NTP concluded that there was equivocal evidence of carcinogenic activity of *p*-nitrotoluene in male F344/N rats, based on increased incidences of subcutaneous skin neoplasia, and some evidence of carcinogenic activity in female F344/N rats, based on increased incidences of clitoral-gland neoplasia (see Table 5-11). There was equivocal evidence of carcinogenic activity of *p*-nitrotoluene in male $B6C3F_1$ mice, based on increased incidences of alveolar/bronchiolar neoplasia, and no evidence of carcinogenicity in female mice.

Table 5-11. Neoplastic lesions identified in rats and mice exposed to *o*-nitrotoluene and *p*-nitrotoluene

Organ or system	*o*-Nitrotoluene		*p*-Nitrotoluene	
	F344/N rats	B6C3F$_1$ mice	F344/N rats	B6C3F$_1$ mice
Mesothelium	✓			
Skin (subcutaneous)	✓		✓	
Mammary gland	✓			
Liver	✓	✓		
Lung	✓			✓
Large intestine (cecum)		✓		
Circulatory system		✓		
Clitoral gland			✓	

Source: NTP 2002a,b.

The NTP also concluded that *o*-nitrotoluene had greater carcinogenic potential than *p*-nitrotoluene (*o*-nitrotoluene was tested in male and female rats at 625, 1,250, and 2,000 ppm in the diet, and *p*-nitrotoluene was tested at 1,250, 2,500, and 5,000 ppm; male and female mice were exposed to 1,250, 2,500, and 5,000 ppm in the diet for both isomers). The authors noted that this was predicted from studies showing that covalent binding of *o*-nitrotoluene to total rat hepatic macromolecules was 3.5 times higher than that of *p*-nitrotoluene and that *o*-nitrotoluene, but not *p*-nitrotoluene, also binds to male F344 rat hepatic DNA (see Section 5.2). In addition, quantitative differences in metabolism of *o*-nitrotoluene and *p*-nitrotoluene have been observed, such as greater metabolism of *o*-nitrotoluene to the nitrobenzyl glucuronide, which is thought to give rise to *o*-aminobenzyl sulfate, the proximal reactive metabolite (see Section 5.2 and Figure 5-2) (NTP 2002a).

Additionally, aromatic amine carcinogens upon metabolic activation yield electrophilic intermediates that bind to DNA yielding *N*-(deoxyguanosin-8-yl)arylamines (Marques *et al.* 1997). DNA binding studies have suggested that while *o*-, *m*-, and *p*-substituted arylamines all bind to DNA, the substitution in the ortho position yields a more stable DNA adduct (Marques *et al.* 1997). The Marques *et al.* study did not look at the comparative stability of the *o*-, *m*-, and *p*-nitrotoluene DNA adducts. However, the results

suggest that further studies on the stability of *o*-, *m*-, and *p*-nitrotoluene DNA adducts are warranted and may help to explain why *o*-nitrotoluene is a more potent chemical carcinogen than *p*-nitrotoluene.

5.5.2 Dinitrotoluenes

In 1984 Rickert *et al.* reviewed the metabolism, mutagenicity, and carcinogenicity of dinitrotoluenes. At the time of their review, three independent chronic bioassays, all of which were feeding studies, had been completed: (1) a National Cancer Institute (NCI) (1978) study of 2,4-dinitrotoluene in F344 rats and B6C3F$_1$ mice, (2) a Chemical Industry Institute of Toxicology (CIIT) study of technical-grade dinitrotoluene in F344 rats, and (3) a U.S. Army-funded (conducted at Midwest Research Institute) study of 2,4-dinitroluene in Sprague-Dawley rats and Swiss mice.

In the NCI study, 2,4-dinitrotoluene (95% pure, contaminants not specified) was administered in the feed at time-weighted average dietary concentrations of 0.02% and 0.008% for 50 male and 50 female F344 rats and at 0.04% and 0.008% for 50 male and 50 female B6C3F$_1$ mice for 78 weeks followed by an additional 26 weeks of observation for rats and 13 weeks for mice. In male rats, a significantly increased incidence of fibroma of the skin and subcutaneous tissue occurred in both dose groups, and a statistically significant incidence of fibroadenoma of the mammary gland occurred in the high-dose female rats. No tumors associated with 2,4-dinitrotoluene exposure were reported in mice.

In the CIIT study, male and female F344 rats (10 per exposure group) were administered technical-grade dinitrotoluene (a mixture containing 2,4-, 2,6-, 3,4-, 2,3-, 2,5- and 3,5-dinitrotoluene at 76.4%, 18.8%, 2.4%, 1.5%, 0.7%, and < 0.1%, respectively) at 0, 3.5, 14, and 35 mg/kg/day for 2 years. High incidences of hepatic neoplasms (hepatocellular carcinomas or neoplastic nodules) were observed in high- and mid-dose males and females. Technical-grade dinitrotoluene also induced cholangiocellular carcinoma in both sexes.

In the third study (U.S. Army funded) reviewed by Rickert *et al.*, a mixture of 98% 2,4- and 2% 2,6-dinitrotoluene was administered in the feed to Sprague-Dawley rats and

Swiss mice for 2 years. In rats, the average intake of 2,4-dinitrotoluene (mg/kg/day) was 0.575, 3.92, or 34.5 for males and 0.706, 5.14, or 45.3 for females. High-dose female rats had significantly increased incidences of hepatocellular carcinoma. In mice, the average intake of 2,4-dinitrotoluene (mg/kg/day) was 13.3, 96.9, or 885 for males and 13.7, 93.8, or 911 for females. An increase in kidney tumors was reported in mid-dose males. It was noted that high-dose males had early deaths.

Initiation-promotion studies in male F344 rats have demonstrated that technical-grade dinitrotoluene and 2,4-dinitrotoluene have initiating and promoting activity in the rat liver, whereas 2,6-dinitrotoluene had only promoting activity (reviewed by Rickert *et al.* 1984b). Leonard *et al*. (1987) noted that 2,4-dinitrotoluene was weakly hepatocarcinogenic in Sprague-Dawley rats, and did not induce hepatic tumors in F344 rats in the NCI study. However, technical-grade dinitrotoluene (which contained 5 to 10 times more of the 2,6-isomer than the 2,4-isomer) caused a 100% liver tumor incidence by 55 weeks in F344 rats in the CIIT study. Because of the contrasting results in the three chronic bioassays, they conducted a feeding study in F344 rats to compare the carcinogenicity activity of technical-grade dinitrotoluene (a mixture of 76.5% 2,4-dinitrotoluene and 18% 2,6-dinitrotoluene), and the two purified isomers (2,4- and 2,6-dinitrotoluene). Rats were fed diets containing the dinitrotoluenes resulting in average intakes (mg/kg/day) of 27 for 2,4-dinitrotoluene, 7 or 14 for 2,6-dinitrotoluene, and 35 for technical-grade dinitrotoluene for 52 weeks. At 52 weeks, hepatocellular carcinomas were observed in the 2,6-dinitroluene-treated animals (100% of high-dose and 85% of low-dose rats) and technical-grade dinitrotoluene-treated animals (47%) but not the 2,4-dinitrotoluene-treated animals.

In addition to the chronic bioassays, 2,4- and 2,6-dinitrotoluene were tested in short-term bioassays for their ability to produce pulmonary tumors. Both compounds were inactive by oral or intraperitoneal administration in strain A (Schut *et al.* 1982, Schut *et al.* 1983) and A/J mice (Stoner *et al.* 1984).

Mutagenicity studies of the dinitrotoluenes in bacterial and yeast systems showed activity for all the isomers (Rickert *et al.* 1984b). The increased activity of putative reduced

metabolites of the dinitrotoluenes together with the inactivity in systems lacking nitroreductase suggests that nitroreduction is necessary for mutagenic activity. Studies in *in vitro* mammalian systems gave uniformly negative results for all isomers of dinitrotoluene, suggesting that extrahepatic metabolism was necessary for activation of these compounds. Studies in the *in vivo–in vitro* rat hepatocyte DNA repair assay yielded results analogous to those obtained by Doolittle *et al.* (1983) for *o*-nitrotoluene (Section 5.3.2). The majority of the activity to induce DNA repair resided with 2,6-dinitrotoluene; the activity was greater in male than in female rats and depended upon an intact intestinal microflora.

The metabolism of 2,6-dinitrotoluene and related isomers is also analogous to that of *o*-nitrotoluene and related isomers (Rickert *et al.* 1984b). Activation of 2,6-dinitrotoluene is thought to involve oxidation of the methyl group to an alcohol, followed by conjugation with glucuronic acid and excretion in bile. As with *o*-nitrotoluene, male rats excrete more of a dose of 2,6-dinitrotoluene in the bile as 2,6-dinitrobenzyl glucuronide than do females. Once in the intestine, the glucuronic acid moiety is cleaved, and one of the nitro groups is reduced before reabsorption. The final activation may involve sulfation at the benzyl alcohol group or via *N*-hydroxylamine formation (Chism and Rickert 1989).

Several studies have investigated the potential for dinitrotoluenes to cause cancer in human subjects. In the first of these (Levine *et al.* 1986), no evidence for a carcinogenic effect was found in ~ 450 workers at two ammunition plants who were exposed to dinitrotoluenes (76% to 98% 2,4-dinitrotoluene and 1% to 19% 2,6-dinitrotoluene, depending on the process stage); however the numbers of observed deaths for specific tissue sites were small. A later study (Stayner *et al.* 1993), which examined nearly 5,000 workers (some of which were part of the Levine *et al.* study) at a munitions factory exposed to technical-grade dinitrotoluenes (76% 2,4-dinitrotoluene, 19% 2,6-dinitrotoluene, and 5% of the remaining four isomers), demonstrated an excess of hepatobiliary cancer in exposed workers (standardized rate ratio = 3.88, 95% CI = 1.04 to 14.41) compared to 7,500 unexposed workers. No exposure-response relationship was demonstrated in this study; the authors noted a lack of workers with long durations of

exposure and a lack of quantitative exposure information as possible reasons for this. A smaller study (Brüning *et al.* 1999, Brüning *et al.* 2002) of 500 underground miners exposed to dinitrotoluenes in the explosive Donarit, which contains 30% technical-grade dinitrotoluene, found a 4.5-fold increased incidence of urothelial cancer (6 cases) and 14.3-fold increased incidence of renal-cell cancer (14 cases). These data suggest the possibility of carcinogenicity of dinitrotoluenes in humans.

5.5.3 o-*Toluidine hydrochloride*

o-Toluidine (*o*-aminotoluene) hydrochloride administered in feed to F344 rats and $B6C3F_1$ mice for 101 to 104 weeks (NCI 1979) increased the incidences of mesothelioma of the abdominal cavity or scrotum in male rats, transitional-cell carcinoma of the urinary bladder in female rats, and several types of sarcoma in both male and female rats. In mice, hemangiosarcoma was induced at various sites in males, and hepatocellular carcinoma or adenoma was induced in females. The NTP concluded that *o*-toluidine hydrochloride was carcinogenic in both rats and mice, producing a significantly increased incidence of one or more types of neoplasia.

In a later study (NTP 1996), the toxicity of *o*-nitrotoluene and *o*-toluidine hydrochloride administered in feed at approximately equimolar doses (5,000 ppm) for 13 or 26 weeks to male F344/N rats was examined because of the structural similarity of these two molecules. Both *o*-nitrotoluene and *o*-toluidine hydrochloride caused mesothelial hyperplasia and mesothelioma in male rats after 13 or 26 weeks of dietary exposure. However, the incidence of mesothelioma was greater and the latency was less in rats administered *o*-nitrotoluene than in rats administered *o*-toluidine hydrochloride. The two chemicals had similar toxic effects on the spleen; however, their morphologic effects on the testis, epididymis, liver, kidney, and urinary bladder differed.

In an epidemiological study, Ward *et al.* (1991) evaluated the incidence of bladder cancer in a chemical plant where the workers were exposed to two potential bladder carcinogens, *o*-toluidine and aniline. An excess number of bladder cancer cases were observed (7 cases observed versus 1.08 expected) among all the 1,749 workers in the plant (standardized incidence ratio [SIR] = 3.60, 90% CI = 2.13 to 5.73). Increased risk of bladder cancer

was strongly associated with increased length of employment in the departments where *o*-toluidine and aniline were used. *o*-Toluidine and its hydrochloride salt are listed in the Report on Carcinogens as *reasonably anticipated to be a human carcinogen* based on limited evidence in humans and sufficient evidence in animals.

5.6 Toxicity

All three isomers of nitrotoluene caused kidney toxicity in male rats, characterized by hyaline droplet nephropathy. Exposure to *o*- or *p*-nitrotoluene also increased renal α_{2u}-globulin levels in male rats (the *m*-isomer was not tested) (NTP 1992, Dunnick *et al.* 1994, NTP 1996). As discussed in Section 4.1, *o*-nitrotoluene also produced hepatotoxicity in male rats, characterized by hepatocyte vacuolization, oval-cell hyperplasia, PGST+ foci, and increased serum bile acids, sorbitol dehydrogenase, and alanine aminotransferase. *o*-Nitrotoluene also resulted in hematopoiesis and pigmentation in the spleen in male and female rats exposed for both 13 weeks and 2 years (NTP 1996, 2002b).

5.7 Summary

5.7.1 Absorption, distribution, metabolism, and excretion

o-Nitrotoluene has been shown to be absorbed after oral administration to rats and mice, and indirect evidence suggests that absorption also occurs in humans exposed to *o*-nitrotoluene. Metabolites of *o*-nitrotoluene,which include *o*-nitrobenzoic acid and *o*-nitrobenzyl alcohol, have been detected in the urine of factory workers and indicate that absorption occurs from skin contact and inhalation. The half-life of *o*-nitrotoluene in plasma of rats is fairly short, approximately 1.5 hours, and the primary route of excretion is urinary, occurring mainly in the first 24 hours after exposure. *o*-Nitrobenzoic acid and *o*-nitrobenzyl glucuronide are major metabolites of *o*-nitrotoluene in both rats and mice, but other metabolites, such as *S*-(*o*-nitrobenzyl)-*N*-acetylcysteine and *o*-aminobenzyl alcohol, are found only in rats. Excretion of *o*-nitrotoluene metabolites also occurs through feces and expired air.

5.7.2 Bioactivation of o*-nitrotoluene*

Deleterious effects of *o*-nitrotoluene generally are more severe in male rats than in females and include changes in hepatic, renal, or splenic histopathology, tumor incidence,

macromolecular covalent binding, or DNA repair. The toxicity of *o*-nitrotoluene, particularly in male rats, likely involves its metabolism by oxidation of the methyl group to an alcohol, conjugation of *o*-nitrobenzyl alcohol with glucuronic acid and excretion in bile, deconjugation of *o*-nitrobenzyl glucuronide and reduction of the nitro group by intestinal bacteria, and final activation of *o*-aminobenzyl alcohol by the formation of *o*-aminobenzyl sulfate. This activation scheme adequately explains the observations on sex and isomer differences in the covalent binding of nitrotoluene-related material in hepatic DNA, and it also explains the sex, isomer, and intestinal flora dependency of the *in vivo–in vitro* DNA repair assay. In rats exposed to *o*-nitrotoluene *in vivo*, DNA adducts and increased DNA repair were detected in males but not females.

5.7.3 Genetic damage and related effects

o-Nitrotoluene did not cause mutations in prokaryotic systems and did not induce chromosomal aberrations in CHO cells or DNA repair in rat or human hepatocytes, and rat spermatocytes or spermatids *in vitro*. However, the compound induced SCE in CHO cells *in vitro*. [The relative lack of genotoxic effects of *o*-nitrotoluene in *in vitro* test systems is likely consistent with the need for metabolism by both mammalian and bacterial enzymes.]

Gene expression studies with mouse L5178Y $Tk^{+/-}$ lymphoma cells *in vitro* identified four genes whose expression was consistently affected by *o*-nitrotoluene. A study of early changes in gene expression in mouse liver after exposure to *o*-nitrotoluene *in vivo* for 2 weeks identified changes in tumor suppressor genes, cell-cycle genes, and apoptosis-promoting genes.

5.7.4 Mechanistic studies and considerations

[The activation of *o*-nitrotoluene to *o*-aminobenzyl sulfate by a combination of mammalian and bacterial metabolism appears adequate to explain the carcinogenicity of *o*-nitrotoluene in male rats; however, based on results of chronic bioassay studies in male and female rats and male and female mice, there appear to be other mechanisms of activation of *o*-nitrotoluene. For example, female rats should be resistant to the hepatocarcinogenic effect of *o*-nitrotoluene if only the activation scheme illustrated in

Figure 5-2 were present; however, females did develop hepatocellular adenomas that were considered to be related to *o*-nitrotoluene administration. In addition, the significantly increased incidences of tumors in other tissues of rats or mice, including mammary gland, skin, lung, large intestine, and hemangiosarcomas in various tissues support the concept that other activation pathways may exist.] Mutations in the p53 and β-catenin genes and production of these proteins were detected in hemangiosarcomas and colon tumors from mice exposed to *o*-nitrotoluene; K-*ras* gene mutations and cyclin D1 protein production also were detected in the colon tumors. Mutations in p53, β-catenin genes and K-*ras* may be a result of the genotoxic effects of *o*-nitrotoluene. The pattern of mutations is consistent with targeting of guanine for adduct formation since mutations in the p53 gene in hemangiosarcomas mainly involved G·C→A·T transitions, and almost all the mutations in the K-*ras* gene in cecal carcinomas were G·C→T·A transversions. Human colorectal cancers also have a high frequency of mutations in the K-*ras* and p53 genes, and the β-catenin and cyclin D1 genes are upregulated. As a result of these genetic effects, both human and mouse colon tumors have alterations in pathways that are considered important for the progression of cells from a normal state to cancer; these pathways include the β-catenin/Wnt signaling pathway, *ras*/MAP kinase pathway, and cell-cycle checkpoint genes (e.g., the cyclin D1 and p53 genes). Major carcinogenic pathways involved in peritoneal mesothelioma formation in mice exposed to *o*-nitrotoluene also were similar to pathways activated in human mesotheliomas, including IGF-1, p38 MAPK, Wnt/B-catenin, and integrin signaling pathways.

5.7.5 Carcinogenicity and genotoxicity of o*-nitrotoluene analogues and metabolites*

The NTP also conducted cancer studies in experimental animals on another nitrotoluene isomer, *p*-nitrotoluene and found *equivocal evidence of carcinogenic a*ctivity in male rats and male mice, *some evidence of carcinogenicity* in female rats and *no evidence of carcinogenic activity* in female mice. The NTP concluded that *o*-nitrotoluene had greater carcinogenic potential than *p*-nitrotoluene and that the differences in carcinogenicity potential between the two isomers may be due to (1) greater stability of the ortho adduct, (2) higher covalent binding of *o*-nitrotoluene to rat hepatic macromolecules, or (3) greater

metabolism of *o*-nitrotoluene to the nitrobenzyl glucuronide, which gives rise to *o*-aminobenzyl sulfate, the proposed proximal reactive metabolite.

5.7.6 Toxicity

o-Nitrotoluene caused toxic effects in the kidney (hyaline droplet nephropathy and increased renal α_{2u}-globulin levels) and liver (hepatocyte vacuolization, oval-cell hyperplasia, PGST+ foci, and others) in male rats and in the spleen (hematopoiesis and pigmentation) in female rats.

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Glossary of Terms

Adenoma: An ordinarily benign neoplasm of epithelial tissue in which the tumor cells form glands or gland-like structures in the stroma.

Autoignition temperature: The minimum temperature required to cause self-sustained combustion without any other source of heat.

Boiling point: The boiling point of the anhydrous substance at atmospheric pressure (101.3 kPa) unless a different pressure is stated. If the substance decomposes below or at the boiling point, this is noted (dec). The temperature is rounded off to the nearest °C.

Carcinoma: Any of the various types of malignant neoplasms derived from epithelial tissue in several sites.

Cholangiocarcinoma: An adenocarcinoma, primarily in intrahepatic bile ducts, composed of ducts lined by cuboidal or columnar cells that do not contain bile.

Covalent binding: A bond in which each atom of a bound pair contributes one electron to form a pair of electrons.

Critical temperature: The temperature at and above which a gas cannot be liquefied, no matter how much pressure is applied.

Density: The density for solids and liquids is expressed in grams per cubic centimeter (g/cm^3) and is generally assumed to refer to temperatures near room temperature unless otherwise stated. Values for gases are generally the calculated ideal gas densities in grams per liter at 25°C and 101.325 kPa.

Diazo-positive compounds: Non-specific markers of exposure to aromatic amines.

Fibroma: A benign neoplasm derived from fibrous connective tissue.

Flash point: The lowest temperature at atmospheric pressure (101.3 kPa) at which a liquid gives off so much combustible vapor at the liquid surface that this vapor, when mixed intimately with air, can be ignited by a flame or spark.

Half-life: The time required for one half of a given material to undergo chemical reactions.

Isomer: One of two or more chemical substances having the same elementary percentage composition and molecular weight but differing in structure.

Hemangiosarcoma: A malignant tumor characterized by rapidly proliferating cells derived from the blood vessels and lining irregular blood-filled spaces.

Henry's Law constant at 25°C: The ratio of the aqueous-phase concentration of a chemical to its equilibrium partial pressure in the gas phase. The larger the Henry's law constant the less soluble it is (greater tendency for vapor phase).

Hepatocyte: A parenchymal liver cell.

Hyperplasia: An increase in the number of cells in a tissue or organ, excluding tumor formation.

Inventory Update Rule (IUR): The purpose of the Inventory Update Rule is to assist EPA in keeping an inventory of chemical substances in commerce in the United States. Initially, the rule required any company that produced or imported any chemical in the TSCA Chemical Substances Inventory List (TSCA Inventory) at a quantity of 10,000 pounds or more to report to EPA some basic information on that chemical. Reporting was required every four years. Amendments to the rule enacted in 2003 raised the threshold limit to 25,000 pounds and increased the reporting period to every 5 years.

K_{oc}: Soil organic adsorption coefficient, which is calculated as the ratio of the concentration of a chemical adsorbed to the organic matter component of soil or sediment to that in the aqueous phase at equilibrium.

Lipoma: A benign neoplasm of adipose tissue, comprised of mature fat cells.

Lipophilic: Having a strong affinity for fats.

Log octanol-water partition coefficient (log K_{ow}): The ratio of concentrations of a substance in octanol and in water, when dissolved in a mixture of octanol and water. For convenience, the logarithm of K_{ow} is used. The octanol/water partition coefficient of a substance is useful as a means to predict soil adsorption, biological uptake, lipophilic storage, and bioconcentration.

Melting point: The melting point of the substance at atmospheric pressure (101.3 kPa). When there is a significant difference between the melting point and the freezing point, a range is given. In case of hydrated substances (i.e., those with crystal water), the apparent melting point is given. If the substance decomposes at or below its melting point, this is noted (dec). The temperature is rounded off to the nearest °C.

Mesothelioma: A neoplasm derived from the lining cells of the pleura and peritoneum that is composed of either epithelial-like cells, spindle cells, or both.

Methemoglobin: A compound formed from hemoglobin by oxidation of the iron atom from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state with essentially ionic bonds, rendering it incapable of functioning reversibly as an oxygen carrier. Methemoglobin is present in small amounts in blood normally, but injury or toxic agents can increase the conversion.

Molecular weight: The molecular weight of a substance is the weight in atomic mass units of all the atoms in a given formula. The value is rounded to the nearest tenth.

Neoplasm: Tumor.

Negative log acid dissociation constant (pK_a): A measure of the degree to which an acid dissociates in water (a measurement of acid strength). The pKa is the negative logarithm (to the base 10) of the acid dissociation constant (Ka); the lower the pKa, the stronger the acid.

Norharman: A co-mutagen found in tobacco tar and in pyrolysate of tryptophan**.**

Perched aquifer: An aquifer that has a confining layer below the groundwater and sits above the main water table.

Physical state: Substances may either be gases, liquids, or solids according to their melting and boiling points. Solids may be described variously as amorphous, powders, pellets, flakes, lumps, or crystalline; and the shape of the crystals is specified if available. Solids also may be described as hygroscopic or deliquescent depending upon their affinity for water.

Pleomorphic: Occurring in various distinct forms.

Poly-3 test: Poly-3 test: A survival-adjusted statistical test that takes survival differences into account by modifying the denominator in the numerical (quantal) estimate of lesion incidence to reflect more closely the total number of animal years at risk. For analysis of a given tumor site, each animal is assigned either (1) a risk weight of one if the animal had a lesion at that site or if it survived until terminal sacrifice or (2) a risk weight that is the fraction of the entire study time that it survived, raised to the 3rd power, if the animal died prior to terminal sacrifice and did not have a lesion at that site. The resulting test is similar to the Cochran-Armitage trend test, with the adjusted tumor rates replacing the observed tumor rates in the test statistic (Portier and Bailer 1989, Bieler and Williams 1993).

Specific gravity: the ratio of the density of a material to the density of a standard material, such as water at a specified temperature; when two temperatures are specified, the first is the temperature of the material and the second is the temperature of water.

Subcutaneous: Beneath the skin.

Tunica vaginalis: The serous membrane covering the front and sides of the testis and epididymis.

Ubiquitination: Post-translational modification of a protein by covalent attachment of one or more ubiquitin monomers, which functions to mark the protein for proteolytic destruction.

Vapor density, relative: A value that indicates how many times a gas (or vapor) is heavier than air at the same temperature. If the substance is a liquid or solid, the value applies only to the vapor formed from the boiling liquid.

Vapor pressure: The pressure of the vapor over a liquid (and some solids) at equilibrium, usually expressed as mm Hg at a specific temperature (°C).