EPA/635/R-07/001
www.epa.gov/iris

TOXICOLOGICAL REVIEW

OF

NITROBENZENE

(CAS No. 98-95-3)

In Support of Summary Information on the Integrated Risk Information System (IRIS)

March 20, 2007

NOTICE

This document is an internal review draft. It has not been formally released by the U.S. Environmental Protection Agency and should not at this stage be construed to represent Agency position on this chemical. It is being circulated for review of its technical accuracy and science policy implications.

U.S. Environmental Protection Agency
Washington, DC

DISCLAIMER

This document is a preliminary draft for review purposes only and does not constitute U.S. Environmental Protection Agency policy. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

CONTENTS —TOXICOLOGICAL REVIEW for

NITROBENZENE (CAS No. 98-95-3)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

AGMK	African green monkey kidney cells
AIC	Akaike Information Criterion
AP	Alkaline phosphatase
ATSDR	Agency for Toxic Substances and Disease Registry
BBMV	Brush border membrane vesicles
BMC	Benchmark concentration
BMCL	95% Lower bound on BMC
BMD	Benchmark dose
BMDL	95% Lower bound on BMD
BMDS	BMD software
BMR	Benchmark response
BRRC	Bushy Run Research Center
BUN	Blood urea nitrogen
CA	Chromosomal aberration
CASRN	Chemical Abstracts Service Registry Number
CIIT	Chemical Industry Institute of Toxicology
con A	Concanavalin A
CREST	Syndrome of calcinosis, Raynaud phenomenon, esophageal motility disorders, sclerodactyly, and telangiectasia
DMPO	5,5-Dimethyl-1-pyrroline-1-oxide
DMSO	Dimethyl sulfoxide
EC	Enzyme Commission (only in combination with numbers, e.g., EC 1.6.99.1)
EC_{XY}	Effective concentration (with subscript indicating point of reference)
EPA	Environmental Protection Agency
ER	Extra risk
ESR	Electron spin resonance
FasL	Fas ligand
GD	Gestation day
GLP	Good Laboratory Practice
GSH	Reduced glutathione
GSSG	Oxidized glutathione dimer
Hb	Hemoglobin
Hct	Hematocrit
HEC	Human equivalent concentration
HSDB	Hazardous Substances Data Bank
IARC	International Agency for Research on Cancer
IgG	Immunoglobulin G
IgM	Immunoglobulin M
i.p.	Intraperitoneal
IPCS	International Programme on Chemical Safety
IRIS	Integrated Risk Information System
IUBMB	International Union for Biochemistry and Molecular Biology
IUR	Inhalation unit risk

KB	Human epidermoid carcinoma cell line
KLH	Keyhole limpet hemocyanin
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
LEC	95% Lower bound of EC
LOAEL	Lowest-observed-adverse-effect level
LPS	Lipopolysaccharide
MCHb	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
metHb	Methemoglobin
NOAEL	No-observed-adverse-effect level
NRC	National Research Council
NTP	National Toxicology Program
OECD	Organization for Economic Cooperation and Development
oxyHb	Oxyhemoglobin
PHA	Phytohemagglutinin
PLN	Popliteal lymph node
PND	Postnatal day
POD	Point of departure
RBC	Red blood cell
RfC	Reference concentration
RfD	Reference dose
S9	9000g microsomal supernatant fraction
SCE	Sister chromatid exchange
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
UDS	Unscheduled DNA synthesis
UF	Uncertainty factor
WBC	White blood cell
WHO	World Health Organization

FOREWORD

The purpose of this Toxicological Review is to provide scientific support and rationale for the hazard and dose-response assessment in IRIS pertaining to chronic exposure to nitrobenzene. It is not intended to be a comprehensive treatise on the chemical or toxicological nature of nitrobenzene.

In Section 6, *Major Conclusions in the Characterization of Hazard and Dose Response,* EPA has characterized its overall confidence in the quantitative and qualitative aspects of hazard and dose response by addressing knowledge gaps, uncertainties, quality of data, and scientific controversies. The discussion is intended to convey the limitations of the assessment and to aid and guide the risk assessor in the ensuing steps of the risk assessment process.

For other general information about this assessment or other questions relating to IRIS, the reader is referred to EPA's IRIS Hotline at 202-566-1676

AUTHORS, CONTRIBUTORS, AND REVIEWERS

CHEMICAL MANAGER/AUTHOR

Todd Stedeford, Ph.D., J.D., DABT
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC

AUTHORS

Ching-Hung Hsu, Ph.D., DABT
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC

Jeanmarie Zodrow, Ph.D.
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC

Belinda Hawkins, Ph.D., DABT
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC

Karen Hogan
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC

Ted Berner, M.S.
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC

George Holdsworth, Ph.D.
Oak Ridge Institute for Science and Education
Oak Ridge Associated Universities
Oak Ridge, TN

Lutz W. D. Weber, Ph.D., DABT
Oak Ridge Institute for Science and Education
Oak Ridge Associated Universities
Oak Ridge, TN

REVIEWERS

This document has been peer reviewed by EPA scientists.

INTERNAL PEER CONSULTATION REVIEWERS

Lynn Flowers, Ph.D., DABT
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC

Karen Hammerstrom
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC

James W. Holder, Ph.D.
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC

Channa Kesheva, Ph.D. (Section 4.4.5)
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC

John Whalan, (Section 5.2)
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC

1. INTRODUCTION

This document presents background information and justification for the Integrated Risk Information System (IRIS) Summary of the hazard and dose-response assessment of nitrobenzene. IRIS Summaries may include an oral reference dose (RfD), inhalation reference concentration (RfC) and a carcinogenicity assessment.

The RfD and RfC provide quantitative information for use in risk assessments for health effects known or assumed to be produced through a nonlinear (possibly threshold) mode of action. The RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime The inhalation RfC is analogous to the oral RfD but provides a continuous inhalation exposure estimate. The inhalation RfC considers toxic effects for the respiratory system (portal of entry) and effects peripheral to the respiratory system (extrarespiratory or systemic effects). Reference values may also be derived for acute (≤24 hours), short term (>24 hours up to 30 days), and subchronic (>30 days up to approximately 10% of the life span), all of which are derived based on an assumption of continuous exposure throughout the duration specified.

The carcinogenicity assessment provides information on the carcinogenic hazard potential of the substance in question and quantitative estimates of risk from oral and inhalation exposure. The information includes a weight-of-evidence judgment of the likelihood that the agent is a human carcinogen and the conditions under which the carcinogenic effects may be expressed. Quantitative risk estimates are derived from the application of a low-dose extrapolation procedure and are presented in two ways to better facilitate their use. First, route-specific risk values are presented. The "oral slope factor" is an upper bound on the estimate of per mg/kg-day of oral exposure. Similarly, a "unit risk" is an upper bound on the estimate of risk per unit of concentration, either per µg/L drinking water or per $\mu g/m^3$ air breathed. Second, the estimated concentration of the chemical substance in drinking water or air when associated cancer risks of 1 in 10,000, 1 in 100,000, or 1 in 1,000,000 is also provided.

Development of these hazard identification and dose-response assessments for nitrobenzene has followed the general guidelines for risk assessment as set forth by the National Research Council (NRC) (1983). Environmental Protection Agency (EPA) guidelines and Risk Assessment Forum technical reports that were used in the development of this assessment include the following: *Guidelines for Mutagenicity Risk Assessment* (U.S. EPA, 1986b), *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991a), *Guidelines for Reproductive Toxicity Risk Assessment* (U.S. EPA, 1996), *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998a), *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005),

Recommendations for and Documentation of Biological Values for Use in Risk Assessment (U.S. EPA, 1988), *Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies* (U.S. EPA, 1994a), *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b), *Use of the Benchmark Dose Approach in Health Risk Assessment* (U.S. EPA, 1995), *Science Policy Council Handbook: Peer Review* (U.S. EPA, 1998b, 2000a), *Science Policy Council Handbook*: *Risk Characterization* (U.S. EPA, 2000b), *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2000c), and *Supplemental Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 2000d), and *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002).

The literature search strategy employed for this compound was based on the Chemical Abstracts Service Registry Number (CASRN) and at least one common name. Any pertinent scientific information submitted by the public to the IRIS Submission Desk was also considered in the development of this document. The relevant literature was reviewed through January 2007.

2. CHEMICAL AND PHYSICAL INFORMATION

Structurally, nitrobenzene consists of a benzene ring with a single substituted nitro group (Figure 2-1). The compound is an oily yellow liquid with an odor of bitter almonds. Synonyms for nitrobenzene include oil of mirbane, essence of mirbane, nitrobenzol, and solvent black 6.

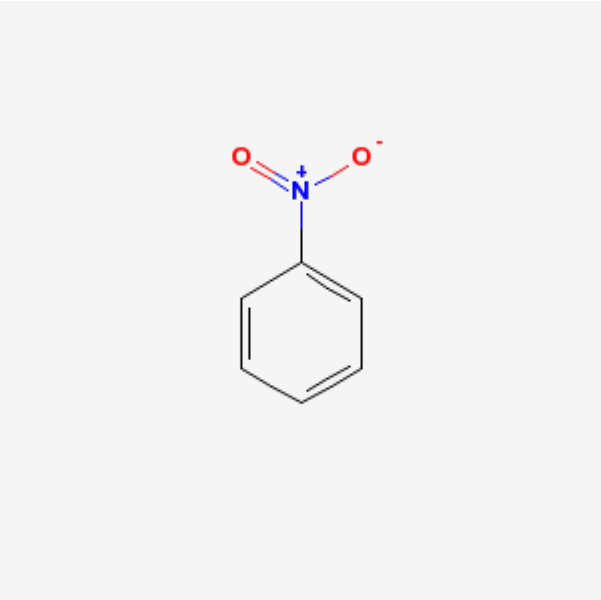


Figure 2-1. Chemical structure of nitrobenzene (KEGG, C06813).

Pertinent physical and chemical properties of the chemical are listed as follows (Hazardous Substances Data Bank [HSDB], 2003; World Health Organization [WHO,] 2003; Agency for Toxic Substances and Disease Registry [ATSDR], 1990):

Chemical formula	$C_6H_5NO_2$
Molecular weight	123.11
Melting point	5.7 ^oC
Boiling point	210.8 ^oC
Density	1.2 g/mL (at 20 ^oC)
Water solubility	1900 mg/L (at 20 ^oC)
Log K_{OW}	1.87
Log K_{OC}	1.56
Vapor pressure	0.15 mm Hg at 25 ^oC (20 Pa at 20 ^oC)
Henry's Law constant	1.31×10^{-5} atm-m^3/mol
Conversion factor	1 ppm = 5.12 mg/m^3; 1 mg/m^3 = 0.2 ppm

Nitrobenzene is manufactured by direct nitration of benzene with nitric acid, using sulfuric acid as catalyst and dehydrating agent. The purified product is used extensively in chemical manufacturing, especially in the synthesis of other industrial chemicals and intermediates. Most important among these is aniline, which is predominantly used in the manufacture of polyurethane (WHO, 2003). Other chemical products of nitrobenzene include benzidine, quinoline, and azobenzene (HSDB, 2003). The compound has been used as a solvent for cellulose ethers and acetates and in petroleum refining. Nitrobenzene is present in a number of commercial products, such as shoe and metal polishes and soaps. An estimated 2,133,800 tons of nitrobenzene were produced worldwide in 1994 (WHO, 2003), about one-third of which was produced in the United States. U.S. production of nitrobenzene has been increasing in recent years, from 435,000 tons in 1986 to 533,000 tons in 1990 to 740,000 tons in 1994 (WHO, 2003).

3. TOXICOKINETICS

3.1. ABSORPTION

3.1.1. Gastrointestinal Tract Absorption Studies

There are no quantitative data on the extent of absorption of nitrobenzene in humans via the oral route; however, it has been shown that nitrobenzene is well absorbed into brush border membrane vesicles (BBMV) from the small intestines of Sprague-Dawley rats (in vitro). Absorption assays with isolated BBMV and nitrobenzene were independent of age, sex, or segment (i.e., proximal third, middle third, or distal third) of small intestine, suggesting that lipophilicity of the compound and lipid composition of the membrane are the determining factors (Alcorn *et al.*, 1991). These basic considerations may be applicable to humans as well.

The WHO (2003) have cited reports of incidents where individuals have been poisoned by ingesting nitrobenzene, either accidentally or intentionally. Some of these case reports provide inferential evidence of the compound's ready passage across the intestinal absorption barrier. For example, Myslak et al. (1971) reported the case of a 19-year-old female who ingested about 50 mL of nitrobenzene approximately 30 minutes prior to the appearance of symptoms. During recovery, samples of her urine were analyzed and revealed the presence of substantial amounts of *p*-amino- and *p*-nitrophenol, metabolites of nitrobenzene (see Section 3.4) demonstrating absorption from the gastrointestinal tract.

Extensive intestinal absorption of nitrobenzene has been demonstrated in experimental animals. For example, a total of six rabbits (sex and strain not stated) were administered [^{14}C]-nitrobenzene and unlabeled nitrobenzene at total doses of 200 mg/kg (two animals) and 250 mg/kg (three animals) by stomach tube. One animal was exposed to 400 mg/kg; however, it died after 2 days (Parke, 1956). Animals were kept in metabolic cages for 30 hours after dosing to permit the collection of feces, urine, and expired air. Exhaled derivatives were trapped in ethanol and/or CO_2 absorbers. Thereafter, the animals were housed in open cages so that their urine and feces could be collected up to 10 days. By 4-5 days after dosing, the author determined that nearly 70% of the radioactivity had been eliminated from the body. This included 1% of the radioactivity expired as CO_2 and 0.6% expired as nitrobenzene (up to 30 hours), 58% excreted as metabolites in the urine (up to 4-5 days), and 9% eliminated by feces (up to 4-5 days).

The action of bacteria normally present in the small intestine of the rat is an important element in the formation of methemoglobin resulting from nitrobenzene exposure. Germ-free rats do not develop methemoglobinaemia when intraperitoneally dosed with nitrobenzene (Reddy et al., 1976). When nitrobenzene (200 mg/kg of body weight in sesame oil) was intraperitoneally administered to normal Sprague-Dawley rats, 30–40% of the haemoglobin in the blood was converted to methaemoglobin within 1–2 h. When the same dose was

administered to germ-free or antibiotic-pretreated rats, there was no measurable methaemoglobin formation, even when measured up to 7 h after treatment. The nitroreductase activities of various tissues (liver, kidney, gut wall) were not significantly different in germ-free and control rats, but the activity was negligible in gut contents from germ-free rats and high in control rats. This led the authors to suggest that a nitrobenzene metabolite such as aniline (which is formed by the bacterial reduction of nitrobenzene in the intestines of rats) is involved in methaemoglobin formation. Confirming and extending the results of Reddy et al. (1976), Rickert et al. (1983) examined the role of bacterial nitroreductases in the gastrointestinal tract in altering the absorption of nitrobenzene. The authors utilized conventional animals and axenic (bacteria-free) animals. Single oral doses of 22.5 or 225 mg/kg [^{14}C]-labeled nitrobenzene were administered to male F344 (CDF[F344]/CrlBR), CD (Crl:CD[SD]BR), and axenic CDF(F344)/CrlGN rats and to male B6C3F1 (B6C3F1/Crl/BR) mice (225 mg/kg only). Animals were housed in metabolic cages for 72 hours after dosing to collect urine, feces, and expired air. In the conventional rats, 56–65% of the administered dose was recovered in the urine, with a maximum of 21.4% recovered in the feces. Six metabolites were found in the bile of conventional rats. Since the metabolites were absent from the bile of axenic rats, the authors concluded that the reduction of nitrobenzene at the nitro group that produced metabolites in conventional rats must have been initiated in the intestines. When corrected for overall recovery, these data provide intestinal absorption estimates of 62–69% in conventional rats. The estimate from the mouse data was lower (43%).

Albrecht and Neumann (1985) gavaged female Wistar rats with [^{14}C]-nitrobenzene (25 mg/kg) in propylene glycol and collected blood, tissue, fecal, and urine samples at various time intervals. Excretion in urine was the major route of elimination with 50% of the administered radioactivity excreted in the urine after 24 hours and 65% after 1 week. In contrast to urine, cumulative fecal excretion of nitrobenzene reached no more than 15.5% of the administered dose within the same time period. This study, taken together with the above observations, indicates that a significant amount of nitrobenzene is absorbed via the gastrointestinal tract.

3.1.2. Pulmonary Absorption Studies

Several reports from the occupational and clinical research setting have addressed the pulmonary absorption of nitrobenzene. Ikeda and Kita (1964) discussed the case of 47-year-old woman who had been exposed via inhalation to paint that contained nitrobenzene. Although her symptoms were less severe, they were nearly identical to an oral exposure case study discussed above by Myslak et al. (1971) . The urinary metabolites, p-amino and p-nitrophenol, demonstrated absorption of nitrobenzene from the lungs and indicated that these metabolites were formed in humans after both oral and inhalation exposures. The report from Ikeda and Kita

(1964) suggests that pulmonary absorption of nitrobenzene had occurred, although it is likely that some dermal absorption had also taken place.

Quantitative estimates of nitrobenzene's pulmonary absorption were provided by Salmowa et al. (1963), who administered a continuous 6-hour exposure of nitrobenzene (5–30 μg/L; 1–6 ppm) to seven human research subjects (adult males, age unstated). Subjects were exposed to nitrobenzene through a mask that also permitted expired air to be collected and analyzed for nitrobenzene. The amount of nitrobenzene absorbed, estimated as the difference between the amount inhaled and the amount exhaled, ranged from 8.4–67.6 mg. The retention of nitrobenzene vapors in the lungs averaged 80%, varying from a mean value of 87% in the first hour to 73% in the sixth hour.

Piotrowski (1967) also exposed four human research subjects (adult males, age unstated) to a range of nitrobenzene concentrations in air (5–30 μg/L; 1–6 ppm). One was exposed for 6 hours daily for 4 successive days. The remaining three were subjected to longer exposures lasting the entire week (Monday through Saturday) and, after a pause on Sunday, were exposed again on Monday of the next week. The absorbed doses of nitrobenzene were estimated from measurements of the concentrations in the air, the volume of the expired air, and the mean pulmonary retention time of 80% as determined by Salmowa et al. (1963). The absorbed doses of nitrobenzene were then compared with the cumulative appearance of nitrobenzene metabolites in the urine. Based on these data, Beauchamp et al. (1982) determined that humans exposed to an airborne nitrobenzene concentration of 10 mg/m^3 for 6 hours would absorb 18.2–24.7 mg of nitrobenzene through the lungs.

3.1.3. Dermal Absorption Studies

Data from a number of sources point to the capacity of nitrobenzene to penetrate the dermal barrier in humans. For example, human research subjects were placed in an exposure chamber containing nitrobenzene vapor for 6 hours while receiving fresh air through a breathing tube and mask (Piotrowski, 1967). The absorption rate per unit of concentration of nitrobenzene was highly variable (0.23–0.30 mg/hr per μg/L), depending on the nitrobenzene concentration in the chamber (5–30 μg/L) and whether the subject was dressed or naked. In naked subjects exposed to a chamber concentration of 10 μg/L nitrobenzene, the absorbed dose ranged from 10–19 mg, compared with 8–16 mg in clothed subjects. Depending on the air concentration (5–30 μg/L), normal working clothes reduced the overall absorption of nitrobenzene by 20–30%. In another study involving human research subjects (age and sex not stated), the capacity of 21 organic compounds, including nitrobenzene, to penetrate the dermal barrier as liquid was surveyed by Feldmann and Maibach (1970), who applied [^{14}C]-labeled compounds in acetone (4 $\mu g/cm^2$) to a 13 cm^2 circular area of the ventral forearm surface of six subjects. The skin site was not protected and the subjects were asked not to wash the area for 24 hours. The authors

also examined the elimination of nitrobenzene following intravenous administration as a comparison to the dermal absorption and elimination studies. For the skin absorption studies, the cumulative amounts of radiolabel measured in urine over 5 days amounted to approximately 1.53 ± 0.84% of the load. The highest rate of absorption was monitored in the first 24-hour period after application, but excretion in the urine was still measurable between 96 and 120 hours after application. The absorption rate (% dose/hour) over the 120 hour period was as follows: 0.022%/hr: 0–12 hours; 0.022%/hr: 12–24 hrs; 0.013%/hr: 24–48 hours; 0.013%/hr: 48–72 hours; 0.011%/hr: 72–96 hours; and 0.006%/hr: 96–120 hours. Continued excretion of [^{14}C]-label at these later time points may have represented redistribution of nitrobenzene or its metabolites from adipose tissue rather than continued absorption. Following intravenous administration of [^{14}C]-nitrobenzene, 60.5% of the radioactive label was detected in the urine by 20 hours after administration. When corrected for the appearance of nitrobenzene in urine following an intravenous injection, an overall dermal absorption factor of approximately 2.6% was determined for nitrobenzene.

3.2. DISTRIBUTION

Albrecht and Neumann (1985) exposed female Wistar rats to 25 mg/kg (0.20 mmol/kg) [^{14}C]-nitrobenzene in propanediol by gavage and reported the appearance of radiolabel predominantly in blood, liver, kidney, and lung, 1 and 7 days after dosing. These findings suggest a wide distribution for nitrobenzene or its metabolites among the major organs and tissues. Radioactive label (radioactivity in tissue, [pmol/mg]/dose [μmol/kg]) recovered from various tissues was blood (229 ± 48) > kidney (204 ± 27) ≫ liver (129 ± 9.5) ≫ lung (62 ± 14) after 1 day of exposure. Only about 50 ± 10% of the nitrobenzene appeared in the urine. Seven days after exposure, tissue levels from highest to lowest were blood (134 ± 19) ≫ kidney (48 ± 2.4) ≫ lung (29 ± 4.1) ≈ liver (26.5 ± 3.5). After seven days, urinary elimination of nitrobenzene reached 65 ± 5.8%. [^{14}C]-Nitrobenzene metabolites were shown to bind with higher affinity to hemoglobin (Hb) and plasma proteins than [^{14}C]-acetanilide (0.15 mmol/kg), although in both cases the reactive metabolite was thought to be nitrosobenzene, and the compound bound to protein sulfhydryls via a sulfinic acid amide bond was identified as aniline. After 1 day, specific binding of nitrobenzene to Hb (1030 ± 137 pmol/mg/dose) and plasma proteins (136 ± 34) was much higher than acetanilide binding to Hb (177 ± 14) and plasma proteins (70 ± 7). By 7 days posttreatment, a marginal decrease in the nitrobenzene binding to Hb (1024 ± 82 pmol/mg/dose) and plasma proteins (101 ± 34) had occurred, as compared with acetanilide binding to Hb (102 ± 24) and plasma proteins (14 ± 3). This is the only study of tissue distribution of nitrobenzene that has been identified.

Goldstein and Rickert (1984) administered a single oral dose of 10 or 40 μCi [^{14}C]-nitrobenzene in corn oil to male CDF (F344)/CrlBR rats and B6C3F1/CrlBR mice with

sufficient carrier nitrobenzene to yield doses ranging from 75–300 mg/kg. The disposition of the bound radiolabel in red blood cells (RBCs) and spleen proteins was then evaluated after lysates or homogenates (spleen) were dialyzed, solubilized, and then separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The amount of covalently bound radiolabel increased dose dependently in RBCs and spleen for both species. Total and bound levels of [^{14}C]-label in RBCs from rats were approximately 6–13 times greater than those from mice at all doses tested. A statistically significant difference between the rat and mouse was observed with time for nitrobenzene binding to RBCs and spleen (Figure 3-1). Spleen weights in rats exposed to 200 mg/kg nitrobenzene increased by up to a factor of two 168 hours after dosing; however, there was no equivalent effect in mice (Figure 3-2).

Goldstein and Rickert (1984) used SDS-PAGE to investigate binding of [14C]nitrobenzene in the erythrocytes and spleen of rats and mice. SDS-PAGE of RBC lysates from rats showed a substantial proportion of the radioactivity coeluting with Hb. The radioactivity bound to spleen homogenates coeluted with methemoglobin (metHb)[1] and an unidentified low molecular weight component. By contrast, there was no sign of significant macromolecular binding of nitrobenzene-derived radiolabel in mice. Goldstein and Rickert (1984) hypothesized that the degree of RBC damage induced by nitrobenzene in mice was insufficient to induce splenic scavenging and clearance from the systemic circulation.

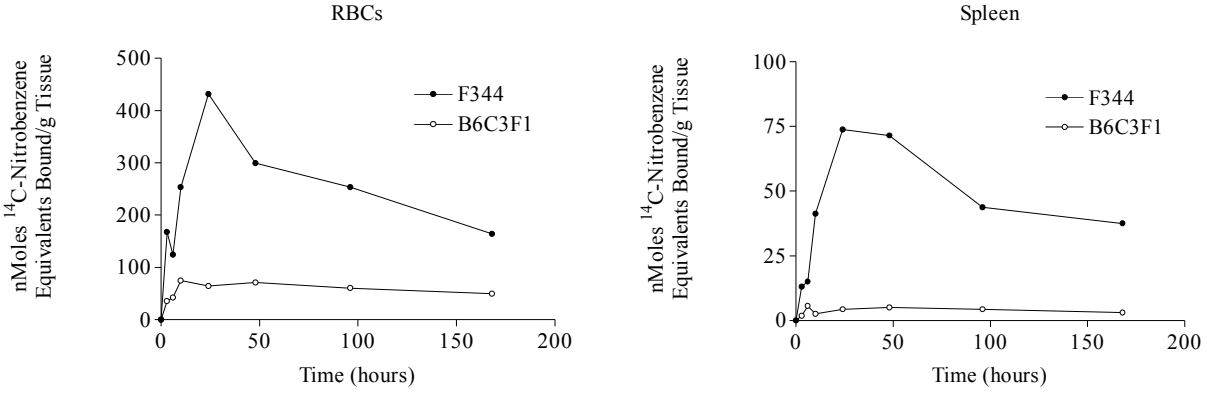


Figure 3-1. Time course of covalently bound [^{14}C]-nitrobenzene in RBCs and spleen of rats and mice.

Animals were administered 200 mg/kg [^{14}C]-nitrobenzene and sacrificed at various time points. Each point represents the mean ± standard error of the mean (SEM) of three to four

[1] "Methaemoglobinaemia arises from the production of non-functional haemoglobin containing oxidised Fe(3+)[i.e., metHb] which results in reduced oxygen supply to the tissues and manifests as cyanosis in the patient. It can develop by three distinct mechanisms: genetic mutation resulting in the presence of abnormal haemoglobin, a deficiency of methaemoglobin reductase enzyme and toxin-induced oxidation of haemoglobin"(Percy et al., 2005). MetHb reduces tissue oxygenation by two mechanisms: iron in the ferric rather than the ferrous form is unable to combine with oxygen and consequently the oxygen-carrying capacity of the blood is reduced and the presence of oxidized iron changes the heme tetramer in such a way as to reduce oxygen release in the tissues (i.e., shifts the oxyhemoglobin dissociation curve to the left as in alkalosis) (Ellenhorn, 1997).

determinations. Statistically significant differences between the F344 rat and B6C3F1 mouse were noted at all doses tested.

Source: Adapted from Goldstein and Rickert, 1984.

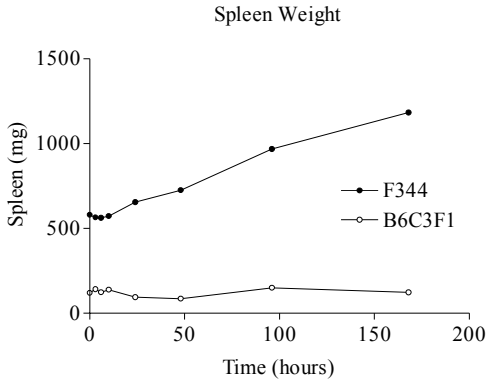


Figure 3-2. Time-related changes in spleen weight in rats and mice following nitrobenzene treatment.
All animals were administered an oral dose of 200 mg/kg nitrobenzene.

Source: Adapted from Goldstein and Rickert, 1984.

3.3. METABOLISM

Metabolism of nitrobenzene in mammals involves both oxidation and reduction reactions. Evidence for this has come from the identification of potential products of nitrobenzene oxidation and reduction reactions in the urine of humans and animals that had been exposed to the compound. Oxidation products of nitrobenzene include *o*-, *m*-, and *p*-nitrophenol; reduction products of nitrobenzene include nitrosobenzene, phenylhydroxylamine, and aniline. The metabolites from aniline include the following oxidative metabolites: *o*-, *m*-, and *p*-aminophenol, nitrocatechols, and aniline (Parke, 1956; Robinson et al., 1951). For all metabolites, involvement in phase II reactions is likely, and the formation and appearance of sulfated or glucuronidated conjugates has been demonstrated (Figure 3-3) (Rickert, 1987).

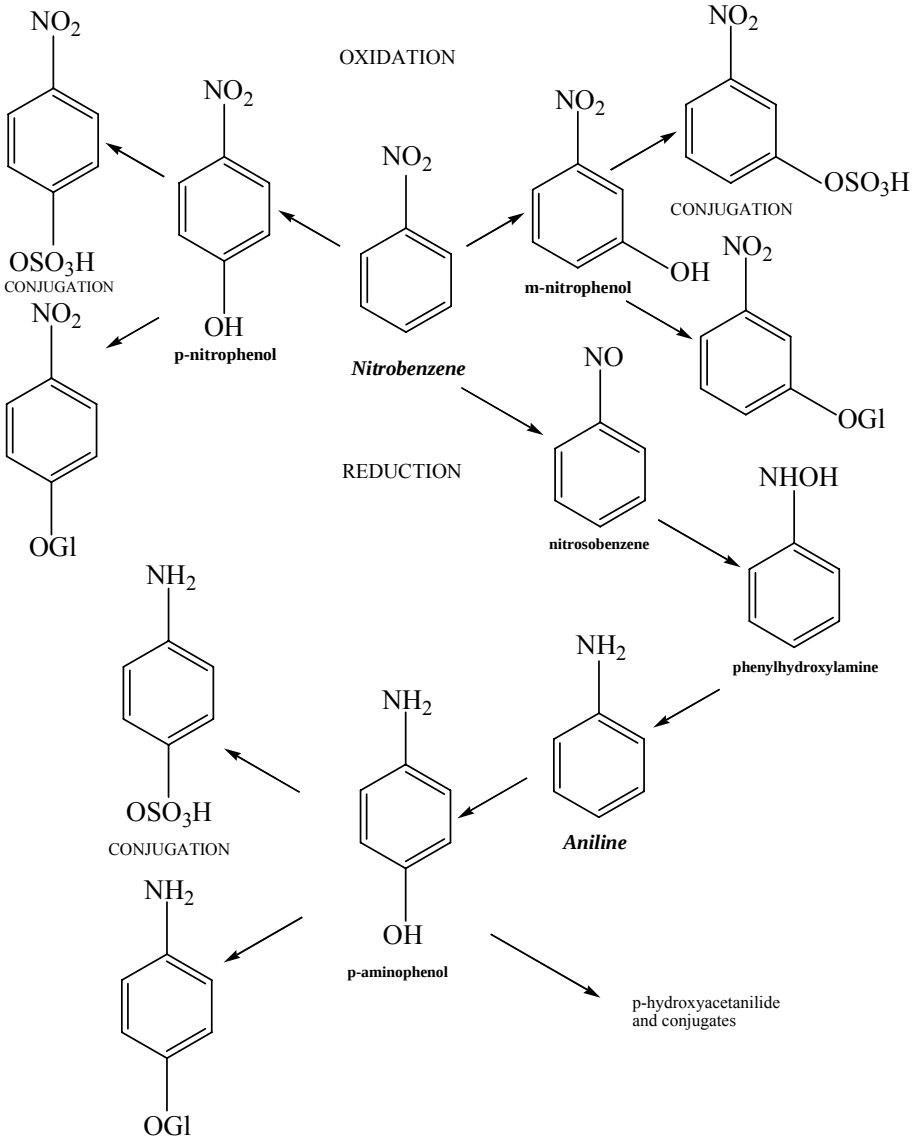


Figure 3-3. Outline of the metabolism of nitrobenzene: a substrate for oxidation and reduction reactions.

Sources: Adapted from WHO, 2003; Rickert, 1987.

The processes driving the metabolism of nitrobenzene in mammals display tissue specificity. Three primary mechanisms have been identified: reduction to aniline by intestinal microflora, reduction by hepatic microsomes and in erythrocytes, and oxidative metabolism by hepatic microsomes. First, nitrobenzene has been shown to undergo a three-step, two-electrons-per-step transfer reduction to aniline in intestinal microflora (Bryant and DeLuca, 1991; Reddy et al., 1976). The intermediates in this process are nitrosobenzene and phenylhydroxylamine. Second, nitrobenzene undergoes a six-step, one-electron-per-step transfer reduction to aniline that takes place in hepatic microsomes and erythrocytes (Levin and Dent, 1982; Reddy et al.,

1976). As illustrated by Holder (1999a), intermediates in the latter process include a nitroanion free radical, nitrosobenzene, hydronitroxide free radical, phenylhydroxlamine, and a theoretical amino-cation free radical. The reductive intermediates have been shown to reverse chemically (i.e., aniline can oxidize back towards nitrobenzene or any step in between), with the direction of flow depending on local redox potentials. The first intermediate in the chain, the nitroanion free radical, may also react nonenzymatically with tissue oxygen to reform nitrobenzene. This "futile loop" generates a superoxide anion in the process (Sealy et al., 1978), which may undergo dismutation by superoxide dismutase to molecular oxygen and hydrogen peroxide (Holder, 1999a; Mason and Holtzman, 1975a, b). Third, oxidative metabolism to the nitrophenols takes place in hepatic microsomes, with probable involvement of the cytochrome P450 family of enzymes. The intermediates in this process are *p*- and *m*-nitrophenols, of which the end products are conjugates of phase II enzymes. The process takes place at an even slower rate than the six-step/one-electron per step microsomal reduction of nitrobenzene. Figure 3-3 shows the range of oxidative and reductive products of nitrobenzene that have been demonstrated (Rickert, 1987).

The metabolic processes undergone by nitrobenzene are important because many of the toxicological effects of the compound are likely to be triggered by metabolites of nitrobenzene. For example, there is abundant evidence that methemoglobinemia is caused by the interaction of Hb with the products of nitrobenzene reduction (i.e., nitrosobenzene, phenylhydroxylamine, and aniline). The current understanding of how metHb is formed from Hb in the presence of these components is discussed below. Similarly, the formation of a superoxide anion during the microsomal reduction of nitrobenzene, with subsequent formation of hydrogen peroxide, may disturb the redox balance of target cells such as hepatocytes, potentially leading to oxidative stress (Gutteridge, 1995) (see Section 4.6.3).

3.3.1. Microbial Reduction of Nitrobenzene (The Three-step/Two-Electrons-per-Step Transfer Process)

Reduction of nitroaromatic compounds by the two-electron reductive pathway is catalyzed by a type I (oxygen-insensitive) nitroreductase (EC 1.6.99.1, common name NADPH dehydrogenase)[2]. This enzyme catalyzes the following general reaction: NADPH + H^+ + acceptor = $NADP^+$ + reduced acceptor (International Union for Biochemistry and Molecular Biology [IUBMB], 2005a). The enzymatic activity for type I nitroreductase is highest in the microflora of the intestinal tract of male Sprague-Dawley rats; however, organ-specific activities have been reported (Figure 3-4).

[2] EC numbers specify enzyme-catalyzed reactions, not specific enzymes.

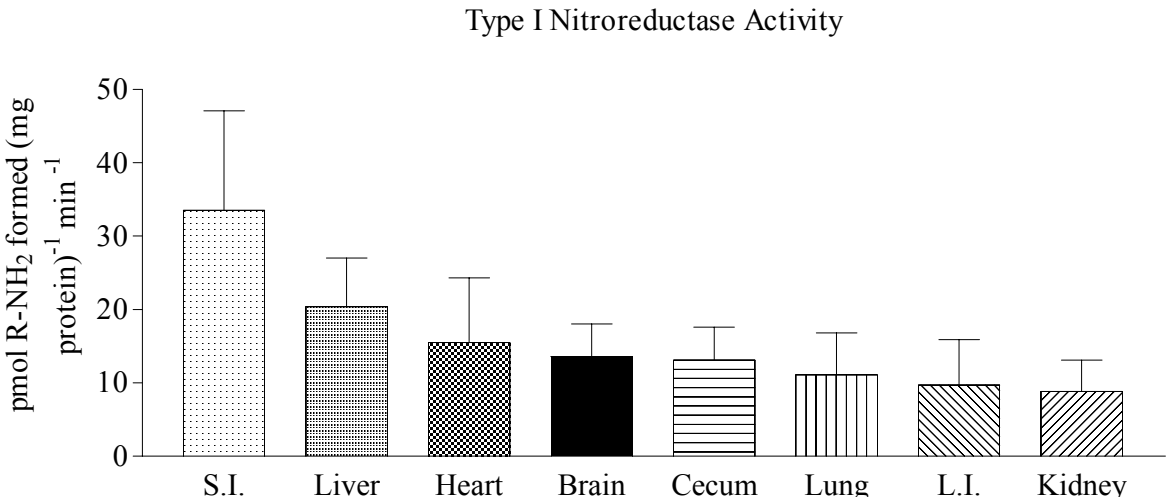


Figure 3-4. Type I nitroreductase activity in male Sprague-Dawley Rats.
Results are expressed as pmol of reduced nilutamide (R-NH_2) formed per milligram protein per minute (mean ± SEM; n≥4). S.I. = small intestine contents, L.I. = large intestine contents.

Source: Adapted from Ask et al., 2004.

Some of the earliest evidence to suggest the importance of microbial nitrobenzene reduction for toxicological outcomes such as metHb formation came from Reddy et al. (1976). These researchers administered 200 mg/kg nitrobenzene in sesame oil intraperitoneally to four groups of male Sprague-Dawley rats, either normal, bacteria-free, bacteria-free then acclimatized in a normal room for 7 days, or normal rats pretreated with antibiotics. Methemoglobinemia developed in normal rats and those bacteria-free animals that had been acclimatized in a normal room (30–40% metHb within 1–2 hours of exposure). When nitrobenzene was given to bacteria-free rats or those pretreated with antibiotics, they did not develop methemoglobinemia. These data emphasize the importance of microbial reduction of nitrobenzene to the onset of methemoglobinemia. Reddy et al. (1976) showed the relative importance of exogenous versus endogenous reductive nitrobenzene metabolism by comparing the rate of synthesis of aniline in homogenates of liver, kidney, gut wall, and gut contents prepared from animals in various treatment groups (Table 3-1). Nitroreductase activity was greatest in the gut contents of control rats. By contrast, this activity was missing in the gut contents of bacteria-free animals.

Table 3-1. Reduction of nitrobenzene by various rat tissue homogenates

Tissue	Aniline formation (nmol/mg protein/hour)[a]		
	Bacteria-free	Bacteria-free (acclimatized)	Control
Liver	2.0 ± 0.2	2.5 ± 0.4	3.3 ± 0.4
Kidney	0.5 ± 0.1	0.8 ± 0.1	0.7 ± 0.4
Gut wall	2.0 ± 0.4	2.0 ± 0.6	2.4 ± 1.0
Gut contents	0.2 ± 0.0	15.2 ± 2.7	11.1 ± 3.3

[a]Results are means ± SEM of determinations in three animals/group, with all determinations in triplicate.

Source: Reddy et al., 1976.

Table 3-2. Methemoglobin formation in the blood of rats dosed intraperitoneally with 200 mg/kg nitrobenzene in corn oil

Time after dosing (hours)	MetHb formation (%)[a]	
	Control rats	Antibiotic-treated rats
1	18.2 ± 5.0	1.7 ± 0.4
2.5	24.7 ± 4.2	2.1 ± 0.2
5	32.7 ± 5.0	1.9 ± 0.4
8	9.9 ± 2.3	0.4 ± 0.1

[a]Results are means ± SEM, three animals/group.

Source: Facchini and Griffiths, 1981.

Facchini and Griffiths (1981) demonstrated that little or no metHb was formed when blood was incubated with nitrobenzene in vitro. Their results, taken together with their in vivo findings with axenic animals (Table 3-2), confirm the importance of microbial reductive metabolism in the formation of metHb, specifically, through the formation of nitrosobenzene, phenylhydroxylamine, or aniline.

Goldstein and Rickert (1984) fed male CDF(F344)/CrlBR rats diets containing pectin (a carbohydrate with nutritional value for microflora) or cellulose (a metabolically inert carbohydrate) for 28 days prior to administering a single 200 mg/kg dose of [^{14}C]-nitrobenzene via gavage. Levels of metHb were monitored in the blood 1, 2, 4, 8, and 24 hours after dosing. Rats receiving the pectin-spiked diet formed substantial amounts of metHb in the blood, with levels peaking at the 4-hour time point. However, no metHb was formed in the blood of animals receiving the cellulose-containing diet. The authors correlated these findings with the greater numbers of anaerobic bacteria present in the cecum of rats receiving the pectin-containing diets. As shown in Table 3-3, [^{14}C]-nitrobenzene was metabolized in vitro in the presence of gut

contents from animals exposed to the subject diets. Metabolites included aniline, nitrosobenzene, and azoxybenzene, with larger amounts measured in those incubations containing pectin-enriched gut contents.

Table 3-3. Formation of metabolites of nitrobenzene in the presence of cecal contents in vitro: influence of diet

Diet	Pectin (%)	Metabolite formation (percent total radioactivity)[a]			
		Aniline	Nitrosobenzene	Azoxybenzene	Nitrobenzene
NIH-07	8.4	36 ± 10[b]	7 ± 0[b]	7 ± 1[b]	34 ± 11[b]
AIN-76A	5 (added)	11 ± 4	3 ± 2	3 ± 2	78 ± 11
AIN-76A	0	3 ± 1	0 ± 0	0 ± 0	95 ± 2

[a]Values are means ± SEM of four determination.
[b]Significantly different from AIN-76A.

Source: Goldstein and Rickert, 1984.

Experiments of Levin and Dent (1982), pertaining to the influence of gut microflora on the metabolism of nitrobenzene, included an in vivo protocol in which normal or antibiotic-treated male F344 (COBS CDF®/CrlBR) rats were gavaged with 225 mg/kg nitrobenzene (containing 0.1 μCi/mg [^{14}C]-nitrobenzene). Rats were kept in metabolic cages for up to 72 hours after treatment, during which urine, feces, and expired air were collected. To the extent possible, the excretory products were characterized and measured by high performance liquid chromatography. As shown in Table 3-4, a statistically significant decrease in *p*-hydroxy-acetanilide (a reductive metabolite of nitrobenzene) and a slight increase in *p*- and *m*-nitrophenol (oxidative metabolites) were observed in antibiotic-treated rats versus controls. Antibiotic pretreatment ameliorated the nitrobenzene-induced methemoglobinemia following a single oral dose of 300 mg/kg. Moreover, antibiotic-treated animals exposed to 300 mg of nitrobenzene per kg had metHb concentrations of 2.1 ± 0.4%, 2.8 ± 0.5%, and 1.9 ± 1.9% at 6, 24, and 96 hours after the dose. However, nitrobenzene-exposed vehicle-control rats still had elevated metHb concentrations (20.0 ± 7.9%) 96 hours after the dose.

Bryant and DeLuca (1991) purified and characterized an oxygen-insensitive nicotinamide adenine dinucleotide (phosphate) (NAD[P]H)-dependent nitroreductase from *Enterobacter cloacae*, which they considered to be typical of enteric bacterial nitroreductases that have been identified in a number of microbial genera. This enzyme was shown to act through an obligatory two-electron transfer mechanism. Figure 3-5 illustrates the three-step/two-electrons-per-step reduction process for nitrobenzene in the intestinal microflora.

Table 3-4. Urinary metabolites of [^{14}C]-nitrobenzene excreted within 72 hours after gavage

Metabolite	Percent of total[a]	
	Control rats	Antibiotic-treated rats
p-Nitrophenol	22.4 ± 0.9	26.5 ± 3.8
m-Nitrophenol	11.4 ± 0.6	16.1 ± 2.0
p-Hydroxy-acetanilide	16.2 ± 1.7	0.9 ± 0.0[b]
Unidentified Peak I	4.5 ± 0.3	5.5 ± 0.9
Unidentified Peak II	3.7 ± 0.6	0.5 ± 0.1[b]
Total recovered	58.2	49.5

[a]Values are means ± standard deviation (SD) for three animals/group.
[b]Significantly different from controls.

Source: Levin and Dent, 1982.

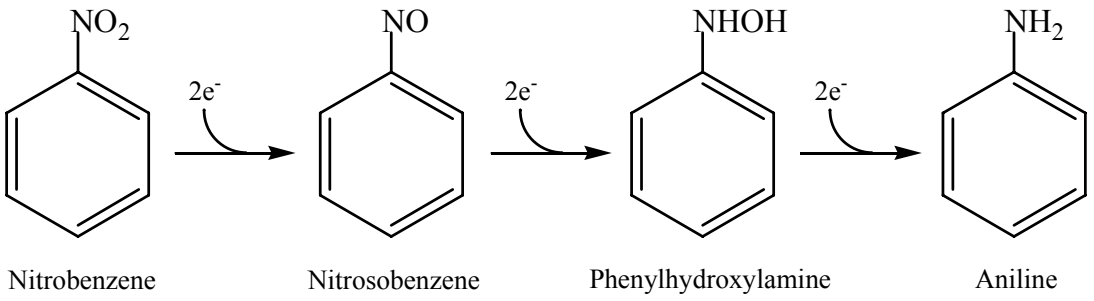


Figure 3-5. Mechanism of bacterial nitrobenzene reduction.

Source: Adapted from Holder, 1999a.

3.3.2. Hepatic and Erythrocytic Reduction of Nitrobenzene (The Six-Step/One-Electron-per-Step Transfer Process)

Reduction of nitroaromatic compounds by the one-electron reductive pathway is catalyzed by a type II (oxygen-sensitive) nitroreductase (EC 1.6.99.3; common name NADH dehydrogenase) (IUBMB, 2005b). A mitochondrial form of type II nitroreductase (EC 1.6.5.3; common name NADH dehydrogenase [ubiquinone]) catalyzes a similar one-electron addition (IUBMB, 2005c). Type II nitroreductases catalyze the following general reaction: NADH + H^+ + acceptor = NAD^+ + reduced acceptor.

Type II nitroreductase activity is highest in the microflora of the intestinal tract of male Sprague-Dawley rats; however, organ-specific activities have been reported (Figure 3-6).

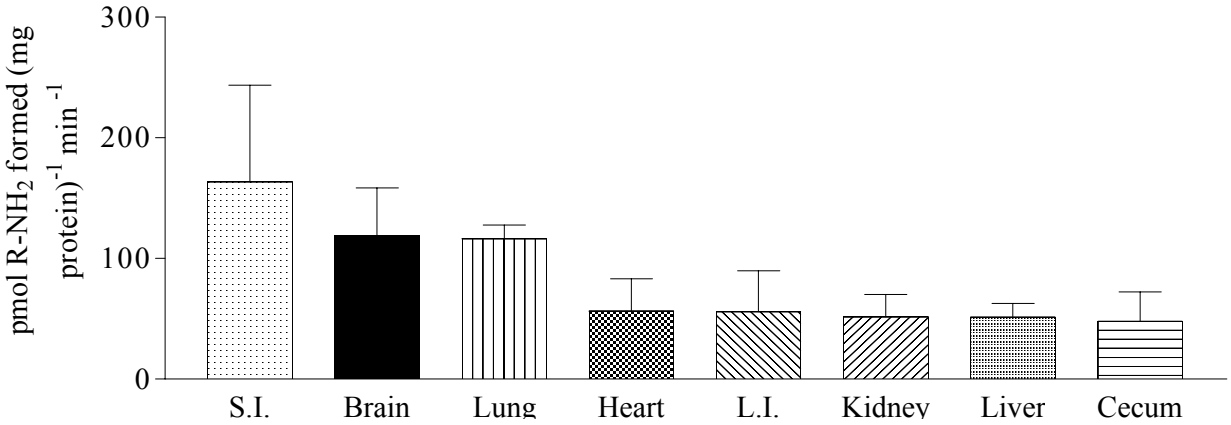


Figure 3-6. Type II nitroreductase activity of male Sprague-Dawley rats.
Results are expressed as pmol of reduced nilutamide (R-NH_2) formed per milligram protein per minute (mean ± SEM; n≥4). S.I. = small intestine contents, L.I. = large intestine contents.

Source: Adapted from Ask et al., 2004.

The findings of Reddy et al. (1976) and Levin and Dent (1982) can be interpreted to suggest differences in the kinetics and mechanisms of action of bacterial versus hepatic microsomal nitroreductases. For example, when Levin and Dent (1982) incubated nitrobenzene (100 μM) under aerobic or anaerobic conditions (e.g., oxygen-scavenging system used) with microsomes or 9000g supernatant fractions prepared from the livers of phenobarbital-induced male F344 (COBS CDF®/CrlBR) rats, metabolism of nitrobenzene by hepatic microsomes was extremely slow under aerobic conditions (0.022 nmol/min-mg protein) compared to anaerobic conditions (0.33 nmol/min-mg protein). In contrast, the rate of reduction of nitrobenzene by cecal microflora, which contains an oxygen-insensitive nitroreductase, was 150 times that in microsomes when expressed as nmol of product/min-g of liver (4.4 ± 0.1) or cecal contents (668 ± 74). The masses of liver and cecal contents in a 200 g rat are approximately equal, so that the cecal contents would represent the major site of reductive metabolism in vivo.

The use of electron spin resonance (ESR) spectrometry by Mason and Holtzman (1975a, b) on the reaction products of in vitro incubations of rat hepatic microsomes, mitochondria, or 165,000g supernatants incubated with nitrobenzene or *p*-nitrobenzoic acid demonstrated the formation of nitroaromatic radicals. The authors suggested that these components were likely to be the first intermediates in the reduction of the respective substrates. The appearance of nitroaromatic radicals would be consistent with a six-step/one-electron-per-step reduction mechanism for the microsomal metabolism of nitroarenes such as nitrobenzene. Sealy et al. (1978) used the same incubation system as Mason and Holtzman (1975a, b) with the substrates nitrofurantoin, nitrofurazone, misonidazole, or nitrobenzoate but added the spin traps 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) or phenyl-N-t-butyl nitrone shortly before the

addition of microsomes. The resulting spectra were consistent with the formation and reaction of superoxide anion with the spin traps to give relatively long-lived nitroxide adducts with a characteristic ESR spectrum. These results suggested that compound-specific nitroanion radicals had been rapidly converted by molecular oxygen to the parent nitroarene with the formation of a superoxide anion. The reconversion to the nitroarenes was an experimental demonstration of the futile cycle by which reduced coenzymes are expended in the presence of endogenous nitrobenzene, with the concommitent production of superoxide radical and possibly hydrogen peroxide. A metabolic chart in Holder (1999a) summarizes the six one-electron reduction step process for nitrobenzene reduction (Figure 3-7).

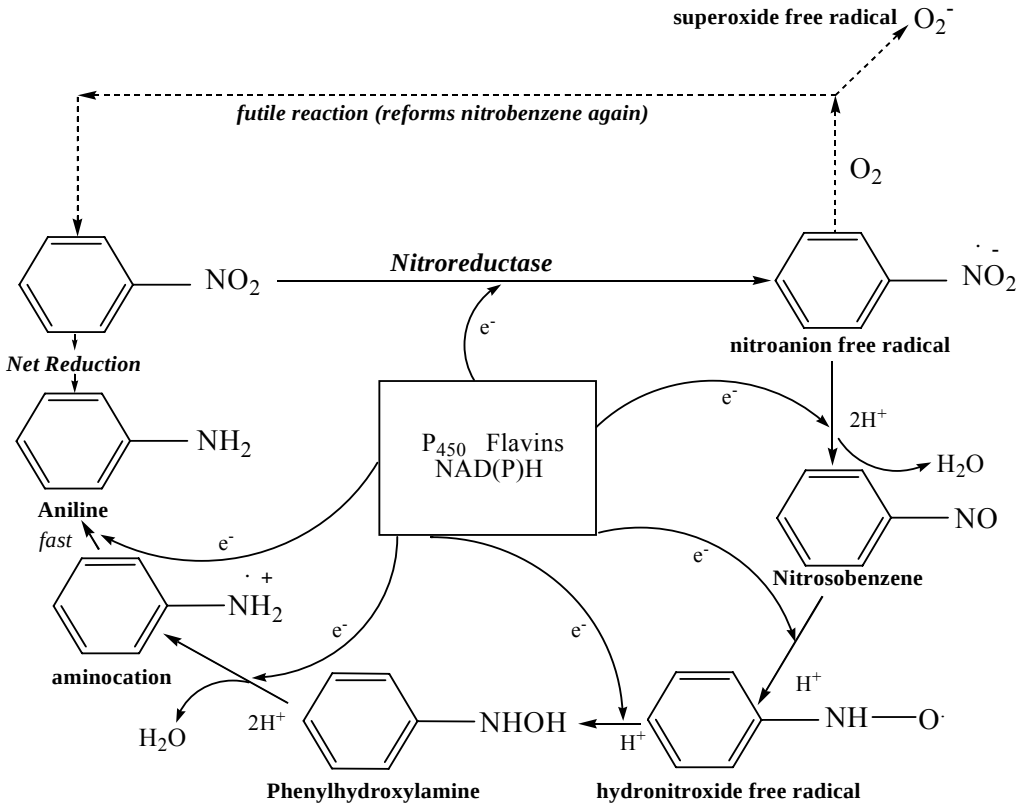


Figure 3-7. Mechanism of microsomal nitrobenzene reduction.

Source: Adapted from Holder, 1999a.

The scheme captures the series of five intermediate compounds and/or radicals to form aniline, with the additional potential for the first product of the process, the nitroanion free radical, to be reoxidized to nitrobenzene with the formation of a superoxide anion. Superoxide dismutase can rapidly convert superoxide anion to hydrogen peroxide, which in turn, may be converted to oxygen and water by catalase, or conjugated with glutathione by glutathione peroxidase, thereby forming glutathione disulfide and water (Table 3-5).

Mason and Holtzman (1975a, b) discussed available information on the biochemical characteristics of hepatic microsomal nitrobenzene reductases. The activities were thought to consist of one or more flavoproteins that represent only single electron-to-electron acceptors. The authors speculated that the microsomal flavoenzymes, NADPH-cytochrome *c* reductase (Enzyme Commission [EC] 1.6.2.4) and NADH-cytochrome b_5 reductase (EC 1.6.2.2), may be the enzymes responsible for the reduction of nitroarenes to their anion radicals (Table 3-5).

Table 3-5. Enzyme systems in erythrocytes

Enzyme	EC number[a]	Reaction
Superoxide dismutase (IUBMB, 2005d)	EC 1.15.1.1	$2\ O_2^{\bullet-} + 2\ H^+ = O_2 + H_2O_2$
Glutathione peroxidase (IUBMB, 2005e)	EC 1.11.1.9	2 glutathione + H_2O_2 = glutathione disulfide + 2 H_2O
Catalase (IUBMB, 2005f)	EC 1.11.1.6	$2\ H_2O_2 = O_2 + 2\ H_2O$
Glutathione transferase (IUBMB, 2005g)	EC 2.5.1.18	RX + glutathione = HX + R-S-glutathione
Glutathione reductase (IUBMB, 2005h)	EC 1.8.1.7	2 glutathione + $NADP^+$ = glutathione disulfide + NADPH + H^+
NADPH-cytochrome *c* reductase (IUBMB, 2005i)	EC 1.6.2.4	NADPH + H^+ + *n* oxidized hemoprotein = $NADP^+$ + *n* reduced hemoprotein
NADH-cytochrome b_5 reductase (IUBMB, 2005j)	EC 1.6.2.2	NADH + H^+ + 2 ferricytochrome b_5 = NAD^+ + 2 ferrocytochrome b_5

[a]EC numbers specify enzyme catalyzed reactions, not specific enzymes (Bairoch, 2000).

Harada and Omura (1980) provided data that addressed this issue by monitoring the formation of aniline, nitrosobenzene, and phenylhydroxylamine in hepatic microsomes that were incubated in the presence of antibodies to NADPH-cytochrome *c* reductase, NADH-cytochrome b_5 reductase, cytochrome b_5, or cytochrome P450 (subfamily not stated). When incubated with antibodies to NADPH-cytochrome *c* reductase and cytochrome P450, the activities of NADPH- and NADH-dependent nitrobenzene reductases were inhibited, with concomitant blockage of the nitrosobenzene and phenylhydroxylamine formation. However, antibodies to NADH-cytochrome b_5 reductase or cytochrome b_5 were ineffective. The initial step in nitrobenzene reduction appeared to be catalyzed by NADPH-cytochrome *c* reductase, with cytochrome P450 playing a role in the final conversion of the intermediates to aniline.

In addition to the hepatic microsomal reduction of nitrobenzene, the reductive metabolism in erythrocytes has been extensively studied due to the propensity of nitrobenzene metabolites to form metHb. Mammalian RBCs are particularly susceptible to oxidative damage because (1) being an oxygen carrier, they are exposed uninterruptedly to high oxygen tension; (2) RBCs have no capacity to repair damaged components; and (3) Hb is susceptible to autooxidation, and its membrane components are susceptible to lipid peroxidation (Rice-Evans,

1990). Several biochemical changes occur in the human RBC during its entire lifespan of about 120 days; for example, there are changes in lipid and protein content of the membrane, in enzyme activities, ion permeability, size, and deformability (Clark and Shohet, 1985; Westerman et al., 1963). At the end of its life span, the erythrocyte is phagocytized by macrophages, predominantly in the spleen. This latter event can lead to splenic congestion in rats following acute treatment with nitrobenzene due to the increased fragility of RBCs and the ultimate increase in splenic scavenging and clearance from the systemic circulation (Goldstein and Rickert, 1984).

The particular redox chemistry associated with nitrobenzene metabolism in RBCs is of special interest because of its association with the development of methemoglobinemia. The work of Reddy et al. (1976) has pointed to an association of metHb formation with the reduction of nitrobenzene to nitrosobenzene, phenylhydroxylamine, and aniline by nitroreductases present within intestinal microflora. Moreover, in vitro incubation of RBCs with nitrobenzene does not result in the formation of metHb (Facchini and Griffiths, 1981). Taken together, these findings suggest that it is the presence and cycling of the reductive products of nitrobenzene within RBCs that causes the conversion of oxyhemoglobin (oxyHb) to metHb (Figure 3-8).

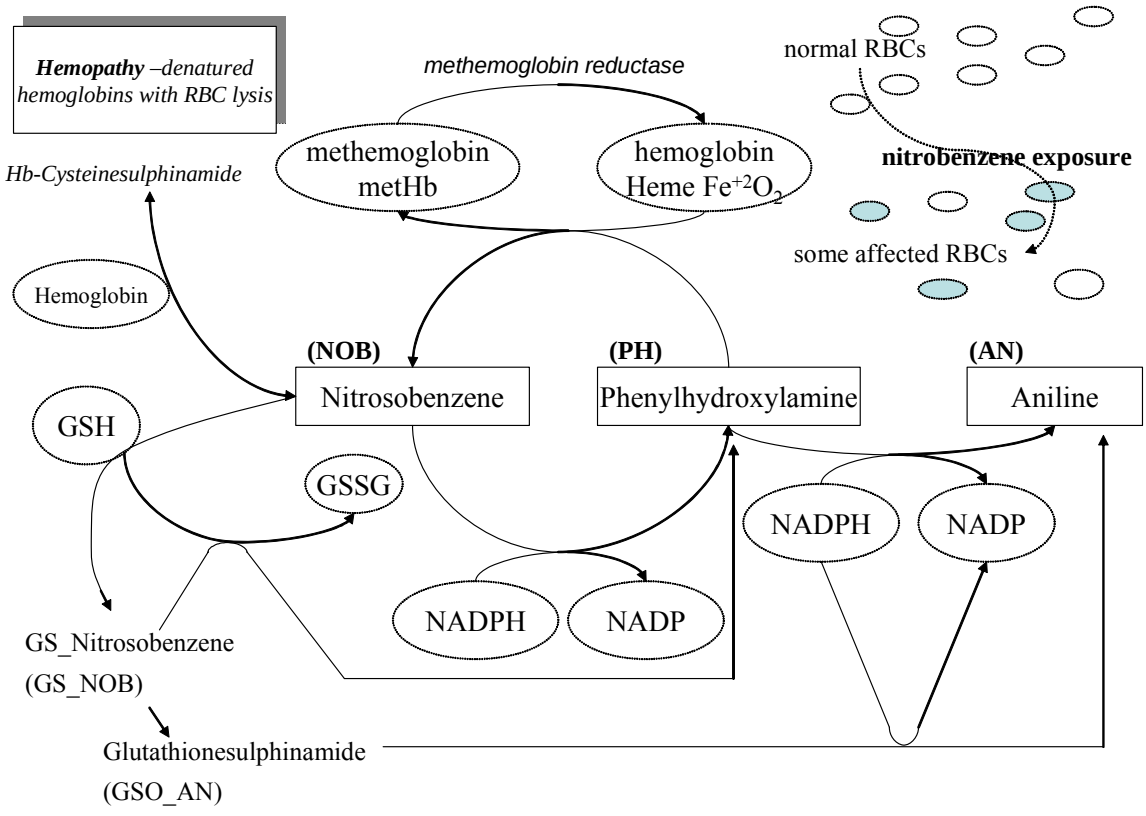


Figure 3-8. Cycling of nitrosobenzene and phenylhydroxylamine in RBCs, resulting in the formation of metHb.

Source: Adapted from Holder, 1999a.

The primary metabolic event in the formation of metHb (Fe^{3+}) from oxyHb (Fe^{2+}) as a result of nitrobenzene exposure is the cycling between phenylhydroxylamine and nitrosobenzene. As explained by Maples et al. (1990), nitrosobenzene can be reduced nonenzymatically by endogenous reducing agents or enzymatically by NADH-cytochrome b_5 reductase to reform phenylhydroxylamine. This completes the redox cycle with the overall expenditure of NADH and the accumulation of metHb. Nitrosobenzene has been shown to participate in a number of reactions that adversely affect the metabolic balance of RBCs. For example, nitrosobenzene has a 14-fold higher binding affinity to the heme moiety of Hb than does molecular oxygen (Eyer and Ascherl, 1987). It is also thought to promote the dissociation of tetrameric Hb to its constituent dimers (Eyer and Ascherl, 1987). Nitrosobenzene can also bind to peptides and proteins carrying cysteine residues, including Hb and reduced glutathione (GSH) (Eyer, 1979). The consequences of the latter interaction potentially include (1) the formation of sulfhemoglobin, (2) the formation of an oxidized dimer of glutathione (GSSG) with reformation of phenylhydroxylamine, or (3) rearrangement to form GSH sulfinamide. Furthermore, an overall depletion of GSH may result from excessive cycling of nitrosobenzene.

Maples et al. (1990) used ESR to demonstrate the formation of a phenylhydronitroxide free radical during the phenylhydroxylamine-initiated reduction of oxyHb. The use of DMPO as a spin trap further demonstrated the transfer of a free electron to cysteine-carrying components such as GSH and Hb, with the formation of their respective thiyl radicals, GS• and HbS•. These moieties are likely to be highly reactive, with the capacity to transfer the unpaired electron to other subcellular components. Continuous recycling of phenylhydroxylamine and nitrosobenzene may lead to increased fragility of RBC membranes, premature scavenging, and destruction within the reticuloendothelial system, followed by engorgement and sinusoid congestion of the spleen (Chemical Industry Institute of Toxicology [CIIT], 1993; Goldstein and Rickert, 1984).

3.3.3. Microsomal Oxidation of Nitrobenzene

Oxidation of nitrobenzene can generally occur via hydroxylation of the benzene ring (usually at positions 3 or 4) forming nitrophenols, or after initial nitroreduction of the exocyclic nitro group to the amine by oxidation to phenylhydroxylamine. These reactions are thought to be mediated by microsomal enzymes.

The appearance of conjugated derivatives of nitrophenols in the urine of female Giant Chinchilla rabbits after receiving an oral dose of nitrobenzene (0.5 g in 25 mL water by stomach tube) implied that the compound can undergo oxidation reactions in addition to the more extensively characterized reduction reactions that are discussed above (Robinson et al., 1951). A greater range of both oxidation and reduction metabolites was formed when rabbits (strain and

sex not stated) were given a single oral dose of [^{14}C]-nitrobenzene and unlabeled nitrobenzene at total doses of 200 mg/kg (two animals) and 250 mg/kg (three animals) (Parke, 1956). Although the mechanism of microsomal oxidation of nitrobenzene has not been well characterized, the involvement of members of the cytochrome P450 family is likely (WHO, 2003). While it is probable that not all active subcellular sites involved in nitrobenzene oxidation have been identified, the overall rate of oxidative metabolism is thought to be very slow. However, oxidation products of nitrobenzene such as *p*- and *m*-nitrophenol have been detected in the urine of subjects exposed to nitrobenzene by inhalation (5–30 μg/L; 1–6 ppm) for 6 hours, suggesting that oxidation reactions do play a role in the metabolism of nitrobenzene in vivo (Salmowa et al., 1963).

3.4. ELIMINATION

The major route of elimination for nitrobenzene in humans and animals is urine (Albrecht and Neumann, 1985; Rickert et al., 1983), with the majority of the dose eliminated within 48 hours. For example, a subject who ingested about 50 mL of nitrobenzene, as reported by Myslak et al. (1971), showed extensive excretion of the nitrobenzene metabolites, *p*-amino- and *p*-nitrophenol, in the urine. These reached maximum levels on day 2 for *p*-aminophenol (198 mg/day) and on day 3 for *p*-nitrophenol (512 mg/day). As discussed in Section 3.1, Ikeda and Kita (1964) detected the same compounds in the urine of a woman who was exposed to nitrobenzene in an occupational setting, primarily by inhalation. However, Salmowa et al. (1963) detected *p*-nitrophenol, but not *p*-aminophenol, in the urine of human research subjects exposed to nitrobenzene via inhalation.

p-Nitrophenol was also detected in the urine of subjects exposed to nitrobenzene through the skin (Piotrowski, 1967). In a quantitative study using human research subjects (Section 3.1), Feldmann and Maibach (1970) applied [^{14}C]-labeled nitrobenzene (50 μg dissolved in acetone) to the forearm skin of six subjects. As noted earlier, an estimated 2.6% of the dose was absorbed through the skin. Excretion of nitrobenzene-derived radiolabel in urine over 5 days was 1.5 ± 0.84% of the dose or about 58% of the absorbed dose. The highest rate of absorption occurred during the first 24 hours after dosing, but radioactivity could be detected in urine for 96–120 hours after application. Following intravenous administration of [^{14}C]-nitrobenzene, 60.5% of the radioactive label was detected in the urine by 20 hours after administration, confirming the high rate of urinary excretion of nitrobenzene in humans.

Robinson et al. (1951) studied the metabolism of nitrobenzene in the Giant Chinchilla rabbit. Their results demonstrated that urine was a major excretion pathway with 45% of the radioactivity following a [^{14}C]-nitrobenzene dose excreted in urine within 72 hours. Parke (1956), using [^{14}C]-nitrobenzene, was able to demonstrate in rabbits that 0.6–0.7% of the

radioactivity from various doses was eliminated via exhaled air as parent compound, up to 1.2% as CO_2, and a very small amount (0.04% at best) as aniline.

As discussed in Section 3.3, the study on nitrobenzene metabolism in rats by Levin and Dent (1982) also determined levels of fecal, urinary, and exhalatory excretion. Values for the recovery of radiolabel in feces and expired air were 16.4 ± 2.2 and 2.3 ± 0.5% for control rats and 12.5 ± 3.6 and 3.4 ± 1.5%, respectively, for antibiotic-treated animals. The observed metabolites were present in urine as sulfate conjugates.

Rickert et al. (1983) exposed male F344 (CDF[F344]/CrlBR) rats, male CD (Crl:CD[SD]BR) rats, and male B6C3F1 (B6C3F1/Crl/BR) mice to single doses of 22.5 (oral) or 225 mg/kg (oral or intraperitoneal [i.p.]) nitrobenzene (containing 20 μCi [^{14}C]-nitrobenzene) in corn oil. Samples of feces, urine, and expired air were collected at various time points up to 72 hours. Urinary metabolites of nitrobenzene were identified after incubation with β-glucuronidase and/or sulfatase. The disposition of radiolabeled products among feces, urine, and expired air 72 hours after dosing is shown in Table 3-6, corroborating urine as the primary route of excretion in all exposed groups. Species and strain differences were evident in the degree of conjugation exhibited by nitrobenzene metabolites (Table 3-7). In F344 rats, all nitrobenzene metabolites were conjugated as sulfates, confirming the observation of Level and Dent (1982). By contrast, the urine of CD rats and B6C3F1 mice contained sulfate and glucuronide conjugates as well as free product. *p*-Aminophenol was detected only in the urine of mice.

Table 3-6. Recovery of radiolabel in F344 and CD rats and B6C3F1 mice 72 hours after exposure to a single oral dose of [^{14}C]-nitrobenzene

Excretory product	Percentage of dose recovered					
	F344 rats			CD rats		B6C3F1 mice
	225 mg/kg oral	225 mg/kg i.p.	22.5 mg/kg oral	225 mg/kg oral	22.5 mg/kg oral	225 mg/kg oral
Urine	63.2 ± 2.1	56.8 ± 0.9	65.8 ± 2.4	60.8 ± 1.1	64.5 ± 0.8	34.7 ± 4.8
Feces	14.2 ± 0.7	13.7 ± 1.8	21.4 ± 1.8[a]	11.8 ± 1.1	11.5 ± 0.1	18.8 ± 0.4[a]
Expired air	1.6 ± 0.1	1.4 ± 0.1	1.0 ± 0.6	2.5 ± 0.3	0.8 ± 0.2	0.8 ± 0.1
Total	79.0 ± 2.2	71.9 ± 2.6	88.2 ± 1.8[a]	75.1 ± 1.1	76.8 ± 1.0	54.3 ± 4.7[a]

[a]Significantly different from F344 rats given 225 mg/kg orally.

Source: Rickert et al., 1983.

Albrecht and Neumann (1985) administered a single dose of 25 mg/kg nitrobenzene by gavage to female Wistar rats. They found that 50% of the dose was eliminated via urine within the first 24 hours, and a total of 65% of the dose was excreted in urine within 1 week. Only 15.5% of the dose was eliminated in the feces within 1 week after dosing.

Table 3-7. Urinary excretion of nitrobenzene metabolites in male rats and mice gavaged with a single oral dose of [^{14}C]-nitrobenzene

Compound	Free/ conjugate	Percentage of dose[a]				
		F344 rat (mg/kg)		CD rat (mg/kg)		B6C3F1mouse (mg/kg)
		225	22.5	225	22.5	25
p-Hydroxyacetanilide	Free	–[b]	–	1.3 ± 0.2	0.9 ± 0.2	0.4 ± 0.0
	Glucuronide	–	–	1.8 ± 0.6	1.1 ± 0.1	3.1 ± 0.3
	Sulfate	19.0 ± 0.9	19.8 ± 2.8	5.8 ± 1.2	1.7 ± 0.9	0.4 ± 0.1
p-Aminophenol	Free	–	–	–	–	0.1 ± 0.1
	Glucuronide	–	–	–	–	0.2 ± 0.2
	Sulfate	–	–	–	–	9.4 ± 1.3
p-Nitrophenol	Free	–	–	2.2 ± 0.6	0.7 ± 0.2	0.8 ± 0.1
	Glucuronide	–	–	0.5 ± 0.1	0.6 ± 0.0	0.1 ± 0.1
	Sulfate	19.9 ± 1.1	23.3 ± 2.1	10.3 ± 2.9	5.6 ± 1.8	6.3 ± 1.1
m-Nitrophenol	Free	–	–	1.2 ± 0.4	0.4 ± 0.1	0.1 ± 0.1
	Glucuronide	–	–	0.5 ± 0.2	0.5 ± 0.1	–
	Sulfate	10.2 ± 0.6	11.6 ± 1.4	6.2 ± 1.7	3.8 ± 1.2	6.1 ± 1.2
Unidentified peak I	Total	9.8 ± 0.7	9.0 ± 0.5	25.3 ± 1.2	31.1 ± 2.1	4.8 ± 0.7
Unidentified peak II	Total	–	–	5.7 ± 4.0	16.4 ± 5.6	2.6 ± 0.2

[a]Values are means ± SEM for three animals/group over a 72-hour period.
[b]– = not detected.

Source: Rickert et al., 1983.

3.5. PHYSIOLOGICALLY BASED TOXICOKINETIC MODELS

No studies were located that addressed the toxicokinetics of nitrobenzene as applicable to physiologically based pharmacokinetic modeling of the compound.

4. HAZARD IDENTIFICATION

4.1. STUDIES IN HUMANS—EPIDEMIOLOGY AND CASE REPORTS

There are no reports of epidemiological studies of the human health impacts of nitrobenzene exposure in the workplace or environment. However, a number of case reports of nitrobenzene poisoning have been published in the biomedical literature. As described in the following sections, nitrobenzene induces a suite of well-characterized toxicological responses irrespective of the route of exposure—oral, inhalation, or dermal. Some toxicokinetic information on nitrobenzene has also emerged from studies in which nitrobenzene was administered to human research subjects (Section 3).

4.1.1. Oral Exposure

Schimelman et al. (1978) reported on a 48-year-old man who was taken to an emergency department 10 minutes after consuming approximately 300 mL of Hoppe's Gunpowder Solvent #9 (30% denatured ethyl alcohol, 30% kerosene, 20–35% essential oils and fatty oil base, 3% ammonia, and 2% nitrobenzene by volume). Upon arrival, the patient was cyanotic, and his respirations were shallow and irregular. Blood was obtained and was dark brown in color, and methylene blue was administered.[3] MetHb level before and after treatment was 75%. The patient underwent seven blood transfusions, after which the level of metHb in the blood gradually declined. Six hours following arrival at the emergency room, his metHb level was 33%. Five days after admission, the patient continued follow-up for a mild poison-induced hemolytic anemia.

Section 3.1 discusses a case report by Myslak et al. (1971) in which a 19-year-old female ~~who~~ consumed approximately 50 mL nitrobenzene. The resulting acute symptoms of toxicity included cyanosis, unconsciousness, and severe methemoglobinemia (82% about 90 minutes after consumption of nitrobenzene), and the patient initially had a distinct smell of bitter almonds on the expired breath. This report is typical of accounts in which subjects have experienced nitrobenzene-induced toxicosis through consuming nitrobenzene-containing substances.

Harrison (1977) described the case of a 19-year-old male who consumed a brown liquid that apparently contained nitrobenzene while pipeting. The time between ingestion and hospital admission was approximately 1.5–2 hours. On examination, the patient was unconscious, his

[3] Therapeutic interventions for methemoglobinemia include the administration of redox scavengers, with ascorbic acid and/or methylene blue. Ascorbic acid infusion results in acidosis, and a resultant shift of the oxygen dissociation curve to the right, which improves oxygen delivery to the tissues. Methylene blue (CASRN 61-73-4) is the antidote of choice for methemoglobinemia. The recommended dose is 1 mg/kg over a period of 5 minutes. At high levels of metHb, methylene blue reduces the half-life of metHb from 15–20 hours to 40–90 minutes. Methylene blue acts as a cofactor to increase erythrocyte reduction of metHb to oxyHb in the presence of NADPH, utilizing the hexose monophosphate shunt pathway. The methylene blue is reduced to leucomethylene blue, which is the electron donor for the nonenzymatic reduction of metHb to Hb (DiSanto and Wagner, 1972).

lips, tongue, and mucous membranes were navy blue, almost black, and his skin was slate gray. A strong smell similar to that of "moth balls" or bitter almond was noted. Profound signs of methemoglobinemia were associated with an initial metHb level of 65% and the characteristic chocolate brown coloration of the blood. The patient underwent gastric lavage and received intravenous administration of methylene blue, ascorbic acid, methylprednisolone, and diazepam. Analysis of gastric aspirate revealed the presence of aniline and nitrobenzene. Approximately 12 hours after admission and following exchange transfusion, the patient's metHb was 25%. Seven days after admission, hemolytic anemia became apparent. Following blood transfusions, the patient ultimately had an uneventful recovery and was discharged after 19 days.

The characteristic signs of acute nitrobenzene poisoning (coma, cyanosis, a smell of bitter almonds on the breath) were evident in a 24-year-old female who had ingested an unreported quantity of nitrobenzene (Ajmani et al., 1986). As in other cases, the patient was responsive to a treatment protocol featuring gastric lavage, intravenous fluids, methylene blue, ascorbic acid, and diuretics. During day 6 of the recovery phase, the subject developed mild jaundice and anemia, yet fully recovered within 2 weeks.

Kumar et al. (1990) described a 21-year-old male who was taken to an intensive care unit approximately 30 minutes after relatives said he consumed 30–40 mL of "varnish," a nitrobenzene-containing dye used in screen printing. On arrival, the patient was in a deeply comatose state with very shallow breathing. Blood samples were obtained that were dark brown in color and a diagnosis of methemoglobinemia was made, secondary to nitrobenzene consumption, when there was no change in the blood sample color after being placed on white filter paper and bubbled with oxygen. Gastric lavage was performed and ascorbic acid and methylene blue were administered intravenously. A second dose of methylene blue was administered after 50 minutes. The patient's metHb measurement was repeated two hours after the second dose of methylene blue and was 5.7%. After the fifth day of admission, the patient was discharged.

Abbinante et al. (1997) reported nine cases of nitrobenzene poisoning in Venezuela between April and July 1993 in people ingesting bitter almond oil containing nitrobenzene. A range of clinical manifestations was observed in affected subjects, including vomiting, dizziness, cyanosis (oral, distal, or general), respiratory depression, convulsions, and generalized weakness. Biochemical findings included anemia, hemolysis, and high levels of metHb. Nuclear magnetic resonance and infrared spectroscopy were used to analyze the almond oil samples and positively confirmed the presence of nitrobenzene.

Two articles by Chongtham et al. (1999, 1997) describe a 24-year-old female whose metHb level was measured as 56.5% as a result of drinking nitrobenzene. The patient was cyanotic, gasping, and had a pulse of 120/minute. In response to the usual range of palliative and

corrective measures (gastric aspiration, lavage, intravenous methylene blue, and ascorbic acid), the subject's metHb level was 5% after 3 days of intensive treatment and care.

Wentworth et al. (1999) described the case of a 2-year-old girl who presented with toxic methemoglobinemia, most likely as a result of consuming a nitrobenzene-containing product. The patient was in shock, with marked cyanosis, a heart rate of 170 beats/minute, blood pressure of 80/50 mm Hg, a respiration rate of 28/minute, and a grade II systolic murmur. While the precise source of the toxicosis remained unknown, nitrobenzene ingestion was suspected and the usual suite of palliative and remedial measures to reduce the patient's 41% metHb level were undertaken. Gupta et al. (2000) reported the case of a 5-year-old boy who died as a result of consuming some screen-printing material that contained nitrobenzene (Gupta et al., 2000). The level of methemoglobinemia was not reported. The patient showed an initial improvement as a result of gastric lavage and oral administration of vitamin C (methylene blue was not given in this case). However, the patient later died of cardiac arrest. Table 4-1 presents a chronological compilation of the cases reported in this section.

Table 4-1. Cases of human poisoning following ingestion of nitrobenzene

Subject(s)	Agent, dose	Symptoms	Treatment	Reference
Male, 5 years	Screen-printing material, unknown quantity	Methemoglobinemia; cardiac arrest and death after initial improvement	Gastric lavage, ascorbic acid	Gupta et al., 2000
Female, 2 years	Unknown substance, unknown quantity	Shock, cyanosis, tachycardia, 41% methemoglobinemia	Methylene blue	Wentworth et al., 1999
Female, 24 years	Nitrobenzene, unknown quantity	Cyanosis, labored breathing, tachycardia	Gastric lavage, methylene blue, ascorbic acid	Chongtham et al., 1999, 1997
Nine cases, adults and children	Bitter almond oil, unknown quantity	Vomiting, dizziness, cyanosis, respiratory depression, convulsions, methemoglobinemia	Not stated	Abbinante et al., 1997
Male, 21 years	Screen-printing varnish, 30-40 mL	Coma, dark brown blood	Gastric lavage, methylene blue, ascorbic acid	Kumar et al., 1990
Female, 24 years	Nitrobenzene, unknown quantity	Coma, cyanosis, bitter almond breath; mild jaundice	Gastric lavage, methylene blue, ascorbic acid, i.v. fluids, diuretics	Ajmani et al., 1986
Male, 48 years	Gunpowder Solvent (2% nitrobenzene), 300 mL	Cyanosis, breathing problems, 75% methemoglobinemia	Methylene blue, blood transfusions	Schimelman et al., 1978
Male, 19 years	Brown liquid, unknown quantity	Unconsciousness, cyanosis, bitter almond breath, 65% methemoglobinemia, hemolytic anemia	Gastric lavage, methylene blue, ascorbic acid, methylprednisolone, diazepam	Harrison, 1977
Female, 19 years	Nitrobenzene, 50 mL	Unconsciousness, cyanosis, bitter almond	Gastric lavage, 2% thionine in glucose	Myslak et al., 1971

		breath, 82% methemoglobinemia	i.v., oxygen, blood transfusions	

4.1.2. Inhalation Exposure

As discussed in Section 3.1, exposure of human research subjects to nitrobenzene vapor resulted in an average absorption of 87% at the blood:gas barrier (Salmowa et al., 1963). However, no case reports were identified that addressed the toxicity of nitrobenzene solely via the inhalation route. For example, the incident described by Ikeda and Kita (1964) most likely also involved dermal contact (Section 3.1). The patient presented with a range of typical symptoms of nitrobenzene toxicosis, including headache, nausea, weakness, hyperalgesia, and cyanosis. The woman had been employed for 17 months in a small paint firm where she painted and polished lids of pans with a red paint containing nitrobenzene as a solvent. The authors determined the nitrobenzene content of the paint solvent to be 97.7% by gas chromatography. Apparently, the workshop was remodeled, and the ventilation became quite poor. The patient started to complain of severe headache, nausea, vertigo, and numbness in the legs approximately 2 months later. After 5 days of bed rest, she returned to work. Nearly 3 months later, the patient experienced the same bout of symptoms, and she was admitted to the hospital the following day. On physical examination, she was emaciated and in a state of distress. Her lips and oral mucosa were cyanotic and the sclerae were slightly jaundiced. The liver and spleen were palpable. During the woman's 2-week stay in the hospital, the nitrobenzene metabolites *p*-amino- and *p*-nitrophenol gradually disappeared from her urine.

4.1.3. Dermal Exposure

A number of case reports exist in which at least a portion of the nitrobenzene dose was absorbed via the dermal route. For example, Stevens (1928) discussed a case in which infant twins were exposed to nitrobenzene contained in a disinfectant that had been applied to their mattress to exterminate bed bugs. The subjects displayed marked cyanosis, rapid pulse rates, and depressed respiration rates, and blood samples revealed the presence of methemoglobinemia. Both subjects made a steady recovery when removed from the source of the contamination.

Levin (1927) discussed the case of a 2-year-old child who was dermally exposed when his mother painted his shoes with a dye containing nitrobenzene. Cyanosis ensued, with rapid pulse and depressed respiration, similar symptoms to those of the infant twins described by Stevens (1928). A sample of blood was extremely dark in color, though metHb was not measured specifically. With the aid of bed rest and occasional oxygen administration, the child recovered once the source of the poisoning had been removed.

Zeligs (1929) reported similar cases involving infants who had been dermally exposed to nitrobenzene or aniline from a laundry mark that had been stamped on their cotton mattress pads.

The infants displayed the typical symptoms of cyanosis and discolored blood. They recovered rapidly when oxygen was administered to aid the restoration of oxyHb levels.

Stevenson and Forbes (1942) reported a case in which an infant developed the characteristic symptoms of nitrobenzene poisoning after the family's living quarters had been treated with an insecticide containing 12.5% nitrobenzene and unstated amounts of kerosene, turpentine, and oil of lilacine, which apparently contaminated the child's crib and mattress. As with the other early cases, it is not clear whether exposure was via inhalation, dermal both routes. The patient presented with marked cyanosis and methemoglobinemia, considerable temperature fluctuations and the appearance of a skin rash. The infant recovered steadily with the aid of oxygen, an intravenous injection of 5% dextrose, and two blood transfusions.

A paper by Zeitoun (1959) discussed 21 cases of cyanotic infants and children who had become sick after being rubbed with fake bitter almond oil that contained nitrobenzene. As in other cases, a range of symptoms including hypoxia, weakness, shock, and, in some cases, excitation or depression accompanied profound methemoglobinemia. Of the 21 cases, 2 subjects died from complications associated with developing bronchopneumonia, while the remaining 19 subjects recovered completely.

A more recent example of methemoglobinemia induced through dermal penetration of nitrobenzene occurred in a 2-month-old baby boy whose mother rubbed his skin with Oleum Dulcis, a topical hair oil containing about 1% nitrobenzene (Mallouh and Sarette, 1993). The typical presentation of bluish coloration of the skin and lips was accompanied by a chocolate colored venous blood sample, in which the metHb level reached 31.5%. The patient was observed without treatment and recovered. A chronological compilation of the case reports involving inhalation and/or dermal exposure to nitrobenzene is presented in Table 4-2.

Table 4-2. Cases of human poisoning with nitrobenzene following inhalation or dermal exposure

Subject(s)	Agent	Symptoms	Treatment	Reference
Male, 2 months	Dermal application of Oleum Dulcis (1% nitrobenzene)	Cyanosis, 31.5% methemoglobinemia	None	Mallouh and Sarette, 1993
Female, 47 years	Paint fumes containing 97.7% nitrobenzene	Cyanosis, headache, nausea, jaundice, hyperalgesia; *p*-aminophenol and *p*-nitrophenol in urine	Glucose i.v., vitamins B1 and B6, iron preparations	Ikeda and Kita, 1964
21 Infants (15 males, 6 females)	Dermal application of false bitter almond oil containing 2–10% nitrobenzene	Shock, tachycardia, cyanosis, hypoxia, coma, weakness, methemoglobinemia; two fatalities	Washing to remove oil, methylene blue, oxygen, ascorbic acid, blood transfusions	Zeitoun, 1959
Infant	Insecticide containing 12.5% nitrobenzene	Cyanosis, methemoglobinemia, skin rash	Oxygen, 5% glucose i.v., blood transfusions	Stevenson and Forbes, 1942

Infants	Laundry marking color containing nitrobenzene	Cyanosis, methemoglobinemia	Oxygen	Zeligs, 1929
Infant twins	Insecticide containing nitrobenzene	Cyanosis, shallow breathing, tachycardia, methemoglobinemia	Removal from exposure source	Stevens, 1928
Male, 2 years	Shoe polish fumes	Cyanosis, shallow breathing, tachycardia, 76% methemoglobinemia	Oxygen, rest	Levin, 1927
16 Cases	Shoe dye fumes	Headache, nausea, dizziness, malaise	NA[a]	Stifel, 1919
Female, adult	Cleaning fluid	Multiple neuritis, contractures, weakness	NA	Adams, 1912 (as cited by Hamilton, 1919)

[a]NA = data not available

4.2. SUBCHRONIC AND CHRONIC STUDIES AND CANCER BIOASSAYS IN ANIMALS—ORAL AND INHALATION

4.2.1. Oral Exposure

4.2.1.1. *Subchronic Studies*

The National Toxicology Program (NTP) sponsored a 90-day oral study of nitrobenzene in which 10 F344 rats/sex/group received 0, 9.38, 18.75, 37.5, 75, and 150 mg/kg-day and 10 B6C3F1 mice/sex/group received 0, 18.75, 37.5, 75, 150, and 300 mg/kg-day by gavage in corn oil (NTP, 1983a). The doses selected were based on the outcome of a 14-day range-finding study in which 10 animals/sex/group received doses from 37.5–600 mg/kg. In the range-finding study, all rats and mice receiving 600 mg/kg-day and all rats and a single mouse receiving 300 mg/kg died prior to planned termination. Toxicological responses to nitrobenzene among the survivors in the range-finding study included depressed body weight gain that was evident in male mice receiving ≥37.5 mg/kg nitrobenzene and in female mice receiving ≥75 mg/kg. Other toxicological endpoints included statistically significant increases in reticulocyte counts and metHb levels. These responses exceeded control levels in treated rats (doses not specified), in male mice at 75 mg/kg and above (reticulocytes) and 150 mg/kg and above (metHb), and in female mice at 75 mg/kg and above (metHb). Histopathologic lesions were observed in brain, liver, lung, kidney, and spleen in rats and mice, though at unstated dose levels.

In the main study, all animals were observed twice daily for clinical signs of toxicity, and body weights and food consumption were monitored weekly. Blood samples were obtained at term to measure hematological parameters, reticulocyte count, and metHb levels, and the weights of the brain, liver, right kidney, thymus, heart, lungs, and right testis were recorded. Necropsies were performed on all animals that died prematurely or were sacrificed at term, and gross examinations of a large suite of organs and tissues were carried out. Tissues were preserved in formalin, and most of those listed were processed for histopathologic examination, primarily all

controls, rats at 75 and 150 mg/kg-day, and mice at the 300 mg/kg-day dose level. Additionally, putative target organs of nitrobenzene toxicity, such as liver, spleen, kidney, lung, brain, bone marrow, testis, epididymis, and uterus, were examined from rats and mice exposed at intermediate dose levels.

Nine male and three female rats at the 150 mg/kg-day dose level died prior to study completion. Clinical signs of toxicity such as ataxia, head tilt, lethargy, and trembling were evident, mostly in animals receiving 150 mg/kg-day and, to a lesser extent, 75 mg/kg-day. Overall, there was little change in body weight gain between control and treated groups, and the final body weights were not significantly different from controls at any dose level. In fact, the only sign of treatment-related body weight reduction was in the single surviving male rat receiving 150 mg/kg nitrobenzene. Organ weights appeared to have been dose dependently affected by nitrobenzene exposure, most notably in the case of liver, kidney, and testis (males). As shown in Tables 4-3 and 4-4, liver weights and their ratios to body weight were dose dependently increased over control levels and achieved statistical significance compared with controls at all dose levels. Right kidney weight was increased over controls at all dose levels, and the ratio of kidney weight to final body weight was significantly increased over controls at the 9.38, 18.75, and 75 mg/kg-day dose levels. Right testis weight and its ratio to body weight were decreased in the 18.75–75 mg/kg dose range.

Table 4-3. Changes in absolute and relative liver, kidney, and testis weights in male F344 rats exposed to nitrobenzene by gavage for 90 days

Dose (mg/kg-day)	Organ weights (mean ± SD)					
	Liver		Kidney		Testis	
	Absolute (mg)	Relative ($\times 10^{-2}$)	Absolute (mg)	Relative ($\times 10^{-3}$)	Absolute (mg)	Relative ($\times 10^{-3}$)
0	11668 ± 1309	3.52 ± 0.22	1025 ± 108	3.10 ± 0.2	1435 ± 96	4.34 ± 0.26
9.38	13269 ± 1555[a]	4.04 ± 0.2[a]	1085 ± 142	3.30 ± 0.2[a]	1435 ± 79	4.39 ± 0.35
18.75	14567 ± 1168[a]	4.37 ± 0.14[a]	1115 ± 83	3.36 ± 0.1[a]	1425 ± 104	4.30 ± 0.23
37.5	15451 ± 1327[a]	4.77 ± 0.22[a]	1070 ± 153	3.30 ± 0.38	1406 ± 71	4.33 ± 0.15
75	15679 ± 2117[a]	5.15 ± 0.15[a]	1083 ± 104	3.44 ± 0.23[a]	873 ± 476[a]	2.78 ± 1.42[a]
150	11264	4.79	1023	4.35	835	3.55

[a]Significantly different from control values, as calculated by the authors.

Source: NTP, 1983a.

Table 4-4. Changes in absolute and relative liver and kidney weights in female F344 rats exposed to nitrobenzene by gavage for 90 days

Dose (mg/kg-day)	Organ weights (mean ± SD)			
	Liver		Kidney	
	Absolute (mg)	Relative ($\times 10^{-2}$)	Absolute (mg)	Relative ($\times 10^{-3}$)
0	6413 ± 613	3.43 ± 0.16	582 ± 56	3.11 ± 0.14
9.38	7402 ± 279[a]	3.76 ± 0.08[a]	615 ± 47	3.13 ± 0.24
18.75	7481 ± 702[a]	3.95 ± 0.18[a]	627 ± 41	3.32 ± 0.14[a]
37.5	8436 ± 587[a]	4.23 ± 0.18[a]	644 ± 52[a]	3.24 ± 0.30
75	9198 ± 713[a]	4.88 ± 0.22[a]	641 ± 68[a]	3.39 ± 0.25[a]
150	9925 ± 436[a]	5.21 ± 0.28[a]	666 ± 40[a]	3.49 ± 0.12[a]

[a]Significantly different from control values, as calculated by the authors.

Source: NTP, 1983a.

There were a number of significant changes in hematological parameters in rats exposed to nitrobenzene via gavage. As shown in Tables 4-5 and 4-6, the principal effects were dose-dependent decreases in hematocrit (Hct), Hb, and RBC count and dose-dependent increases in reticulocyte counts and metHb. In males, these changes achieved statistical significance compared with controls at a dose of 9.38 mg/kg-day for metHb and Hb and 18.75 mg/kg-day for the other parameters. In females, the changes achieved statistical significance compared with controls at 37.5 mg/kg-day and above for the RBC count and at 9.38 mg/kg-day for the other parameters. The authors reported little change in white blood cell (WBC) count and differential except in those rats receiving 150 mg/kg-day, in which a marked leukocytosis appeared to be accompanied by lymphocytosis and a greater number of polymorphonuclear cells.

Table 4-5. Hematological parameters, reticulocytes, and metHb levels in male F344 rats exposed to nitrobenzene via gavage for 90 days

Dose (mg/kg-day)	Hb (g/dL)[a]	Hct (%)[a]	RBCs ($\times 10^6$)[a]	Reticulocytes (%)[a]	MetHb (%)[a]
0	16.24 ± 0.42	47.82 ± 3.2	9.06 ± 0.41	2.23 ± 0.44	1.13 ± 0.58
9.38	15.73 ± 0.29[b]	44.19 ± 4.98	9.01 ± 0.23	2.62 ± 0.45	2.75 ± 0.58[b]
18.75	15.54 ± 0.37[b]	41.84 ± 1.88[b]	8.70 ± 0.37[b]	3.72 ± 0.65[b]	4.22 ± 1.15[b]
37.5	14.72 ± 0.30[b]	37.66 ± 0.93[b]	7.97 ± 0.34[b]	4.75 ± 0.62[b]	5.62 ± 0.85[b]
75	14.87 ± 0.41[b]	38.08 ± 1.96[b]	7.61 ± 0.41[b]	6.84 ± 0.72[b]	7.31 ± 1.44[b]
150	16.2	38	6.31	15	12.22

[a]Values are means ± SD.
[b]Significantly different from controls, as calculated by the authors.

Source: NTP, 1983a.

Table 4-6. Hematological parameters, reticulocytes and metHb levels in female F344 rats exposed to nitrobenzene via gavage for 90 days

Dose (mg/kg-day)	Hb (g/dL)[a]	Hct (%)[a]	RBCs ($\times 10^6$)[a]	Reticulocytes (%)[a]	MetHb (%)[a]
0	15.82 ± 0.22	42.27 ± 3.41	8.39 ± 0.49	2.60 ± 0.37	0.94 ± 0.03
9.38	15.53 ± 0.29[b]	39.37 ± 1.26[b]	8.05 ± 0.28	3.69 ± 0.32[b]	2.06 ± 0.45[b]
18.75	15.49 ± 0.39[b]	39.59 ± 1.79[b]	8.01 ± 0.35	4.75 ± 0.68[b]	3.62 ± 1.09[b]
37.5	15.43 ± 0.38[b]	38.95 ± 0.62[b]	7.83 ± 0.35[b]	6.28 ± 0.90[b]	5.27 ± 0.76[b]
75	14.86 ± 0.52[b]	37.52 ± 1.11[b]	7.33 ± 0.30[b]	8.72 ± 1.49[b]	6.85 ± 2.25[b]
150	15.62 ± 0.60	35.88 ± 1.30[b]	5.86 ± 0.35[b]	32.07 ± 3.56[b]	12.77 ± 1.83[b]

[a]Values are means ± SD.
[b]Significantly different from controls, as calculated by the authors.

Source: NTP, 1983a.

At necropsy, rats receiving 150 mg/kg-day nitrobenzene had enlarged spleens. Males at this dose level had enlarged livers, and those receiving 75 mg/kg-day and 150 mg/kg-day showed signs of testicular atrophy.

Histopathologic examination of the major organs and tissues revealed compound-related effects in the spleen, which appeared to be congested. Splenic corpuscles were small and the red pulp contained hemosiderin. The incidence of these and other histopathologic lesions in relation to dose is shown in Tables 4-7 and 4-8. The extent to which some observed histopathologic effects in the liver were compound-related is unclear, because hematopoietic foci and hepatocellular necrosis were evident in both treated and control rats. Hyaline droplets were noted in the cortical tubule cells of the kidney, and some pigmented granules were evident in the cells of a few treated rats. There were obvious compound-related histopathologic effects on the seminiferous tubules of the testis of male rats. In some cases, the tubules contained spermatogonia and spermatocytes, while in others there were very few or no spermatids, spermatozoa, and Sertoli cells. Some tubules appeared to contain only a lacy fibrinous material, and others contained multinucleate giant cells. Histopathologic changes in the brains of treated rats included hemorrhage, vacuolization, and a wide range of inconsistent degenerative changes.

Table 4-7. Significant histopathology in male F344 rats exposed to nitrobenzene for 90 days via gavage

Tissue examined	Nitrobenzene dose (mg/kg-day)					
	0	9.38	18.75	37.5	75	150
Spleen						
Congestion	1/10	4/10	7/10	6/10	10/10	10/10
Lymphoid depletion	0/10	0/10	0/10	1/10	9/10	10/10
Liver						
Congestion	0/10	0/10	0/10	0/10	0/10	6/10
Testis						
Atrophy	0/10	0/10	0/10	1/10	9/10	9/9
Hypospermatogenesis	0/10	0/10	0/10	0/10	10/10	9/9
Multinucleate giant cells	0/10	0/10	0/10	0/10	10/10	8/9
Brain stem						
Hemorrhage	0/10	1/10	4/10	4/10	5/10	2/10
Vacuolization	7/10	0/10	4/10	0/10	3/10	0/10
Degeneration	0/10	0/10	0/10	0/10	0/10	4/10
Malacia	0/10	0/10	0/10	0/10	0/10	4/10

Source: NTP, 1983a.

Based on the changes in absolute and relative organ weights, the dose-dependent increases in reticulocyte count and metHb concentration and the increased incidence of splenic congestion, all of which were evident at the lowest administered dose, a LOAEL of 9.38 mg/kg-day is appropriate for the subchronic oral effects of nitrobenzene in F344 rats in this study.

Table 4-8. Significant histopathology in female F344 rats exposed to nitrobenzene for 90 days via gavage

Tissue examined	Nitrobenzene dose (mg/kg-day)					
	0	9.38	18.75	37.5	75	150
Spleen						
Congestion	2/10	5/10	10/10	10/10	10/10	10/10
Lymphoid depletion	0/10	0/10	2/10	4/10	8/10	10/10
Kidney						
Pigmentation	0/10	0/10	0/10	0/10	5/10	9/10
Brain stem						
Hemorrhage	4/10	2/10	3/10	1/10	1/10	7/10
Vacuolization	6/10	3/10	1/10	1/10	1/10	5/10
Degeneration	0/10	0/10	0/10	0/10	0/10	4/10
Malacia	0/10	0/10	0/10	0/10	0/10	3/10

Source: NTP, 1983a.

As with the nitrobenzene-exposed rats, the mice exhibited signs of toxicity reflective of neurological impairment, increased liver and kidney weights, and decreased testis weight in male mice or decreased thymus in female mice. Three male B6C3F1 mice receiving 300 mg/kg-day died prior to study completion, most likely as a result of nitrobenzene exposure. Some surviving

animals at this dose level showed clinical signs of toxicity, including ataxia, hyperactivity, and irritability. However, there were no compound-related changes in body weight gain at any dose level. Absolute and relative organ weight changes were confined to liver, kidney, and testis in male mice and to the liver, kidney, and thymus in females. For example, liver weight and its ratio to body weight were dose dependently increased in male mice, the increases achieving statistical significance at the 150 and 300 mg/kg-day dose levels. Relative kidney weight was significantly increased at 75 and 300 mg/kg-day in males. Absolute and relative testis weights were decreased at dose levels of 300 mg/kg-day. Treatment-related increases in absolute liver weights in female mice were increased significantly at 18.75 mg/kg-day and above, with relative liver weights achieving statistical significance at a dose level of 37.5 mg/kg-day and above. Absolute and relative thymus weights were also elevated in nitrobenzene-receiving female mice. These changes are documented in Tables 4-9 and 4-10.

Table 4-9. Changes in absolute and relative liver, kidney, and testis weights in male B6C3F1 mice exposed to nitrobenzene by gavage for 90 days

Dose(mg/kg-day)	Organ weights in mg (mean ± SD)					
	Liver		Kidney		Testis	
	Absolute (mg)	Relative ($\times 10^{-2}$)	Absolute (mg)	Relative ($\times 10^{-3}$)	Absolute (mg)	Relative ($\times 10^{-3}$)
0	1527 ± 286	4.71 ± 0.44	272 ± 35	8.44 ± 0.39	116 ± 7.9	3.66 ± 0.60
18.75	1597 ± 137	4.78 ± 0.27	276 ± 23	8.27 ± 0.45	111 ± 12	3.32 ± 0.34
37.5	1591 ± 129	4.74 ± 0.33	288 ± 22	8.59 ± 0.52	120 ± 8.3	3.60 ± 0.30
75	1709 ± 245	5.02 ± 0.51	300 ± 19[a]	8.84 ± 0.30[a]	113 ± 9.7	3.35 ± 0.31
150	1871 ± 172[a]	5.49 ± 0.33[a]	294 ± 20	8.61 ± 0.31	113 ± 16	3.33 ± 0.52
300	2223 ± 126[a]	6.53 ± 0.55[a]	312 ± 28[a]	9.14 ± 0.58[a]	84 ± 14[a]	2.45 ± 0.42[a]

[a]Significantly different from control values, as calculated by the authors.

Source: NTP, 1983a.

Table 4-10. Changes in absolute and relative liver, kidney, and thymus weights in female B6C3F1 mice exposed to nitrobenzene by gavage for 90 days

Dose (mg/kg-day)	Organ weights (mean ± SD)					
	Liver		Kidney		Thymus	
	Absolute (mg)	Relative ($\times 10^{-2}$)	Absolute (mg)	Relative ($\times 10^{-3}$)	Absolute (mg)	Relative ($\times 10^{-3}$)
0	1179 ± 58	4.41 ± 0.22	175 ± 14	6.53 ± 0.27	44.14 ± 7.82	1.65 ± 0.26
18.75	1278 ± 113[a]	4.64 ± 0.32	179 ± 22	6.52 ± 0.71	51.22 ± 9.94	1.87 ± 0.39
37.5	1276 ± 74[a]	4.79 ± 0.32[a]	180 ± 11	6.74 ± 0.46	47.06 ± 9.47	1.76 ± 0.35
75	1256 ± 75[a]	4.69 ± 0.19[a]	166 ± 15	6.19 ± 0.44	50.41 ± 8.97	1.89 ± 0.38

Dose (mg/kg-day)	Organ weights (mean ± SD)					
	Liver		Kidney		Thymus	
	Absolute (mg)	Relative ($\times 10^{-2}$)	Absolute (mg)	Relative ($\times 10^{-3}$)	Absolute (mg)	Relative ($\times 10^{-3}$)
150	1374 ± 51[a]	5.05 ± 0.14[a]	181 ± 17	6.65 ± 0.56	47.21 ± 13.2	1.73 ± 0.46
300	1566 ± 124[a]	5.79 ± 0.28[a]	189 ± 19	7.00 ± 0.41[a]	51.45 ± 9.19	1.91 ± 0.37

[a]Significantly different from control values, as calculated by the authors.

Source: NTP, 1983a.

Hematological responses observed in mice were similar to those in rats, with dose-dependent increases in reticulocytes and metHb and progressively lower levels of Hb, Hct, and RBC. These changes are documented in Tables 4-11 and 4-12.

Table 4-11. Hematological parameters, reticulocytes, and metHb levels in male B6C3F1 mice exposed to nitrobenzene via gavage for 90 days

Dose (mg/kg-day)	Hb (g/dL) [a]	Hct (%)[a]	RBCs ($\times 10^6$)[a]	Reticulocytes (%)[a]	MetHb (%)[a]
0	15.20 ± 0.66	41.77 ± 2.29	9.27 ± 0.75	5.02 ± 1.0	1.07 ± 0.32
18.75	14.59 ± 0.66	39.76 ± 2.89	8.87 ± 0.50	5.81 ± 0.88	2.16 ± 0.32[b]
37.5	15.02 ± 0.92	41.13 ± 3.48	9.17 ± 0.76	6.95 ± 0.82[b]	3.42 ± 0.61[b]
75	14.63 ± 0.35[b]	39.56 ± 2.66	8.68 ± 0.52	7.85 ± 0.74[b]	4.75 ± 1.03[b]
150	14.44 ± 0.47[b]	37.62 ± 1.94[b]	8.25 ± 0.37[b]	9.30 ± 1.12[b]	5.98 ± 0.97[b]
300	15.45 ± 0.52	36.26 ± 3.30[b]	7.79 ± 0.29[b]	10.45 ± 1.58[b]	6.72 ± 1.28[b]

[a]Values are means ± SD.
[b]Significantly different from controls, as calculated by the authors.

Source: NTP, 1983a.

Table 4-12. Hematological parameters, reticulocytes, and metHb levels in female B6C3F1 mice exposed to nitrobenzene via gavage for 90 days

Dose (mg/kg-day)	Hb (g/dL)[a]	Hct (%)[a]	RBCs ($\times 10^6$)[a]	Reticulocytes (%)[a]	MetHb (%)[a]
0	15.66 ± 0.61	44.33 ± 3.41	9.54 ± 0.67	4.17 ± 0.35	0.87 ± 0.23
18.75	15.70 ± 0.60	44.24 ± 2.32	9.52 ± 0.35	5.54 ± 0.51[b]	1.20 ± 0.22[b]
37.5	15.24 ± 0.83	43.86 ± 2.30	9.21 ± 0.60	6.29 ± 0.61[b]	1.45 ± 0.34[b]
75	14.98 ± 0.50[b]	41.66 ± 1.71[b]	9.06 ± 0.44	6.72 ± 0.60[b]	1.82 ± 0.30[b]
150	14.96 ± 0.33[b]	40.98 ± 2.24[b]	8.81 ± 0.35[b]	7.31 ± 0.48[b]	2.25 ± 0.40[b]
300	15.99 ± 0.59	38.66 ± 2.69[b]	8.11 ± 0.61[b]	11.08 ± 1.96[b]	3.54 ± 1.39[b]

[a]Values are means ± SD.
[b]Significantly different to controls, as calculated by the authors.

Source: NTP, 1983a.

There were few signs of treatment-related lesions in the mice at necropsy, although some evidence of a darkening in coloration of such organs as kidney, lung, spleen, adrenal, and lymph nodes was noted in animals exposed to 300 mg/kg-day nitrobenzene. As summarized in Tables 4-13 and 4-14, there were not many histopathologic changes, and those that were observed may have been unrelated to the effects of the compound. However, enlargement of hepatocytes in the centrilobular zone in male and female mice exposed to 300 mg/kg-day was noteworthy.

Table 4-13. Significant histopathology in male B6C3F1 mice exposed to nitrobenzene for 90 days via gavage

Tissue examined	Nitrobenzene dose (mg/kg-day)					
	0	18.75	37.5	75	150	300
Spleen						
Lymphoid depletion	0/10	0/10	0/10	0/10	0/10	1/10
Liver						
Cytomegaly	0/10	0/10	0/10	1/10	2/10	10/10
Testis						
Atrophy	0/10	3/10	2/10	0/10	5/10	5/10
Hypospermatogenesis	0/10	0/10	0/10	0/10	0/10	4/10
Multinucleate giant cell	0/10	0/10	0/10	0/10	0/10	2/10
Brain stem						
Hemorrhage	3/10	1/10	3/10	0/10	0/10	2/10
Degeneration	0/10	0/10	0/10	0/10	0/10	1/10

Source: NTP, 1983a.

Table 4-14. Significant histopathology in female B6C3F1 mice exposed to nitrobenzene for 90 days via gavage

Tissue examined	Nitrobenzene dose (mg/kg-day)					
	0	18.75	37.5	75	150	300
Spleen						
Lymphoid depletion	0/10	0/10	0/10	0/10	2/10	5/10
Liver						
Cytomegaly	0/10	0/10	0/10	0/10	0/10	8/10
Adrenal						
Fatty change	0/10	0/10	0/10	0/10	0/10	8/10
Brain stem						
Hemorrhage	2/10	2/10	1/10	2/10	0/10	3/10

Source: NTP, 1983a.

The statistically significant increase in metHb concentration observed in both sexes of B6C3F1 mice at the lowest dose level tested points to a dose of 18.75 mg/kg-day as LOAEL for the subchronic effects of nitrobenzene in this species when administered via the oral route. Support for this designation is provided by the clear-cut trend in increased reticulocytes, which was statistically significant from controls in females receiving 18.75 mg/kg-day. While the increase in reticulocytes did not achieve statistical significance at the lowest dose level in males, the value appeared to be part of a dose-dependent trend toward a statistical significance that was evident at higher dose levels. This supports the choice of 18.75 mg/kg-day as LOAEL for this response in B6C3F1 mice.

Shimo et al. (1994) gavaged six F344 rats/sex/group with 0, 5, 25, and 125 mg/kg-day nitrobenzene for 28 days. An additional set of control and 125 mg/kg rats were allowed to recover for 14 days after the completion of treatment. As determined from the English data

tables, animals were evaluated for generalized signs of toxicity, and body weight changes and food consumption were monitored in all groups. Blood samples were taken at term for hematological and clinical chemistry parameters. Major organs were weighed at term, and tissue samples were fixed and processed for histopathologic examination.

Clinical signs in high-dose rats included decreased movement, pale skin, and abnormal gait. Additionally, the authors plotted the body weight changes against time and showed a marked treatment-related reduction in body weight increase, even though food consumption was little changed among the groups. Striking changes in hematological parameters were evident in nitrobenzene-treated rats, with dose-dependent reductions in RBC count, Hct, Hb concentration, and a dose-dependent increase in mean corpuscular volume (MCV). By contrast, the WBC count increased dramatically with dose. However, these changes were not noted in those animals allowed to recover for 14 days after dosing (Table 4-15).

Table 4-15. Hematological and clinical chemistry parameters in rats treated with nitrobenzene for 28 days, with or without a recovery period of 14 days

Parameter	28-Day dosing study[a]				14-Day recovery group[a]	
	Control	5 mg/kg	25 mg/kg	125 mg/kg	Control	125 mg/kg
Males						
RBC ($\times 10^4$/mm^3)	761 ± 117	670 ± 54	524 ± 36[b]	412 ± 54[b]	727 ± 93	724 ± 100
Hb (g/dL)	16.9 ± 0.6	16.6 ± 0.6	14.5 ± 0.5[b]	14.2 ± 0.5[b]	16.7 ± 0.7	17.7 ± 0.6[c]
Hct (%)	41.6 ± 6.3	35.6 ± 3.3	32.3 ± 2.4[c]	34.9 ± 3.4[c]	38.2 ± 4.9	45.7 ± 6.6[c]
MCV (fL)	54.7 ± 0.8	53.0 ± 0.9	61.3 ± 2.7[b]	84.8 ± 5.5[b]	52.7 ± 1.4	63.0 ± 1.4
WBC ($\times 10^2$/mm^3)	44 ± 14	45 ± 8	122 ± 44[b]	1426 ± 521[b]	46 ± 5	40 ± 16
BUN (mg/dl)	17.8 ± 1.1	16.1 ± 1.5[c]	14.1 ± 2.4[b]	12.7 ± 1.2[b]	16.8 ± 2.5	17.5 ± 1.1
AST (IU/l)	111± 14	81 ± 6[b]	86 ± 6[b]	105 ± 17	89 ± 9	94 ± 10
ALT (IU/l)	40 ± 6	43 ± 5	38 ± 4	47 ± 9	35 ± 7	37 ± 4
Females						
RBC ($\times 10^4$/mm^3)	708 ± 63	718 ± 129	635 ± 126	458 ± 43[b]	694 ± 79	674 ± 86
Hb (g/dL)	17.5 ± 0.9	16.3 ± 1.0	15.5 ± 0.6[b]	14.5 ± 0.8[b]	16.8 ± 0.4	18.0 ± 1.2
Hct (%)	38.1 ± 3.2	37.8 ± 6.5	37.7 ± 7.4	35.4 ± 3.4	36.7 ± 4.6	39.5 ± 5.1
MCV (fL)	53.8 ± 1.2	52.7 ± 0.5	59.5 ± 1.6[b]	77.2 ± 1.6[b]	52.8 ± 0.8	58.3 ± 5.2[c]
WBC ($\times 10^2$/mm^3)	40 ± 12	43 ± 8	73 ± 44	1990 ± 298[b]	42 ± 4	47 ± 6
BUN (mg/dl)	17.5 ± 2.2	14.2 ± 1.0[b]	12.8 ± 2.2[b]	12.3 ± 3.4[c]	18.9 ± 3.9	16.8 ± 1.9
AST (IU/l)	96± 5	79 ± 5[b]	85 ± 9[c]	94 ± 10	77 ± 5	79 ± 5
ALT (IU/l)	39 ± 5	36 ± 4	42 ± 8	53 ± 14[c]	40 ± 2	31 ± 5[b]

[a]Values are means ± SD for six animals/group.
[b]$p<0.01$ versus controls as calculated by the authors.
[c]$p<0.05$ versus controls as calculated by the authors.

Source: Shimo et al., 1994.

Treatment-related changes in clinical chemistry parameters were also evident; a consistent dose-dependent decrease in blood urea nitrogen (BUN) was evident in both males and females (Table 4-15). Serum transaminase activities (e.g., aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) were inconclusive. AST activity was significantly decreased in male and female rats receiving either 5- or 25-mg/kg nitrobenzene; however, the biological relevance of the observed decrease is questionable, especially since no change was observed between the high dose (125 mg/kg) animals and controls. Similarly, a statistically significant increase in ALT was observed only in female rats receiving the high dose. This finding was also of questionable relevance as the values were only 14% higher than controls. Hematology parameters and serum BUN concentrations returned to control levels after a 14-day recovery period.

Absolute changes in organ weights exhibited similar trends between male and female rats with increases noted for the spleen, liver, and kidney, and decreases found with the thymus and adrenals of both sexes, and testis in males. A strong dose-dependent increase in absolute spleen weight was observed with males and females with a nearly 4-fold increase at the highest dose for both sexes. Absolute liver weight increased dose dependently in female rats up to 80% with the highest dose, whereas a 19% increase was observed in male rats at the highest dose. In contrast to the spleen and liver, increases in absolute kidney weights did not exhibit clear dose dependent responses. In male rats, an 8% increase was observed with the 25-mg/kg group; however, kidney weights from high dose animals (125 mg/kg) were consistent with controls. In contrast, absolute kidney weight in female rats was only increased (13%) at the highest dose with all other groups being similar to controls. Following the 14-day recovery period, the absolute spleen weights for male and female rats were still increased by 37% in males and 26% in females, whereas absolute liver and kidney weights returned to control values. Decreases in absolute thymus weight occurred with both male (27% ↓) and female (30% ↓) rats at the highest dose, but returned to control values at the end of the 14-day recovery period. Absolute testis weights were significantly reduced (70% ↓) in high-dose males and remained reduced by 46% at the end of the recovery period.

Histopathological evaluation of tissues was used to corroborate changes in tissue weight and clinical chemistry with severity of response (Grade: ‘no change’ < ‘moderate’ < ‘severe’). In male rats, graded responses for splenic congestion, increased brown pigmentation in red pulp, and increased extramedullary hematopoiesis exhibited a dose-dependent increase in grade, with 100% of animals being scored as ‘severe’ at the highest dose tested. Female rats exhibited a similar dose dependent increase in severity of scores for the above indices with 100% of animals being graded as ‘severe’ for splenic congestion and increased extramedullary hematopoiesis. Increased brown pigmentation in red pulp was graded as ‘severe’ in 2, and ‘moderate’ in 3

female rats. Liver scores were graded as 'no change' in all groups, except for the high dose animals in both sexes. In high dose males and females, increased extramedullary hematopoiesis was 'moderate' in 5 males and 2 females, and exhibited 'no change' in 1 male and 3 females. Brown pigmentation in Kupffer cells was 'moderate' in 5 males and 4 females, and 'severe' in 1 male and 1 female. Absolute kidney weights in males were inconsistent with the histopathological finding. 'No changes' were found for brown pigmentation in tubular epithelium, except for the high dose group, with 5 animals being graded as 'moderate' and 1 being graded as 'severe' (Cf. no change in absolute kidney weight at 125 mg/kg). In contrast, female histopathology of the kidney correlated with the absolute weight in that 100% of animals were graded with 'moderate' brown pigmentation in tubular epithelium at the highest dose (Cf. 13% increase in absolute kidney weight at 125 mg/kg). All other animals were consistent with controls ('no changes'). Decreased absolute testis weight correlated with 'severe' degeneration of seminiferous tubular epithelium and 'severe' atrophy of seminiferous tubule in 100% of male rats receiving 125-mg/kg nitrobenzene.

A synopsis of the oral toxicity studies in animals is presented in Table 4-16.

Table 4-16. Summary of effects observed in oral dosing studies with nitrobenzene*

Species, strain	Number	Dosing	Effect[a]	NOAEL (mg/kg-day)[b]	LOAEL (mg/kg-day)[b]	Reference
Rat, F344	6/sex	0, 5, 25, 125 mg/kg-day, gavage, 4 wk	RBC ↓, Hct ↓, MCV ↓, WBC ↑	5 (M, F)	25 (M, F)	Shimo et al., 1994
Rat, F344	10/sex	0, 9.4, 18.8, 37.5, 75, 150 mg/kg-day, gavage, 90 d	Liver weight ↑ Kidney weight↑ MetHb ↑ & Hb ↓ Reticulocytes ↑ Splenic congestion	NA NA (M) 9.4 (F) NA 9.4 (M) NA (F) NA	9.4 (M, F) 9.4 (M) 18.8 (F) 9.4 (M, F) 18.8 (M) 9.4 (F) 9.4 (M, F)	NTP, 1983a
Mouse, B6C3F1	10/sex	0, 18.8, 37.5, 75, 150, 300 mg/kg-day gavage, 90 d	Liver weight ↑ MetHb ↑ Hb ↓ Reticulocytes ↑ Liver cytomegaly	75 (M) NA (F) NA (M, F) 37.5 (M, F) 18.8 (M) NA (F) 150 (M, F)	150 (M) 18.8 (F) 18.8 (M, F) 75 (M, F) 37.5 (M) 18.8 (F) 300 (M, F)	

*NOAELs and LOAELs determined by nitrobenzene assessment authors.

[a]Only endpoints with evident dose responses were selected. ↓ or ↑ = a decrease or increase in the respective endpoint.
[b]M = male; F = female; F_0 = parental generation.

4.2.1.2. *Chronic Studies*

No studies were identified that addressed the chronic toxicity of nitrobenzene when administered via the oral route.

4.2.2. Inhalation Exposure

4.2.2.1. *Subchronic Studies*

CIIT (1984) reported a subchronic study in which F344 rats, CD rats, and B6C3F1 mice, 10/sex/group, were exposed via inhalation to 0, 5, 16, or 50 ppm nitrobenzene, 6 hours/day, 5 days/week for 90 days. During the in-life phase of the 90-day study, behavioral signs were observed twice daily, and body weights were monitored weekly. At the end of the 90-day exposure period, animals were fasted overnight, then sacrificed following i.p. injection with pentobarbital. Samples of blood were taken to measure hematological and clinical chemistry parameters. Animals were examined for gross abnormalities at necropsy, and the weights of certain key target organs, such as the spleen, liver, kidney, testes, and brain, were recorded. Eight-hour urine samples were obtained from all animals after 60 days of exposure. Among the parameters assessed were color, turbidity, specific gravity, pH, protein, glucose, ketones, bilirubin, blood, and the presence of cells, casts, and crystals. Histopathologic examination was carried out in a full range of excised organs and tissues, including the epithelium lining the air passages of the nose and lungs.

There were no compound-related effects on body weight, mortality, or the occurrence of behavioral signs in the subchronic 90-day study. However, increases in spleen weights were evident in all strains and sexes of rats and mice exposed to nitrobenzene at the high concentration and at 16 ppm in male F344 and CD rats. By contrast, there was a statistically significant reduction in testis weight in male F344 rats exposed to 50-ppm nitrobenzene. Examination of the internal organs of exposed animals at necropsy confirmed that the liver, spleen, and testis were the primary target organs of nitrobenzene. For example, in high-concentration rats of either strain, males presented with testicular atrophy, enlarged spleens, and the presence of irregular blotches on the surface of the liver. Similarly, both sexes of B6C3F1 mice had enlarged spleens in response to nitrobenzene at 50 ppm.

A number of statistically significant changes occurred in the hematological parameters under investigation, but all were not obviously related to exposure concentration. However, in the rats, there was an increased incidence of hemolytic anemia in response to increased

concentrations of nitrobenzene. Most marked among the potential compound-related changes in hematological or clinical chemistry parameters were the increased concentrations of serum metHb (Table 4-17), and increases in the concentration of bilirubin in male F344 rats receiving 16 and 50 ppm nitrobenzene. Histologic sections of organs and tissues of nitrobenzene-receiving rats and mice demonstrated treatment-related lesions in the spleen, testis, liver, epididymides, kidney, and bone marrow, plus other possible target organs of nitrobenzene, such as the adrenals, lymph nodes, and lungs. For example, in F344 rats, lesions in the spleen consisted of acute sinusoidal congestion, proliferative capsular lesions, and increases in extramedullary hematopoiesis. These effects were dose-dependent with 10/10 animals of either sex affected in F344 rats exposed to 50 ppm.

Table 4-17. Concentrations of metHb in plasma of F344 and CD rats and B6C3F1 mice in response to nitrobenzene inhalation

Species/strain/sex		Concentration of nitrobenzene (ppm)			
		0	5	16	50
		Concentration of metHb in plasma (%)			
F344	males	1.2 ± 0.4	3.0 ± 1.0[a]	4.4 ± 1.3[a]	10.1 ± 1.2[a]
	Females	1.6 ± 0.8	3.2 ± 0.9	3.9 ± 1.3[a]	10.5 ± 1.5[a]
CD	males	0.6 ± 0.2	0.9 ± 0.6	3.2 ± 0.7[a]	10.1 ± 2.0[a]
	Females	2.1 ± 1.2	2.3 ± 0.6	3.7 ± 0.2	9.6 ± 2.5[a]
B6C3F1	males	0.7 ± 0.6	1.6 ± 0.4	2.1 ± 1.3	5.8 ± 1.7[a]
	Females	1.3 ± 0.9	0.8 ± 0.5	2.0 ± 0.6	5.1 ± 0.8[a]

[a] $p<0.05$, as calculated by the authors.

Source: CIIT, 1984.

Histopathologic effects of nitrobenzene on the liver in F344 rats included disorganization of the hepatic cord architecture and centrilobular degeneration of the hepatocytes in 7/10 high-concentration males but only in 1/10 high-concentration females. Other histopathologic effects evident in F344 rats included basophilia of the medullary cells of the adrenal in 5/10 high-concentration males and in 3/10 high-concentration females, plus an increased incidence of bronchial hyperplasia in both sexes receiving the highest dose. All male F344 rats displayed degeneration of tubular epithelial cells in the testis. The condition was described by the authors as representing a cessation of maturation at the level of primary and secondary spermatocytes and was usually accompanied by interstitial edema and hyperplasia of Leydig cells. There were no mature sperm in the epididymis of these F344 rats. Instead, the presence of some apparently proteinaceous material was noted in the ducts. Kidney effects of nitrobenzene in F344 rats were

characterized by a toxic nephrosis associated with an accumulation of droplets in the cytoplasm of proximal tubular epithelial cells. The droplets were described in the report as hyaline and eosinophilic, and the lesions increased in incidence and severity with dose in both sexes of F344 rats. The report makes no mention of whether or not kidney sections were stained for the male rat-specific protein, α_{2u}-globulin. In the absence of this information, and in view of the appearance of kidney lesions in both sexes of F344 rat, the kidney responses cannot be assigned to α_{2u}-globulin-associated nephropathy (U.S. EPA, 1991b).

Many of the target organs indicative of nitrobenzene toxicity in F344 rats also were target organs in CD rats, including spleen, liver, kidney, epididymis, bone marrow, and nasal turbinates. For example, the splenic lesions consisted of sinusoidal congestion, increased extramedullary hematopoiesis, and numbers of hemosiderin-laden macrophages infiltrating the red pulp. An increase in the thickness of the splenic capsule was noted in 4/10 males and 3/10 female CD rats exposed to 50-ppm nitrobenzene. CD rats also displayed a marked bilateral testicular atrophy in response to nitrobenzene, as indicated by a loss of seminiferous epithelium, interstitial cell hyperplasia, edema, and the absence of mature sperm in the epididymal lumen. These features were evident in 1/10 subjects receiving 5-ppm nitrobenzene, 2/10 receiving 16 ppm, and 9/10 receiving nitrobenzene at the highest concentration. Toxic effects of nitrobenzene were particularly apparent in the nasal passages of CD rats. These lesions were characterized by the occurrence of lymphoid hyperplasia, inflammation, and the presence of interstitial and granulomatous pneumonitis, together with the presence of macrophages and lymphocytes in perivascular areas. In a manner similar to F344 rats, CD rats displayed dose-dependent toxic nephrosis, with 10/10 male and 5/10 female rats exposed to 50-ppm nitrobenzene displaying this condition.

The adrenal gland, liver, and spleen were also target organs of nitrobenzene in B6C3F1 mice, as judged by the range of histopathologic lesions observed in the study. In the liver, instances of centrilobular hyperplasia were noted in mid- (4/9) and high-concentration males (9/9), compared with 7/9 high-dose females displaying these lesions. Table 4-18 provides a summary of the identified LOAELs for rats and mice.

Table 4-18. Summary of effects observed in subchronic inhalation studies with nitrobenzene*

Species, strain	Number	Dosing	Effect[a]	NOAEL (ppm)[b]	LOAEL (ppm)[b]	Reference

Rat, F344	10/sex	0, 5, 16, 50 ppm, 6 hr/d, 5 d/wk, 90 d	Methemoglobinemia ↑ Organ weight, ↑ Splenic congestion ↑ Testicular pathology ↑	NA (M) 5 (F) 5 (M, F) NA (M, F) NA	5 (M) 16 (F) 16 (M, F) 5 (M, F) 5	CIIT, 1984
Rat, CD			Methemoglobinemia ↑ Spleen weight, ↑ Splenic congestion ↑ Liver weight, ↑ Testicular pathology ↑	5 (M) 16(F) 16 (M) 5 (F) NA 5 (M, F) 5 (M)	16 (M) 50 (F) 50 (M) 16 (F) 5 (M, F) 16 (M, F) 16 (M)	
Mouse, B6C3F1			Methemoglobinemia ↑ Splenic congestion ↑	16 (M, F) 5 (M) NA (F)	50 (M, F) 16 (M) 5 (F)	

*NOAELs and LOAELs determined by nitrobenzene assessment authors.

[a]Only endpoints with evident dose responses were selected. ↑ = an increase in the respective endpoint.

[b]M = male; F = female; S-D = Sprague-Dawley.

4.2.2.2. *Chronic Studies*

A chronic inhalation study of nitrobenzene was conducted in F344 rats, Sprague-Dawley (CD) rats, and B6C3F1 mice (Cattley et al., 1994; CIIT, 1993). A total of 70 male and female F344 rats and 70 male Sprague-Dawley (CD) rats were exposed to 0, 1, 5, or 25 ppm nitrobenzene, and a total of 70 male and female B6C3F1 mice were exposed to 0, 5, 25, or 50 ppm nitrobenzene, 6 hours/day, 5 days/week, excluding holidays, for 2 years, resulting in a total of 505 exposures. Animals were observed for clinical signs twice daily, with body weights determined weekly for the first 13 weeks and twice weekly thereafter. Ten rats/sex/strain/group were terminated 15 months into the study to provide samples for an interim evaluation of hematological parameters. For the scheduled interim and final sacrifices, animals were fasted overnight, weighed, and then anaesthetized using an intraperitoneal injection of pentobarbital prior to exsanguination. Among the hematological parameters evaluated were WBC counts, RBC counts, Hb, Hct, MCV, MCHb, red cell distribution width, and platelet count. In addition, a percentage metHb value was determined, and the relative and absolute differential cell counts were determined microscopically. A wide range of tissues from high-concentration and control

animals and all gross lesions were processed for histopathologic examination. Tissues considered to be specific targets of nitrobenzene, such as liver, spleen, and nose, were examined microscopically in all exposure groups. Additional tissues were examined where significant findings of toxicity had become evident in the high dose group.

Effects of nitrobenzene on clinical signs, body weight changes, and survival appeared to be sporadic and unrelated to dose. For example, during the first 2 weeks of exposure, nine B6C3F1 mice died (across all exposure groups) and were replaced with substitutes of the same age and shipment. Animals that died after the first 2 weeks of the study were not replaced. As noted by the authors, the percentages of animals surviving to term were 60% and 45% in male and female mice, 75% and 80% in male and female F344 rats, and 40% in the male CD rats, thus providing sufficient statistical power to support conclusions about the incidence of any late developing neoplastic lesions that became apparent at necropsy (Cattley et al., 1994; CIIT, 1993).

A summary of the positive findings of tumor formation in the study in animals with two years of exposure is shown in Table 4-19. Animals sacrificed at 15 months (interim) were not included in the analysis because they were deliberately removed from the study, rather than being removed due to nitrobenzene-induced effects. In male F344 rats, the incidence of combined adenomas and carcinomas in liver displayed a statistically significant trend and an increased incidence with dose (16/46 in males receiving 25 ppm compared with 1/43 in controls). However, this effect was not apparent in female F344 rats. Similarly, statistically significant trends for dose-dependent increases in combined adenomas and carcinomas in kidney and thyroid were observed in male F344 rats but not in females. However, there was a dose-dependent trend and statistically significant increase in the incidence of endometrial polyps in female F344 rats (19/49 in rats exposed to 25 ppm versus 9/48 in controls). The only compound-related tumorigenic effect in CD rats was in males that showed statistically significant increases in the incidences of combined adenomas and carcinomas in liver (5/23 in 25 ppm rats versus 0/23 in controls). As set forth in Table 4-19, there was a possible compound-related increase in the incidence of combined adenomas and carcinomas in the follicular cells of the thyroid in male B6C3F1 mice. Other neoplastic responses to nitrobenzene observed in the mice included the formation of adenocarcinomas of the mammary gland and an increased incidence of combined adenomas and carcinomas of the lungs in males.

A number of noncarcinogenic responses to nitrobenzene were observed in the study (Cattley et al., 1994; CIIT, 1993). Both male and female F344 rats in the 25-ppm group displayed treatment-related statistically significant reductions in RBCs, Hct, and Hb concentration, with mean levels that were lower in animals sacrificed at term compared with animals sacrificed at 15 months. Concentrations of metHb increased with increasing nitrobenzene exposure, though time-related trends in this parameter were less clear-cut. Most

notable among the hematological responses in CD rats were the increases in metHb in the 15-month interim blood samples, as shown in Table 4-20. These achieved statistical significance (p<0.01) versus controls at all dose concentrations employed in the study. No histopathology was performed on the spleens of CD rats at interim or final sacrifice to determine if effects in the spleen accompanied the statistically significant increase in metHb levels. It should be noted, however, that at final sacrifice, metHb levels were only increased in the 25-ppm exposure group, which indicated a compensatory response to metHb formation.

Table 4-19. Summary of neoplastic and nonneoplastic findings following 2-year inhalation exposure to nitrobenzene

Site of increased tumorigenicity	Sex with positive carcinogenic response	Comments on neoplastic and/or nonneoplastic lesions[a]
B6C3F1 mouse		
Lung: A/B adenoma or carcinoma	M	Neoplastic ♂ Significantly positive nitrobenzene exposure-related trend in incidence;[b] Statistically significant difference in incidence for all treated groups versus controls.[c] Nonneoplastic ♂ A significantly positive nitrobenzene exposure-related trend in incidence for A/B hyperplasia and bronchiolization was observed;[b] a statistically significant difference from incidence of A/B hyperplasia in controls (1%) versus 25 ppm- (12%) and 50 ppm-dose (20%) animals occurred;[c] a statistically significant difference from incidence of bronchiolization in controls (0%) versus 5 ppm- (87%), 25 ppm- (89%), and 50 ppm-dose (94%) animals occurred.[c] Nonneoplastic ♀ A significantly positive nitrobenzene exposure-related trend in incidence for bronchiolization was observed;[b] a statistically significant difference from incidence of A/B hyperplasia in controls (0%) versus 25 ppm-dose (8%) animals occurred;[c] a statistically significant difference from incidence of bronchiolization in controls (0%) versus 5 ppm- (92%), 25 ppm- (98%), and 50 ppm-dose (100%) animals occurred.[c]
Thyroid: follicular cell adenoma	M	Neoplastic ♂ Significantly positive nitrobenzene exposure-related trend in incidence;[b] statistically significant difference in incidence for 50 ppm-dose group versus controls.[c] Nonneoplastic ♂ A significantly positive nitrobenzene exposure-related trend in incidence for follicular cell hyperplasia was observed;[b] a statistically significant difference from incidence of follicular cell hyperplasia in controls (2%) versus 25 ppm- (11%) and 50 ppm-dose (19%) animals occurred.[c] Nonneoplastic ♀ A significantly positive nitrobenzene exposure-related trend in incidence for follicular cell hyperplasia was observed;[b] a statistically significant difference from incidence of follicular cell hyperplasia in controls (4%) versus 50 ppm-dose (13%) animals occurred.[c]

Table 4-19. Summary of neoplastic and nonneoplastic findings following 2-year inhalation exposure to nitrobenzene

Site of increased tumorigenicity	Sex with positive carcinogenic response	Comments on neoplastic and/or nonneoplastic lesions[a]
Mammary gland	F	Neoplastic ♀ Statistically significant difference in incidence for 50 ppm-group versus controls;[c] 25 ppm- and 5 ppm- groups were not examined.
Liver: hepatocellular adenoma	F	Neoplastic ♀ Significantly positive nitrobenzene exposure-related trend in incidence.[b] Nonneoplastic ♀ A significantly positive nitrobenzene exposure-related trend in incidence for centrilobular hepatocytomegaly was observed;[b] a statistically significant difference from incidence of centrilobular hepatocytomegaly in controls (0%) versus 50 ppm-dose (11%) animals.[c] Nonneoplastic ♂ A significantly positive trend in incidence of centrilobular hepatocytomegaly and multinucleated hepatocytes was observed;[b] a statistically significant difference from incidence of centrilobular hepatocytomegaly in controls (1%) versus 5 ppm- (23%), 25 ppm- (68%), and 50 ppm- (89%) animals occurred;[c] a statistically significant difference from incidence of multinucleated hepatocytes in controls (3%) versus 5 ppm- (22%), 25 ppm- (69%), and 50 ppm- (88%) animals occurred.[c]
F344/N rat		
Liver: hepatocellular adenoma or carcinoma	M	Neoplastic ♂ Significantly positive nitrobenzene exposure-related trend in incidence[b]; statistically significant difference in 25 ppm- group versus control.[c] Nonneoplastic ♂ A significantly positive trend in incidence of eosinophilic foci and centrilobular hepatocytomegaly was observed;[b] a statistically significant difference from incidence of eosinophilic foci in controls (42%) versus mid- (63%) and high-dose (81%) animals occurred;[c] a statistically significant difference from incidence of centrilobular hepatocytomegaly in controls (0%) versus 5 ppm- (11%) and 25 ppm- (81%) animals occurred.[c]

Table 4-19. Summary of neoplastic and nonneoplastic findings following 2-year inhalation exposure to nitrobenzene

Site of increased tumorigenicity	Sex with positive carcinogenic response	Comments on neoplastic and/or nonneoplastic lesions[a]
	F	Neoplastic ♀ Significantly positive nitrobenzene exposure-related trend in incidence.[b] Nonneoplastic ♀ A significantly positive trend in incidence of eosinophilic foci was observed;[b] a statistically significant difference occurred in the 25 ppm-dose (23%) versus controls (9%).[c]
Thyroid: follicular cell adenoma or adenocarcinoma	M	Neoplastic ♂ Significantly positive nitrobenzene exposure-related trend in incidence.[b] Nonneoplastic ♂ A significantly positive trend in incidence of follicular cell hyperplasia was observed.[b]
Kidney: tubular adenoma or carcinoma	M	Neoplastic ♂ Significantly positive nitrobenzene exposure-related trend in incidence;[b] statistically significant difference in incidence for 25 ppm- group versus controls.[c] Nonneoplastic ♂ A significantly positive trend in incidence of tubular hyperplasia was observed;[b] a statistically significant difference occurred in the 25 ppm- group (19%) versus controls (3%).[c]
Endometrial stromal polyp	F	Nonneoplastic ♀ Significantly positive nitrobenzene exposure-related trend in incidence;[b] a statistically significant difference in incidence for 25 ppm- group versus controls.[c]

Table 4-19. Summary of neoplastic and nonneoplastic findings following 2-year inhalation exposure to nitrobenzene

Site of increased tumorigenicity	Sex with positive carcinogenic response	Comments on neoplastic and/or nonneoplastic lesions[a]
Sprague-Dawley rat		
Liver: hepatocellular adenoma or carcinoma	M	Neoplastic ♂ Significantly positive nitrobenzene exposure-related trend in incidence;[b] statistically significant difference in incidence for 25 ppm- group versus controls.[c] Nonneoplastic ♂ A significantly positive trend in incidence of eosinophilic foci and centrilobular hepatocytomegaly was observed;[b] a statistically significant difference from incidence of centrilobular hepatocytomegaly in controls (5%) versus 5 ppm- (20%) and 25 ppm- (60%) animals occurred.[c]

[a]The sex of the animal is the same as the sex that exhibited a positive carcinogenic response, unless indicated otherwise (male ♂ or female ♀).
[b]Cochran-Armitage trend test, $p<0.05$, as calculated by the study authors.
[c]Fisher Exact test, $p<0.05$, as calculated by the study authors.

Sources: Cattley et al., 1994; CIIT, 1993.

In mice, RBCs and Hct were significantly lower in 50-ppm males than in controls (8.70 ± 0.12 versus $9.61 \pm 0.29 \times 10^6$ cells/μL and 41.64 ± 0.52 versus $45.06 \pm 1.15\%$, respectively). In common with the rats, however, there were statistically significant increases in metHb concentrations in high-dose mice of both sexes compared with controls (Table 4-20).

Table 4-20. Percentage metHb formation in response to inhaled nitrobenzene

Treatment group	MetHb (%)			
	Interim sacrifice (15 months)		Terminal sacrifice (24 months)	
	Males	Females	Males	Females
B6C3F1 mice				
0	NA[a]	NA	1.97 ± 0.24	1.39 ± 0.20
5	NA	NA	1.94 ± 0.34	1.37 ± 0.18
25	NA	NA	3.02 ± 0.41	2.22 ± 0.26[b]
50	NA	NA	3.97 ± 0.48[c]	2.79 ± 0.24[c]
F344 rats				
0	2.90 ± 0.31	2.35 ± 0.36	3.88 ± 0.33	2.68 ± 0.37
1	3.21 ± 0.18	3.33 ± 0.40	3.31 ± 0.32	2.13 ± 0.16
5	3.18 ± 0.43	3.17 ± 0.39	4.19 ± 0.53	2.54 ± 0.30
25	4.73 ± 0.52[c]	5.90 ± 0.96[c]	5.27 ± 0.33[c]	5.00 ± 0.45[c]
CD rats				
0	1.18 ± 0.34	NA	2.75 ± 0.52	NA
1	4.08 ± 0.80[c]	NA	2.87 ± 0.34	NA
5	6.22 ± 1.60[c]	NA	2.35 ± 0.32	NA
25	5.85 ± 0.83[c]	NA	4.60 ± 0.53[c]	NA

[a]NA = not applicable.
[b]$p<0.05$.
[c]$p<0.01$.

Source: Cattley et al., 1994.

Numerous noncancerous histopathologic lesions resulted from nitrobenzene inhalation, though some of these responses were not clear-cut because of a high incidence of the same effect in controls, which left the possibility that the response might be a nonspecific lesion due to age. For example, chronic nephropathy and extramedullary hematopoiesis of the spleen occurred in controls and at all concentration levels in both sexes of F344 rats and in male Sprague-Dawley

rats. However, a number of histopathologic effects of nitrobenzene appeared to be compound-related, including those in the nose, spleen, liver, kidney, and testis (Table 4-21).

Pigmentation of the olfactory epithelium was dose-dependently increased in male and female rats, with incidences of 99% in male F344 rats versus 60% of controls, 95% in male CD rats versus 67% of controls, and 100% in female F344 rats versus 55% of controls in the high exposure groups. Splenic pigmentation was assessed in male and female F344 rats. In male F344 rats, an exposure-related increase was observed (100% of 25 ppm exposed animals versus 80% of controls). In contrast, 99% of female rats were found with this endpoint in the highest exposure group compared to 90% of controls. Liver effects exhibited a mixed response with respect to exposure-dependent changes. Hepatic eosinophilic foci were observed in a dose-dependent manner in 81% and 23% of male and female F344 rats at the highest dose (25 ppm) compared to 38% and 8.6% of controls, respectively. Male F344 rats exhibited an exposure-dependent increase in spongiosis hepatis (83% of animals at 25 ppm versus 36% of controls), whereas this endpoint was observed with only the high exposure groups in 57% of male CD rats compared to 40% of controls, and 9% of female F344 rats versus 0% of controls. The number of male rats presenting with centrilobular hepatocytomegaly at necropsy was increased at 5- and 25-ppm nitrobenzene, with 81% of F344 rats and 60% of CD rats afflicted at the highest exposure level compared to 0% and 5% of controls, respectively; however, this endpoint was not detected in female F344 rats, regardless of exposure level. Changes in the kidney were restricted to the high exposure group in male F344 rats, with less clear exposure-related changes in female F344 rats. Tubular hyperplasia was detected in 19% of male F344 rats versus 3% of controls, and only 3% of female F344 rats at 5- and 25-ppm nitrobenzene and none of the controls. Testicular changes were assessed in male CD rats. Clear exposure-dependent changes were observed for bilateral atrophy of the testis (57% at the highest dose; 18% of controls) and bilateral hypospermia of the epididymis (54% at the highest dose; 13% of controls).

In mice, tissue sites displaying increased incidence of non-neoplastic lesions included lung, olfactory epithelium, and, in the males, thyroid follicular cells and hepatocytes (Table 4-22). Histopathological endpoints for the lung included hyperplasia and bronchiolization.[4] In male mice, a clear exposure-dependent increase in hyperplasia was found, up to 20% in high exposure animals versus 1.5% of controls. In contrast, female mice displayed a mixed response, with findings of hyperplasia in 3% of animals at 5 ppm, 8% at 25 ppm, and 2% at 50 ppm versus controls. Bronchiolization of the alveoli was significantly increased at all exposure levels (male

[4] Bronchialization of the alveoli represents a metaplastic response of the peripheral airway epithelium to the field effect of chemical exposure and shares characteristics of other premalignant lesions. This response is typically graded from simple metaplasia (*e.g.*, single layer of bronchiolar epithelial cells [ciliated and non-ciliated columnar epithelium] lining both sides of the alveolar septae) to marked atypia (*e.g.*, multiple layers of bronchiolar epithelial cells exhibiting loss of cellular orientation and cilia, extreme variation in cell size and shape, high nuclear/cytoplasmic ratio, marked parachromatin clearing [chromatin condensation], marked variation in size and shape of sister nuclei) (Jensen-Taubman, et al. 1998).

mice: 5 ppm, 87%; 25 ppm, 89%; and 50 ppm, 94%; female mice: 5 ppm, 92%; 25 ppm, 98%; and 50 ppm, 100%). This endpoint was not detected in any controls. Additional effects of nitrobenzene on the respiratory tract were noted with significant increases in the number of animals presenting with pigmentation and degeneration of the olfactory epithelium. Pigmented olfactory epithelium was detected in 74% and 48% of high dose male and female mice, respectively. Similarly, an exposure-dependent increase in degenerated olfactory epithelium occurred in mice of both sexes, with 62% of males and 69% of females being affected in the high exposure groups. A differential response was observed between male and female mice with histopathological endpoints in the thyroid and liver. In the thyroid, an exposure-dependent increase in follicular cell hyperplasia, up to 19% at 50 ppm was found in male mice versus 2% of controls, whereas this effect was only observed in females up to 13% compared to 4% of controls) at the highest exposure (50 ppm). In the liver, male mice presented with exposure-dependent changes in centrilobular hepatocytomegaly and multinucleated hepatocytes, up to 89% and 88%, respectively. In contrast, centrilobular hepatocytomegaly was undetectable in female mice, except for the highest dose (11% above controls), as were multinucleated hepatocytes (3% above controls).

Table 4-21. Significant Noncancer Histopathological Changes in Rats as a result of Exposure to Nitrobenzene via Inhalation for 2 Years

	Exposure Concentration (ppm)							
	Males				Females			
Target Tissue	**0**	**1**	**5**	**25**	**0**	**1**	**5**	**25**
F-344 rats								
Liver								
Eosinophilic foci	26/69	25/69	44/70*	57/70*	6/70	9/66	13/66	16/70*
Centrilobular hepatocytomegaly	0/69	0/69	8/70*	57/70*	0/70	0/66	0/66	0/70
Spongiosis hepatis	25/69	24/69	33/70	58/70*	0/70	0/66	0/66	6/70*
Kidney								
Tubular hyperplasia	2/69	2/68	2/70	13/70*	0/70	0/66	2/66	2/70
Nose								
Pigmented olfactory epithelium	40/67	53/67	67/70	68/69*	37/67	54/65	60/65	66/66*
Spleen								
Pigmentation	55/69	63/69	64/70	70/70*	62/69	61/66	60/66	68/69*
CD rats								
Liver								
Centrilobular hepatocytomegaly	3/63	1/67	14/70*	39/65*				
Spongiosis hepatis	25/63	25/67	25/70	37/65*				
Nose								
Pigmented olfactory epithelium	42/63	49/64	60/66	58/61*				
Testis								
Bilateral atrophy	11/62	17/66	22/70	35/61*				

Epididymis Bilateral hypospermia	8/60	13/65	15/67	32/59*				

*Significantly different from control values, as calculated by the authors.

Source: CIIT (1993); Cattley et al. (1994)

Table 4-22. Significant Noncancer Histopathological Changes in B6C3F1 Mice as a result of Exposure to Nitrobenzene via Inhalation for 2 Years

	Exposure Concentration (ppm)							
	Males				Females			
Target Tissue	**0**	**5**	**25**	**50**	**0**	**5**	**25**	**50**
Liver								
Centrilobular hepatocytomegaly	1/68	15/65	44/65*	57/64*	0/51	0/61	0/64	7/62*
Multinucleated hepatocytes	2/68	14/65	45/65*	56/64*	0/51	0/61	0/64	2/62*
Lung								
Hyperplasia	1/68	2/67	8/65*	13/66*	0/53	2/60	5/64*	1/62
Bronchiolization	0/68	58/67*	58/65*	62/66*	0/53	55/60*	63/64*	62/62*
Thyroid								
Follicular cell hyperplasia	1/65	4/65	7/65*	12/64*	2/49	1/59	1/61	8/61
Nose								
Pigmented olfactory epithelium	0/67	7/66	46/65*	49/66*	0/52	6/60*	37/63*	29/61*
Degenerated olfactory epithelium	1/67	1/66	32/65*	41/66*	0/52	19/60*	47/63*	42/61*

*Significantly different from control values, as calculated by the authors.

Source: CIIT (1993); Cattley et al. (1994). ND = No data

A synopsis of the effects observed from chronic nitrobenzene inhalation in animals is presented in Table 4-23.

Table 4-23. Summary of effects observed from chronic inhalation with nitrobenzene*

Species, strain	Number	Dosing	Effect[a]	NOAEL (ppm)[b]	LOAEL (ppm)[b]	Reference
Rat, F344	70/sex	0, 1, 5, 25 ppm 6 hr/d, 5 d/wk, 2 y	Methemoglobinemia ↑ Liver, eosinophilic foci ↑ Adenoma/carcinoma ↑	5 (M, F) 1(M), 5(F) 5 (M, F)	25 (M, F) 5(M), 25(F) 25 (M, F)	CIIT, 1993
Rat, CD	70 males		Methemoglobinemia ↑ Hepatocytomegaly ↑ Adenoma/carcinoma ↑	NA 1 5	5 5 25	
Mouse, B6C3F1	70/sex	0, 5, 25, 50 ppm 6 hr/d, 5 d/wk, 2 y	Methemoglobinemia ↑ Bronchiolization ↑ Adenoma/carcinoma ↑	25 (M, F) NA 5 (M, F)	50 (M, F) 5 (M, F) 25 (M, F)	

*NOAELs and LOAELs determined by nitrobenzene assessment authors.
[a]Only endpoints with evident dose responses were selected. ↑ = increase in the respective endpoint
[b]M = male; F = female; S-D = Sprague-Dawley.
[c]Measured at 15-month interim sacrifice.

4.2.3. Dermal Exposure

4.2.3.1. *Subchronic Studies*

NTP sponsored a 90-day skin painting toxicological study with nitrobenzene in F344 rats and B6C3F1 mice (NTP, 1983b). The authors treated F344 rats and B6C3F1 mice (10 animals/sex/group) with 50, 100, 200, 400, and 800 mg/kg-day nitrobenzene in acetone, the responses being compared with those in animals painted with acetone alone. All 800 mg/kg rats, plus 9/10 male and 8/10 female mice exposed at this level died before the end of the experiment. Furthermore, surviving animals in the other exposure groups (dose levels not stated) displayed profound clinical signs of acute toxicity, including ataxia, dyspnea, circling, lethargy, and insensitivity to pain. Only female mice showed a dose-related increase in metHb concentration. Among the histopathologic findings, there was a marked degeneration of the testes in the males of both species and all nitrobenzene-receiving rats displayed congestion of the spleen. The incidence of congestion of the lungs was dose-dependently increased in males and females of both species. Vacuolization of the brain or brain stem was another characteristic histopathologic finding, the effects becoming apparent in rats exposed to nitrobenzene at 100 mg/kg or higher, in male mice exposed to 800 mg/kg, and female mice exposed to 400 and 800 mg/kg nitrobenzene. Tables 4-24, 4-25, 4-26, and 4-27 document these histopathologic changes.

Table 4-24. Incidence of histopathologic lesions in male F344 rats exposed to nitrobenzene for 90 days via dermal exposure

	Dose (mg/kg-day)					
Target tissue	**0**	**50**	**100**	**200**	**400**	**800**
Lung						
Congestion	1/10	1/10	7/10	4/10	4/10	10/10
Spleen						
Congestion	0/10	10/10	10/10	10/10	10/10	10/10
Hematopoiesis	10/10	10/10	10/10	10/10	10/10	10/10
Lymphoid atrophy	0/10	0/10	7/10	7/10	10/10	10/10
Liver						
Congestion	0/10	1/10	0/10	0/10	0/10	6/10
Kidney						
Congestion	0/10	0/10	0/10	0/10	0/10	7/10
Testis						
Atrophy	0/10	0/10	0/10	0/10	10/10	10/10
Hypospermatogenesis	0/10	0/10	0/10	0/10	10/10	10/10
Multinucleate giant cells	0/10	0/10	0/10	0/10	9/10	10/10
Brain						
Hemorrhage	1/10	4/10	0/10	0/10	2/10	2/10

Source: NTP, 1983b.

Table 4-25. Incidence of histopathologic lesions in female F344 rats exposed to nitrobenzene for 90 days via dermal exposure

Target tissue	Dose (mg/kg-day)					
	0	**50**	**100**	**200**	**400**	**800**
Lung						
Congestion	1/10	1/10	3/10	1/10	6/10	9/10
Spleen						
Congestion	8/10	10/10	10/10	9/10	10/10	10/10
Hematopoiesis	0/10	10/10	10/10	10/10	10/10	10/10
Lymphoid atrophy	0/10	0/10	0/10	1/10	9/10	10/10
Liver						
Congestion	0/10	0/10	0/10	0/10	0/10	4/10
Kidney						
Congestion	0/10	0/10	0/10	0/10	4/10	4/10
Uterus						
Atrophy	0/10	0/10	0/10	0/10	0/10	6/10
Brain						
Hemorrhage	0/10	1/10	5/10	2/10	1/10	2/10
Cerebrum						
White matter vacuolization	0/10	0/10	10/10	10/10	4/10	3/10
Cerebellum						
White matter vacuolization	0/10	0/10	8/10	4/10	7/10	6/10
Brain stem						
Hemorrhage	0/10	1/10	1/10	4/10	7/10	6/10
Vacuolization	0/10	0/10	10/10	8/10	4/10	3/10

Source: NTP, 1983b.

Table 4-26. Incidence of histopathologic lesions in male B6C3F1 mice exposed to nitrobenzene for 90 days via dermal exposure

Target tissue	Dose (mg/kg-day)					
	0	50	100	200	400	800
Lung						
Congestion	2/10	6/10	4/10	4/10	10/10	9/10
Spleen						
Congestion	0/10	0/10	0/10	0/10	0/10	10/10
Hematopoiesis	1/10	3/10	3/10	9/10	9/10	10/10
Lymphoid atrophy	0/10	0/10	0/10	0/10	0/10	3/10
Liver						
Congestion	0/10	0/10	0/10	1/10	10/10	10/10
Pigmentation	0/10	0/10	0/10	0/10	0/10	6/10
Thymus						
Atrophy	0/10	0/10	0/10	0/10	0/10	7/7
Testis						
Atrophy	0/10	0/10	0/10	0/10	5/10	10/10
Hypospermatogenesis	0/10	0/10	0/10	0/10	2/10	10/10
Multinucleate giant cells	0/10	0/10	0/10	0/10	0/10	4/10
Brain						
Hemorrhage	1/10	1/10	3/10	1/10	0/10	2/10
Brain stem						
Hemorrhage	1/10	1/10	2/10	1/10	1/10	6/10
Degeneration	0/10	0/10	0/10	0/10	0/10	3/10
Skin						
Inflammation	0/10	0/10	0/10	0/10	8/10	3/10

Source: NTP, 1983b.

Table 4-27. Incidence of histopathologic lesions in female B6C3F1 mice exposed to nitrobenzene for 90 days via dermal exposure

Target tissue	Dose (mg/kg-day)					
	0	50	100	200	400	800
Lung						
Congestion	4/10	3/10	2/10	4/10	8/10	10/10
Spleen						
Congestion	0/10	0/10	1/10	0/10	2/10	9/10
Hematopoiesis	7/10	4/10	3/10	7/10	10/10	9/10
Lymphoid atrophy	0/10	0/10	1/10	0/10	0/10	3/10
Liver						
Cytomegaly	0/10	0/10	0/10	0/10	0/10	8/10
Thymus						
Atrophy	0/10	0/10	0/10	0/10	0/10	9/9
Ovary						
Atrophy	0/10	0/10	0/10	0/10	0/10	3/10
Uterus						
Atrophy	0/10	0/10	0/10	1/10	1/10	5/10
Adrenal Cortex						
Fatty change	0/10	6/10	9/10	10/10	8/10	2/10
Brain						
Hemorrhage	0/10	1/10	0/10	1/10	3/10	2/10

Brain stem						
Hemorrhage	1/10	0/10	0/10	0/10	2/10	4/10
Degeneration	0/10	0/10	0/10	0/10	1/10	3/10
Skin						
Inflammation	0/10	0/10	0/10	0/10	9/10	7/10

Source: NTP, 1983b.

A summary of the animal toxicity studies with nitrobenzene following dermal administration is presented in Table 4-28.

Table 4-28. Summary of effects observed in dermal dosing studies with nitrobenzene*

Species, strain	Number	Dosing	Effect[a]	NOAEL (mg/kg-day)[b]	LOAEL (mg/kg-day)[b]	References
Rat, F344	10/sex	0, 50, 100, 200, 400, 800 mg/kg-day, 90 d	Splenic congestion ↑ Lung congestion ↑ Brain pathology ↑ Testicular pathology ↑	NA 50 (M, F) 50 (F) 200 (M)	50 (M, F) 100 (M, F) 100 (F) 400 (M)	NTP, 1983b
Mouse, B6C3F1	10/sex	0, 50, 100, 200, 400, 800 mg/kg-day, 90 d	Splenic Hematopoiesis ↑ Testicular pathology ↑ Mortality ↑	100 (M, F) 200 (M) NA	200 (M, F) 400 (M) 800 (M, F)	

*NOAELs and LOAELs determined by nitrobenzene assessment authors.
[a]Only endpoints with evident dose responses were selected. ↑ = an increase in the respective endpoint.
[b]M=male; F=female; NA=not applicable.

4.3. REPRODUCTIVE/DEVELOPMENTAL STUDIES—ORAL AND INHALATION

4.3.1. Oral Exposure

Levin et al. (1988) investigated adverse effects of nitrobenzene on spermatogenesis that might be associated with impaired testicular function by surgically routing the vas deferens of male F344 rats to the bladder. This permitted spermatogenesis to be continually monitored during and after exposure to nitrobenzene. Six rats/group were subjected to this surgical procedure and, after a recovery period of 6 weeks, gavaged with a single dose of 300-mg/kg nitrobenzene in corn oil. Controls received corn oil alone. Animals were housed in metabolic cages and assessed for the release of sperm to the urine for up to 100 days. Two other groups of rats, 45 exposed and 30 controls, were gavaged in a manner similar to the surgically altered

subjects. These were serially sacrificed for histopathologic examination at various time points, up to 100 days. Output of sperm held steady after nitrobenzene administration for about 20 days, then dropped to zero within 12 days and persisted at this level until day 48. Fifty days after treatment, sperm began to reappear in the urine of treated animals, ultimately achieving about 78% of control levels. Histopathologically, treated animals displayed degeneration of the seminiferous epithelium within 3 days of treatment, an effect characterized by the appearance of pachytene-derived giant cells and loss of the more mature elements of the seminiferous epithelium. As discussed by the authors, the pachytene spermatocytes (found in Stages VI–XIII) were the most sensitive to the effects of the compound. Clear histopathologic signs of regeneration were apparent at about 21 days after treatment. However, at least some signs of the abnormal cellular architecture and tubular organization described above always remained. For example, approximately 10% of the tubules examined showed little evidence of spermatogenesis even at 8 weeks posttreatment, with mature spermatids rarely apparent. The authors interpreted their results in accordance with the known processes and time frame by which spermatogenesis occurs in F344 rats and presented a nomogram that correlated the spermatogenic cycle of the rats with the proposed chronology of nitrobenzene-induced lesions.

Bond et al. (1981) administered a single oral dose of 0, 50, 75, 110, 165, 200, 300, or 450 mg/kg nitrobenzene in corn oil to six male F344 rats/group. Three rats at each dosage were sacrificed 2 and 5 days following nitrobenzene administration. Samples of blood were obtained by cardiac puncture to measure metHb, and 25 tissues and organs were excised for histopathologic examination. The liver, testes, and brain from all animals in the study were examined histopathologically, whereas histologic sections of other tissues were examined only in the high dose and control groups. Hepatic centrilobular necrosis appeared inconsistently in rats given various doses of nitrobenzene, while hepatocellular nucleolar enlargement was consistently detected in rats given doses of nitrobenzene as low as 110 mg/kg. Lesions occurred in the seminiferous tubules of the testicles, with marked necrosis of primary and secondary spermatocytes following a single oral dose of 300 mg/kg (Bond et al., 1981). Furthermore, within 3 days of nitrobenzene administration, multinucleated giant cells were observed, and decreased numbers of spermatozoa were observed in the epididymis. Histopathologic analyses indicated that nitrobenzene had no apparent effects on spermatogonia or the epididymal epithelium. In parallel to the observed histopathologic lesions in liver and testes, methemoglobinemia was increased to 25% immediately after dosing at 300 mg/kg, with a subsequent slow decline over the next 10 days. In a control experiment, the administration of sodium nitrite also induced methemoglobinemia but had no histopathologic effects on the testes and liver, suggesting that the histopathologic effects of nitrobenzene occurred through a direct action of the compound or its metabolites at the tissue site rather than as a secondary effect of metHb formation.

Two further studies confirmed the association between orally administered nitrobenzene and the onset of toxic effects in the testes and epididymides. In the first study, Matsuura et al. (1995) gavaged 10-week-old male Sprague-Dawley rats with 30 or 60 mg/kg nitrobenzene, 5 days/week for 3 weeks. Parameters evaluated included the weights and histopathology of the testes and epididymides, together with an analysis of the count, motility, viability, and morphology of the sperm. Nitrobenzene at the high dose (60 mg/kg) induced a relative decrease in the weight of the epididymis, decreases in sperm motility and viability, and an increase in the incidence of morphologically abnormal sperm. Degeneration and decreases in spermatids and pachytene spermatocytes were specified as primary effects of nitrobenzene at this dose level. In the second study, Koida et al. (1995) gavaged several groups of five male Sprague-Dawley rats of different ages (6, 8, 10, and 40 weeks old) with 50 mg/kg-day nitrobenzene in sesame oil for 2 or 4 weeks. All subjects were examined for changes in testis and epididymis weights (compared with controls), differential morphology and histopathology, and altered sperm counts. In general, treatment was associated with reduced sperm counts and depressed sperm activity, with some histopathologic changes evident in the reproductive organs of younger animals.

Kawashima et al. (1995a) administered nitrobenzene (60 mg/kg-day in sesame oil by gavage) to male Sprague-Dawley rats for periods of time from 7–70 days, after which the animals were mated with untreated females and then terminated the following day. Comparative changes in testicular and epididymal weights, sperm count, motility, and viability were evaluated, along with the fertility and copulation indices of treated groups. Significant reductions in testicular (>50%) and epididymal weights, sperm count, and motility were observed in those animals exposed to nitrobenzene for 14 days, while sperm viability and the fertility index were severely reduced in those males exposed to nitrobenzene for 21 days or more. There was a concomitant increase in the incidence of abnormal sperm. While the copulation indices of treated males appeared unchanged with duration of exposure, the numbers of virgin females becoming pregnant by treated males declined markedly with duration of exposure. No mating females became pregnant in groups that were mated with males treated for 28 days or longer, an effect that appeared to result from the production of sperm with poor motility and reduced viability.

Kawashima et al. (1996, 1995b) used computer-imaging systems to evaluate the motility of sperm from rats gavaged with nitrobenzene. For example, they described an experimental protocol in which, in the first study, male Sprague-Dawley rats were gavaged with 60 mg/kg-day nitrobenzene for up to 2 weeks (Kawashima et al., 1995b). Sperm from treated and control rats were evaluated in an image processor that used motion analysis software to quantify such parameters as curvilinear distance, curvilinear velocity, and amplitude of lateral head displacement. The values of each motility parameter were lower in the sperm of nitrobenzene-exposed rats. These researchers also used computer-assisted sperm analysis to evaluate sperm

motility in Sprague-Dawley rats exposed to up to 60 mg/kg-day by gavage for up to 28 days (Kawashima et al., 1996). All sperm motility parameters in rats exposed to 30 and 60 mg/kg-day were lower than in controls, irrespective of exposure duration. Such parameters as curvilinear velocity, straight-line velocity, and motility rate were lower in rats exposed at the lowest dose level (15 mg/kg-day) for 28 days.

Other abstracts of studies by Japanese research teams attested to the impact of nitrobenzene on sperm viability and motility when administered to rats via the oral route (Kito et al., 1999, 1998; Kato et al., 1995). In one example, (Kato et al., 1995) exposed rats (number and strain not stated) to nitrobenzene at concentrations up to 60 mg/kg and used a vital dye (ethidium homodimer) to show loss of sperm viability compared with equivalent samples from untreated rats. Viable sperm from nitrobenzene-receiving animals showed reduced motility. In a more recent full-length research report, nitrobenzene was used as one of several recognized testicular toxicants to evaluate the utility of different parameters in sperm motion analysis (Ban et al., 2001). Curvilinear velocity and mean amplitude of lateral head movement were considered to be among the more sensitive indicators of impaired sperm motility.

Linder et al. (1992) had likewise included nitrobenzene as a positive control in a survey of compounds for spermatotoxic effects in male Sprague-Dawley rats. The experimental protocol featured oral administration of the compound as a single dose of 300 mg/kg. A number of well-characterized spermatotoxic tests were employed, including counts of sperm heads, sperm velocity, sperm morphology, and the histopathology of the testis and epididymis. Marked changes observed in nitrobenzene-receiving rats included degenerating and missing pachytene spermatocytes in Stages VII to XIV, some multinucleated giant cells, the existence of testicular debris, and an increase in the number of morphologically abnormal sperm.

Mitsumori et al. (1994) reported a reproductive toxicity study on nitrobenzene that employed a complex protocol proposed by the Organization for Economic Cooperation and Development (OECD). Ten Sprague-Dawley rats/sex/group were gavaged with 0, 20, 60, or 100 mg/kg-day nitrobenzene in sesame oil for a 14-day premating period, a mating period of up to 14 days, a gestation period of 22 days, and a subsequent lactation period of 4 days, making a potential overall dosing period of 54 days, at which point all animals (males, females, and pups) were necropsied. Because the observed mating period was no more than a single day for most mating pairs, the actual dosing duration for males and females was 40–41 days but could have lasted as long as 54 days for some. Clinical signs were observed daily, and body weights and food consumption were monitored weekly. A complete range of hematological and clinical chemistry parameters was measured in blood and serum samples collected from the males prior to termination. At necropsy, weights of liver, kidneys, thymus, adrenals, spleen, testes, epididymides, and ovaries were noted. The numbers of corpora lutea and implantation sites were

counted in females. Excised pieces of brain, heart, liver, kidneys, adrenals, spleen, ovaries, testes, and epididymides were fixed and processed for histopathologic examination.

High-dose animals displayed a number of clinical signs as a result of nitrobenzene administration, including piloerection, salivation, emaciation, and an apparent anemia from day 13 onwards. A number of behavioral/neurological signs were evident and body weight and food consumption were reduced by 17% in the high-dose males from day 21 onwards. Male rats displayed profound dose-related changes in the levels of some hematological parameters, including decreases in RBCs, Hb, and Hct, and increases in metHb, MCH, WBCs, reticulocytes, and erythroblasts. For a number of these parameters, statistically significant differences to controls were observed in the low-dose group (Table 4-29). At necropsy, the relative liver, kidney, and spleen weights were statistically significantly increased, and those of testes and epididymides were significantly decreased in the 60 and 100 mg/kg-day animals compared with controls. However, in rats exposed to 20 mg/kg-day nitrobenzene there was a slight upward fluctuation in relative testis and epididymis weights compared with controls (Table 4-30).

Table 4-29. Hematological findings in male Sprague-Dawley rats exposed via gavage to nitrobenzene

Parameter	Dose (mg/kg-day)[a]			
	0	20	60	100
RBC (10^{12}/L)	8.96 ± 0.23	7.75 ± 0.40[b]	6.44 ± 0.44[b]	5.28 ± 0.44[b]
Hb (g/L)	15.3 ± 0.6	13.6 ± 0.6[b]	13.3 ± 0.7[b]	12.9 ± 1.0[b]
MetHb (%)	0.70 ± 0.69	3.64 ± 3.14[c]	4.79 ± 1.09[b]	6.76 ± 2.07[b]
Packed cell volume (%)	45.0 ± 1.8	40.7 ± 1.8[b]	38.5 ± 2.2[b]	36.5 ± 2.3[b]
Mean cell volume (fL)	50.2 ± 1.1	52.5 ± 1.7	59.8 ± 2.4[b]	69.3 ± 5.2[b]
MCH (pg)	17.1 ± 0.4	17.5 ± 0.5	20.8 ± 0.8[b]	24.5 ± 1.0[b]
Reticulocytes (per 1000 RBCs)	34.1 ± 21.1	64.2 ± 23.0	116.6 ± 24.4[b]	223.0 ± 60.9[b]
Erythroblasts (per 200 WBCs)	2.3 ± 2.6	7.0 ± 4.9	18.7 ± 16.6[c]	19.6 ± 14.6[b]
WBCs (10^9/L)	4.65 ± 1.49	4.69 ± 1.0	4.12 ± 1.28	16.42 ± 7.70[c]

[a]Values are means ± SD.
[b]$p<0.01$ versus controls, as calculated by the authors.
[c]$p<0.05$ versus controls, as calculated by the authors.

Source: Mitsumori et al., 1994.

A wide range of histopathologic consequences of nitrobenzene treatment was observed, especially in animals receiving 60 and 100 mg/kg-day of the compound. These included atrophy of the seminiferous tubules, hyperplasia of Leydig cells, and loss of intraluminal sperm in the epididymides. Such histopathologic lesions as centrilobular swelling of hepatocytes, hemosiderin deposition in Kupffer cells, and increased extramedullary hematopoiesis in the liver

and spleen were seen in all exposed groups. Neuronal necrosis/gliosis in the cerebellar medulla was evident in rats exposed to 60 and 100 mg/kg-day nitrobenzene. Perhaps the most important findings of the study related to the reproductive/developmental parameters that were evaluated. Principal among these findings was that, though there were no statistical differences to controls in the copulation and fertility indices at any dose level, only 2/9 pregnant females in the high-dose group survived to term with the subsequent deaths of the two survivors occurring on days 1 and 3 of lactation.

Table 4-30. Relative organ weights of male Sprague-Dawley rats gavaged with nitrobenzene

Organ (g/100g body weight)	Dose (mg/kg-day)[a]			
	0	20	60	100
Liver	2.87 ± 0.24	3.38 ± 0.17[b]	3.94 ± 0.30[b]	4.15 ± 0.20[b]
Kidney	0.64 ± 0.04	0.67 ± 0.05	0.73 ± 0.05[b]	0.84 ± 0.07[b]
Spleen	0.18 ± 0.01	0.29 ± 0.04[b]	0.51 ± 0.07[b]	0.67 ± 0.14[b]
Testes	0.79 ± 0.04	0.83 ± 0.07	0.32 ± 0.04[b]	0.37 ± 0.07[b]
Epididymides	0.28 ± 0.02	0.31 ± 0.04	0.23 ± 0.05[b]	0.20 ± 0.02[b]

[a]Values are means ± SD.
[b]$p<0.01$ versus controls, as calculated by the authors.

Source: Mitsumori et al., 1994.

A synopsis of NOAELs and LOAELs, as identified by the nitrobenzene assessment authors, from Mitsumori et al (1994) is presented in Table 4-31.

Table 4-31. Summary of effects observed in an oral reproductivestudy with nitrobenzene

Species, strain	Number	Dosing	Effect[a]	NOAEL (mg/kg-day)[b]	LOAEL (mg/kg-day)[b]	Reference
Rat, Sprague-Dawley	10/sex	0, 20, 60, 100 mg/kg-day, gavage, up to 54 d	Organ weights, ↑	NA	20 (M, F_0)	Mitsumori et al., 1994
			Testicular pathology ↑	20	60 (M, F_0)	
			Copulation, fertility	100	NA	
			Developmental toxicity	100	NA	

[a]Only endpoints with evident dose responses were selected. ↑ = an increase in the respective endpoint.
[b]M=male; F=female; F_0=parental generation; NA=not applicable.

Sertoli cells control spermatogenesis via the secretion of different proteins varying cyclically according to the stage of spermatogenesis. In order to assess the possibility of identifying chemical-induced, stage-specific changes in protein secretion, McLaren et al. (1993a) employed a novel experimental approach to examine the in vivo effects of nitrobenzene (single oral dose of 300 mg/kg) and *m*-dinitrobenzene using seminiferous tubules from male Wistar rats at different stages of the spermatogenic cycle. Tissue extracts then were cultured in vitro for 24 hours with [^{35}S]-methionine. Incorporation of [^{35}S]-methionine served as a marker for the secretion of newly formed polypeptides in response to challenges with nitrobenzene or *m*-dinitrobenzene, a well-characterized Sertoli cell toxicicant. In other experiments, seminiferous tubules were exposed to nitrobenzene and *m*-dinitrobenzene in vitro in the presence of [^{35}S]-methionine. Using two-dimensional SDS-PAGE or isoelectric focusing, the authors were able to identify six marker proteins, normally produced in the tubules, whose secretion was changed as a result of exposure to nitrobenzene or *m*-dinitrobenzene. For the most part, the abundance of these marker proteins was reduced in response to nitrobenzene, as compared with controls. One component, however, MP-4, a structural protein in Sertoli cells, had not been apparent previously in the secretions of seminiferous tubule cells from control animals but appeared in detectable amounts in the polypeptide secretions from nitrobenzene-exposed seminiferous tubules. Further work demonstrated that the toxicological effects of nitrobenzene, such as those outlined above, did not occur in isolates from immature rats, thus suggesting an age specificity of the nitrobenzene- and *m*-dinitrobenzene-induced responses (McLaren et al., 1993b).

Morrissey et al. (1988) evaluated rodent sperm, vaginal cytology, and reproductive organ weight data from a series of NTP 13-week gavage studies, one of which was on nitrobenzene (NTP, 1983a). As tabulated by Morrissey et al. (1988), the effects of nitrobenzene on the reproductive organs and the incidence of abnormal sperm were assessed at dose levels of 0, 9.4, 37.5, and 75 mg/kg in rats and at 18.75, 75, and 300 mg/kg in mice. Though no dose-specific data were provided in the report, the authors stated that the absolute and relative weights of epididymides and testes were reduced in animals receiving nitrobenzene. In addition, sperm motility was adversely affected, and the incidence of abnormal sperm was increased.

A number of experimental approaches has been used to determine the mechanism by which nitrobenzene induces testicular toxicity. For example, Allenby et al. (1990) used in vitro experimental protocols to investigate possible mechanisms for how nitrobenzene may affect spermatogenesis. The effects of incubating Sertoli cell isolates or cocultures from Alpk:AP (Wistar derived) rats with a range of concentrations of nitrobenzene or *m*-dinitrobenzene (the latter compound being a well-characterized Sertoli cell toxicant serving as a positive control) were investigated. A number of parameters were monitored, including the exfoliation of germ cells, the secretion to the medium of lactate, pyruvate, inhibin (a gonadal glycoprotein hormone

that inhibits pituitary FSH secretion), and, in general, any apparent changes in cellular morphology. Vacuolization of the Sertoli cells was observed in the presence of 1 mM nitrobenzene, with lower concentrations of the compound stimulating the release of lactate and pyruvate, indicators of cell damage. Similarly, the release of inhibin was enhanced in the presence of low concentrations of nitrobenzene, allowing the conclusion that the compound is a Sertoli cell toxicant, though less effective than *m*-dinitrobenzene. The same scientists (Allenby et al., 1991) also compared the ability of nitrobenzene and *m*-dinitrobenzene to induce inhibin release from seminiferous tubule cultures obtained from rats of the Sprague-Dawley-derived strain or Sertoli cell cultures obtained from AlpK:AP_FSD (Wistar derived) rats. Adult Sprague-Dawley rats (approximately 70 days old) were used for in vivo experiments. Nitrobenzene and *m*-dinitrobenzene caused a statistically significant increase in the release of inhibin from isolated seminiferous tubules and, more variably, from isolated Sertoli cells. When animals were administered a single dose of either nitrobenzene (300 mg/kg), *m*-dinitrobenzene (25 mg/kg), or methoxyacetic acid (650 mg/kg), levels of inhibin were detectable in the testicular interstitial fluid 1 to 3 days postexposure, although a statistically significant decrease in testicular weight was not apparent until 3 days, suggesting that inhibin release may serve as an early indicator of impairment of spermatogenesis.

Shinoda et al. (1998) used terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling and DNA gel electrophoresis to investigate the extent to which germ cell degeneration represented necrosis or apoptosis. The in-life phase of the experiment featured a single oral dose of 250-mg/kg nitrobenzene to male Sprague-Dawley rats, the subjects being terminated at various time points up to 7 days posttreatment. Germ cell degeneration was evident as early as 24 hours after dosing, and electron micrographs showed spermatocytes undergoing changes thought to be characteristic of apoptosis. Degenerating spermatocytes contained fragmented DNA. Linking their data to those of Allenby et al. (1991, 1990), Shinoda et al. (1998) speculated that nitrobenzene exposure could alter secretion of one or more Sertoli cell factors that might trigger germ cell apoptosis.

Richburg and Nañez (2003) studied molecular mechanisms of nitrobenzene-induced testicular toxicity via the Fas/Apo-1/CD95 and Fas ligand (FasL) signaling system, in which FasL activates Fas. Following the engagement of FasL with Fas, an intrinsic apoptotic program is initiated in the target cell. In testis, Sertoli cells express FasL and select germ cells express Fas. This is a paracrine signaling system[5] by which Sertoli cells can initiate killing of Fas-expressing germ cells (Richburg and Boekelheide, 1996). Two mouse spontaneous mutations, *lpr* and *gld*, are loss-of-function mutations of Fas and FasL, respectively (Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992). In the study by Richburg and Nañez (2003), similar mice

[5] Paracrine signaling involves communication between a signal-releasing cell "A" and a nearby cell "B" that receives the signal. Autocrine signaling involves the release of a signal by cell "A" that signals cell "A".

(CBA/KlJms-*Tnfrsf61pr-cg* [lpr^{cg}] and B6.SMNC3H-$Fas^{gld,gld}$ [*gld*]) were utilized to determine the role of Fas and FasL at initiating germ cell apoptosis at 0, 6, 12, and 24 hours following a challenge with nitrobenzene (8-week-old mice, 800 mg/kg; 4-week-old mice, 600 mg/kg). The authors found that lpr^{cg} and *gld* mice still displayed nitrobenzene-induced apoptosis of germ cells and concluded that nitrobenzene-induced germ cell apoptosis was not mediated by the Fas and FasL system but more likely by an autocrine pathway within the germ cells.

Kawaguchi et al. (2004) investigated differences in fertility and sperm motion in male rats treated with α-chlorohydrin, known to produce spermatotoxicity, and nitrobenzene, known to produce testicular toxicity. Ten-week-old male Crj:CD(SD) IGS rats were treated with either saline solution or 60 mg/kg-day nitrobenzene by gavage for 3 or 18 days. Male rats were mated with 8-week-old female rats, same strain, on day 3 and days 14–17. In the 18-day treated group, but not the 3-day group, a statistically significant decrease in absolute and relative weights of both testes and epididymides was observed. No histopathologic lesions were observed in the 3-day group; however, in the 18-day group, nitrobenzene caused severe atrophy of the seminiferous tubules, along with decreased concentrations of sperm and prominent cellular debris in the tubular lumina of the caput/corpus and cauda epididymidis. A statistically significant increase in the number of detached sperm heads was observed in the cauda epididymidis of 18-day treated animals. The movement of sperm in the 18-day nitrobenzene group was less vigorous than at other time periods and was attributed to the marked decrease of spermatogenesis in the testes. The fertility index was not affected by nitrobenzene treatment. The authors concluded that the full adverse effect on male fertility (viz., complete absence of sperm in the cauda epididymis) could be detected only after a full spermatogenic cycle (i.e., 21–28 days after treatment).

4.3.2. Inhalation Exposure

Tyl et al. (1987) exposed 26 pregnant female Sprague-Dawley rats/group to gaseous nitrobenzene at 0, 1, 10, or 40 ppm, 6 hours/day on gestation days (GDs) 6–15. Clinical signs were monitored daily, and maternal body weights were recorded on GDs 0, 6, 9, 12, 15, 18, and 21. All dams were terminated on GD 21 and subjected to a gross necropsy. The range of evaluated maternal and fetal reproductive and developmental parameters included the numbers of corpora lutea, maternal liver and uterine weights, the numbers of live and dead fetuses, the numbers of resorption sites, fetal weights and sex distribution, the incidence of fetal malformations, and visceral and skeletal abnormalities.

The results showed that there were no compound-related clinical signs, though maternal body weight gain was reduced by 19% in the high dose group compared with controls between GDs 6 and 15. However, this parameter had returned to control values by GD 21. Spleen weights increased dose dependently from 0.60 g in controls to 0.84 g in 40-ppm dams, achieving

statistical significance in the 10 and 40-ppm dose groups. Gestational parameters, such as the numbers of corpora lutea, resorptions and dead fetuses, live fetuses per litter, the pre- or postimplantation loss rates (as a percent), sex ratio, or fetal body weights, were all unaffected by treatment. Similarly, there were no indications of concentration-dependent developmental toxicity or teratogenicity. There was no effect on fetal body weights, and the incidence of skeletal variations also did not indicate fetal toxicity. The single exception was a significant increase in the incidence of parietal skull plates with an area of nonossification in the 40-ppm group, as shown in Table 4-32. However, it is unclear whether this isolated effect represents a teratogenic effect of nitrobenzene or whether it is a consequence of maternal toxicity observed in the high-concentration group. In general, the reproductive and developmental toxicity effects of nitrobenzene on Sprague-Dawley rats appeared to be mild, at least to the extent of their effects on female reproductive physiology.

Table 4-32. Incidence of skeletal variations in Sprague-Dawley fetuses exposed to nitrobenzene in utero

Nitrobenzene concentration (ppm)	Incidence by fetus and litter			
	Parietal skull plates (non-ossification)[a]	Bilobed thoracic centrum 9[a]	Split anterior arch of atlas[a]	Poorly ossified premaxillary[a]
0	9/167 (f) 8/25 (l)	6/167 (f) 6/25 (l)	1/167 (f) 1/25 (l)	3/167 (f) 3/25 (l)
1	15/172 (f) 9/25 (l)	3/172 (f) 3/25 (l)	7/172 (f) 7/25 (l)[b]	19/172 (f) 11/25 (l)[b]
10	21/174 (f) 11/25 (l)	3/174 (f) 3/25 (l)	5/174 (f) 5/25 (l)	13/174 (f) 7/25 (l)
40	29/181 (f) 19/26 (l)[b]	1/181 (f) 1/26 (l)[b]	6/181 (f) 5/26 (l)	12/181 (f) 6/26 (l)

[a](f) = incidence among all fetuses of one dose group; (l) = litters affected per all litters of one dose group.
[b]$p<0.05$.

Source: Tyl et al., 1987.

Dodd et al. (1987) carried out a two-generation reproductive/developmental toxicity study on nitrobenzene in which initially 30 Sprague-Dawley rats/sex/group were exposed to 0, 1, 10, or 40 ppm nitrobenzene, 6 hr/day, 5 days/week for 10 weeks via inhalation, prior to a mating period of up to 2 weeks. This study also has appeared as a Toxic Substances Control Act Test Submission (Bushy Run Research Center [BRRC], 1985). After mating, the F_0 males were sacrificed, while the pregnant females were exposed to nitrobenzene through GD 19 and again after delivery on postnatal days (PNDs) 5–20 at which point the pups were weaned. The F_0 females were sacrificed prior to necropsy on PND 21. On this day, 30 pups/sex/group (F_1 generation) were selected (one male and one female from each litter, where possible) and allowed a 2-week growth period during which no nitrobenzene was administered. Subsequently, a repeat of the F_0 exposure and treatment protocol was undertaken, with the exception that, after mating, some F_1 males were not sacrificed. These males were allowed to enter a recovery phase,

and after 9 weeks of nonexposure they were mated with virgin, unexposed Sprague-Dawley females to examine potential reversibility of effects on the male gonads. The results of this mating, and all associated reproductive and developmental parameters of this offspring and the F_2 progeny, were noted, as described below. During the in-life phase of the study, clinical signs of all rats were observed daily, while body weights were recorded weekly. After parturition, litters were examined for the numbers of pups, their sex, the numbers of stillbirths and live births, the appearance of external abnormalities, and all incidences of toxicity and/or mortality. Pup weights were noted on a litter basis on PND 0, then individually on PNDs 4, 7, 14, and 21. The 30 animals/sex/group that were entered into the F_1 mating study were weighed weekly. F_1 males selected for the recovery phase and subsequent mating were weighed every 2 weeks. At termination, all animals were subjected to a full necropsy, and the weights of putative target organs, such as the testis and epididymis, were recorded. Tissues preserved for histopathologic examination from the 40 ppm and control animals included the vagina, uterus, ovaries, testis, epididymides, seminiferous tubules, prostate, and all tissues with gross lesions. Sections of the testis were examined in males exposed at all concentration levels.

As indicated in Table 4-33, there were marked reductions in the fertility indices as a result of matings among the 40-ppm animals compared with controls. Most notably, this reduction was also apparent in the matings that involved unexposed females with those high-concentration F_1 males that had been allowed a 9-week period of recovery. In all matings that resulted in live offspring, gestational parameters, such as the number of uterine implantations, resorptions, and postimplantation losses, were unaffected by nitrobenzene in either generation. However, marked spermatocyte degeneration and atrophy of the seminiferous tubules were observed in both generations of high-concentration males, including those that entered the 9-week recovery period. Morphologically, the lesions were characterized by severe multifocal and diffuse atrophy of the seminiferous tubules in 14/30 animals in the 40-ppm group and by the appearance of giant syncytial spermatocytes in the seminiferous tubules of 22/30 subjects of the F_0 generation. Giant syncytial spermatocytes were much less evident in F_1 males (1/30), and the active stages of spermatocyte degeneration in the seminiferous tubules were less frequent. However, the epididymides of 40-ppm males in the F_0 and F_1 generations displayed degenerative spermatocytes and a reduced number of spermatids. By contrast, there were no apparent lesions in the histopathology of the female reproductive organs at this concentration.

Table 4-33. Fertility indices for the F_0, F_1, and recovery generations: number of pregnancies per number of females mated

Groups	Fertility index			
	Exposure groups (ppm)			
	0	1	10	40
F_0	30/30	27/30	29/30	16/30[a]
F_1	30/30	27/30	26/30	3/30[a]
F_1/recovery	29/30	ND[b]	ND[b]	14/30[a]

[a]$p<0.01$ compared with control.
[b]ND = not determined

Source: Dodd et al., 1987.

Dodd et al. (1987) considered the histopathologic lesions to be less striking in the F1 males of the recovery group compared with other high-concentration males and correlated this finding with the higher fertility index in their matings compared with those of the regular F1 males. From their data, a NOAEL of 10 ppm for the reproductive and fertility effects of nitrobenzene in Sprague-Dawley rats was suggested.

Biodynamics Inc. (1983) carried out a reproductive/developmental study in which 12 pregnant female New Zealand white rabbits were exposed to nitrobenzene at 0, 10, 40, or 80 ppm, 6 hours/day on GDs 7–19. All dams were terminated on GD 20. The weights of livers and kidneys of all subjects were recorded, and fertility data, such as the number of corpora lutea, live and dead fetuses, late or early resorptions, and implantation sites, were monitored. There were no maternal effects of nitrobenzene, including dose-related changes in body weight or observable clinical signs. The absolute and relative weights of kidneys were similar among all groups, while any increases in liver weights were not statistically significant. One of the few findings of any toxicological importance in the study was the statistically significant increase in the concentration of metHb on GDs 13 and 19, a well recognized effect of nitrobenzene. However, the study did not indicate any nitrobenzene-related changes in any of the fertility parameters measured.

Biodynamics Inc. (1984) carried out a follow up study in which 22 pregnant female New Zealand white rabbits were exposed to nitrobenzene concentrations of 0, 10, 40, and 100 ppm, 6 hours/day on days 7–9 of gestation. All surviving dams were sacrificed on GD 30, and, as in the range-finding experiment of Biodynamics Inc. (1983), the suite of reproductive and developmental toxicity parameters evaluated included such fertility data as the numbers of corpora lutea, implantation sites, resorptions, and live fetuses. However, in this experiment, recovered fetuses were given a gross external examination, and all were evaluated for either soft tissue malformations or skeletal malformations and variations. Maternal toxicity was evidenced

by some upward fluctuations in relative liver weight (to about 12%) and 40% and 60% increases in mean metHb levels in 40 and 100-ppm groups, respectively. However, the only evidence of any reproductive or developmental toxicity effects was in the slightly higher incidence of resorptions in high-concentration dams (11 litters with resorptions versus 7 in controls). These high-dose resorption data were stated to be at or near the historical value observed in New Zealand white rabbits for this testing laboratory. No teratological effects of nitrobenzene were observed.

BRRC (1984) carried out a reproductive, developmental, and toxicological study of the effects of inhaled nitrobenzene in 26 pregnant female CD rats/group. Exposure to nitrobenzene vapor was at nominal concentrations of 0, 1, 10, or 40 ppm, 6 hours/day on GDs 6–15. All dams were sacrificed on GD 21. The weights of the liver, kidney, spleen, and uterus of all subjects were recorded, and fertility data, such as the numbers of corpora lutea, live and dead fetuses, late or early resorptions, and implantation sites, were monitored. Recovered fetuses were given a gross external examination, and all were evaluated for either soft tissue malformations or skeletal malformation and variations. The authors reported some evidence of maternal toxicity, including transient fluctuations in body weight and elevated absolute and relative spleen weights in mid- and high-dose dams. However, all reproductive, developmental, and teratological parameters were unaffected by treatment.

A synopsis of developmental toxicity studies with nitrobenzene following inhalation exposure is presented in Table 4-34.

Table 4-34. Summary of effects observed in developmental inhalation studies with nitrobenzene*

Species, strain	Number	Dosing	Effect[a]	NOAEL (ppm)[b]	LOAEL (ppm)[b]	Reference
Rat, S-D	26 pregnant	0, 1, 10, 40 ppm, 6 hr/d, GDs 6–15, sacrifice on GD 21	Fertility ↓ Developmental Skull ossification ↑	40 40 20	NA NA 40	Tyl et al., 1987
Rat, S-D	30/sex two-generation	0, 1, 10, 40 ppm, 10 wk before mating & through mating, gestation	Testicular pathology ↑ Fertility ↓ Developmental toxicity	10 (M, F_1) 10 (M, F_1) 40	40 (M, F_1) 40 (M, F_1) NA	BRRC, 1985; Dodd et al., 1987
Rabbit, New Zealand	12 (22) pregnant	0, 10, 40, 80 (100) ppm, 6 h/d, GDs 7–19, sacrifice on GD 20 (30)	Fertility ↓ Developmental toxicity	80 (100) 80(100)	NA NA	Biodynamics, 1983, 1984

*NOAELs and LOAELs determined by nitrobenzene assessment authors.

[a]Only endpoints with evident dose responses were selected. ↓ or ↑ = a decrease or increase in the respective endpoint.
[b]M = male; F = female; F_1 = first filial generation; S-D = Sprague-Dawley; NA = not applicable.
[c]Measured at 15-month interim sacrifice.

4.4. OTHER STUDIES

4.4.1. Acute and Short-Term Toxicity Data

DuPont (1981) reported a short-term inhalation study in which 16 male Crl:CD rats/group were restrained and exposed (head only) 6 hours/day, 5 days/week for 2 weeks to either 0, 12, 39, or 112 ppm nitrobenzene. A subset of the exposed animals was terminated directly at the completion of dosing (10 exposures), whereas others were allowed to recover for 14 days after treatment. Blood was obtained from the tail vein on the day of the final exposure and at the end of the recovery period. A wide range of hematological parameters was monitored, along with such clinical chemistry parameters as the activities of alkaline phosphatase (AP), glutamate pyruvate transaminase, AST, and the concentrations of BUN, creatinine, total protein, and cholesterol. Depending on the dose level, a number of the animals displayed clinical signs of exposure to nitrobenzene. Signs were severe, reflecting a degree of toxicity that led to death among animals of the high-concentration group. For example, rats in the mid- and high-concentration groups were cyanotic, and, from day 7 onwards, high-concentration males appeared semi-prostrated during exposure, with labored breathing, hind-limb ataxia, and reduction in body weight. In fact, after the scheduled 10 total exposures, the high-concentration group was reduced to three survivors, of which only one survived through the recovery period. Among the hematological responses, the mid- and high-concentration animals displayed statistically significant reductions in Hb concentration and RBC count, while the platelet count, MCV, and mean corpuscular Hb (MCHb) were increased. MetHb was markedly and dose-dependently higher in nitrobenzene-receiving rats versus controls, with mean percentage values of 0.86, 1.7, 4.1, and 18.1 for rats exposed to 0, 12, 39, and 112 ppm, respectively. Urinalysis indicated a decrease in osmolality, but there was a treatment-related increase in urine volume and urobilinogen concentration, a breakdown product of Hb. After the 14-day recovery period, many of these symptoms were found to persist. Among the histopathologic responses, there was a dose-dependent increase in the deposition of hemosiderin in the spleen of mid- and high-concentration animals. High-dose rats displayed hemorrhage of the brain, plus lesions of the spinal cord; atrophy of the germinal cells; a range of histopathologic effects in the testis and epididymis; pulmonary edema; and lymphoid cell atrophy. In evaluating their data, the authors noted a trend towards increases in the organ/body weight ratios for such organs as spleen, liver, kidney, and heart, though they considered these changes to be unrelated to the toxic effects of

nitrobenzene. By contrast, there were significant reductions in the testis and epididymis weights that appeared to be related to treatment and that persisted in those animals allowed to undergo a period of recovery.

Sprague-Dawley (CD) rats and B6C3F1 mice were more sensitive to the effects of inhaled nitrobenzene than F344 rats in a 2-week exposure study reported by Medinsky and Irons (1985). Ten rats and mice of both sexes were exposed to concentrations of 0, 10, 35, or 125 ppm nitrobenzene, 6 hours/day, 5 days/week for 2 weeks. Five animals of each species, strain, and sex were sacrificed at 3 and 14 days after the last exposure, though many of the B6C3F1 mice and Sprague-Dawley rats either died or were moribund prior to the end of the exposure period. A total of 24 organs and tissues was examined for signs of gross lesions, and the spleen, left kidney, liver, testes, and brain were weighed. Hematological parameters and clinical chemistry measurements were also evaluated.

In the 125 ppm group, it was necessary to sacrifice all mice of both sexes after 2–4 days of exposure, and all Sprague-Dawley rats were sacrificed after 5 days of exposure. By contrast, all F344 rats of both sexes survived the full 2-week exposure period with minimal signs of distress. Concentration-dependent increases in relative liver, kidney, and spleen weights were observed in both sexes of F344 rats, and increased relative spleen weights were observed in Sprague-Dawley rats. Statistically significant increases in relative liver and kidney weights in F344 male rats and relative spleen weights in Sprague-Dawley rats were observed even in the low (10 ppm) concentration groups. Decreased testes weights were observed at the high (125 ppm) concentration F344 rats, a response that persisted throughout the 14-day recovery period. The cause of death in the high-concentration Sprague-Dawley rats was presumably due to perivascular hemorrhage, accompanied by edema and malacia in the cerebellar peduncle. Similar lesions were found in high-concentration group B6C3F1 mice. Histopathologic lesions were observed in the brain, liver, kidney, lung, and spleen of Sprague-Dawley rats and B6C3F1 mice exposed to nitrobenzene. As tabulated by the authors, these lesions included, in the brain, cerebellar perivascular hemorrhage; in the liver, centrilobular necrosis, centrilobular hydropic degeneration, and necrosis of hepatocytes; in the lung, bronchial epithelial hyperplasia, vascular congestion, and perivascular edema; in the kidney, hydropic degeneration of cortical tubular cells; in the testis, testicular degeneration, dysspermiogenesis, and the appearance of multinucleated giant cells; and in the spleen, acute congestion, extramedullary hyperplasia, and the appearance of hemosiderin-laden macrophages in red pulp. Histopathologic lesions observed in F344 rat tissues as a result of exposure to 125 ppm nitrobenzene included, in the spleen, acute congestion, extramedullary hyperplasia, focal capsular hyperplasia, and the appearance of hemosiderin-laden macrophages in red pulp; in testis, edema, increased numbers of

multinucleated giant cells, Sertoli cell hyperplasia, and severe dysspermiogenesis; and, in the kidney, a hyaline nephrosis that was especially marked in male rats. Testicular degeneration was observed in the high-concentration mice and in one animal in the 35-ppm concentration group. The most sensitive organ, based upon the histopathology findings, was the spleen. Lesions in the spleen were observed in all animals of all concentration groups. In F344 rats, there was a concentration-dependent increase in the number of hemosiderin-laden macrophages infiltrating the red pulp, increased extramedullary hematopoiesis, and acute sinusoidal congestion 3 days after the last exposure. Similar lesions were observed in Sprague-Dawley rats and B6C3F1 mice. A concentration-dependent increase in blood metHb was noted in F344 rats 3 days after the end of exposure, but this effect was not observed after 14 days (Table 4-35). Blood metHb ranged from 13–31% in B6C3F1 mice that were sacrificed early.

Table 4-35. Percent metHb in rats exposed to nitrobenzene vapors

Group	Fischer 344 rats		Sprague-Dawley rats	
	Male	**Female**	**Male**	**Female**
Sacrifice at term + 3 days				
Control	0	3.6 ± 2.2	6.9 ± 1.3	4.8 ± 0.7
10 ppm	1.9 ± 0.7	4.8 ± 0.8	6.1 ± 0.5	6.3 ± 0.6
35 ppm	6.6 ± 0.2	6.6 ± 0.8	8.7 ± 1.0	7.3 ± 1.4
125 ppm	11.7 ± 1.2	13.4 ± 2.1	14.0 ± 1.3	31.3 ± 2.5[a]
Sacrifice after recovery period				
Control	4.5 ± 0.3	4.1 ± 0.5	4.6 ± 0.3	5.6 ± 0.6
10 ppm	4.1 ± 0.1	3.1 ± 0.3	9.2 ± 1.6	5.2 ± 1.0
35 ppm	5.6 ± 2.2	5.1 ± 1.9	5.8 ± 0.9	5.0 ± 0.5
125 ppm	4.8 ± 1.9	4.5 ± 1.5	[b]	[b]

[a]Rats were euthanized after 5 days of exposure.
[b]No high-concentration rats survived in this group.
Note that statistical significance was not provided by the authors.
Source: Medinsky and Irons, 1985.

A synopsis of the acute inhalation studies with nitrobenzene is presented in Table 4-36.

Table 4-36. Summary of effects observed in acute inhalation studies with nitrobenzene*

Species, strain	Number	Dosing	Effect[a]	NOAEL (ppm)[b]	LOAEL (ppm)[b]	Reference
Rat, Crl:CD	16 male	0, 12, 39, 112 ppm, 6 hr/d, 5 d/wk, 2 wk	Methemoglobinemia Mortality	NA NA	12 112	DuPont, 1981
Rat, S-D	10/sex	0, 10, 35, 125 ppm, 6 hr/d, 5 d/wk, 2 wk	Spleen weight ↑ Mortality	NA NA	35 125	Medinsky & Irons, 1985
Rat, F344			Organ weights ↑ Testis weight ↓	NA 35	35 125	
Mouse, B6C3F1			Testicular pathology	35	125	

*NOAELs and LOAELs determined by nitrobenzene assessment authors.
[a]Only endpoints with evident dose responses were selected. ↓ or ↑ = a decrease or increase in the respective endpoint.
[b]M = male; F = female; F_1 = first filial generation; S-D = Sprague-Dawley; NA = not applicable.
[c]Measured at 15-month interim sacrifice.

Few data are available for the oral median lethal dose (LD_{50}) for nitrobenzene, although Lewis (1992) reported a value of 590 mg/kg in mice. HSDB (2003) gives values of 600–640 mg/kg nitrobenzene in rats. DuPont (1981) reported a 4-hour median lethal concentration (LC_{50}) of 556 ppm in male Sprague-Dawley rats exposed (head only) to nitrobenzene vapor.

A number of research reports describe the use of acute or short-term exposure regimens to examine sublethal toxicological effects of nitrobenzene. Those addressing the absorption, distribution, metabolism, and excretion of the compound and its metabolites have been described in Section 3 of this Toxicological Review. Other toxicological responses of experimental animals to short-term nitrobenzene exposure are described in the following paragraphs.

As discussed in Section 4.3.1, the single oral dose experiments of Bond et al. (1981) resulted in histopathologic lesions in liver, testes, and brain and in the immediate development and subsequent slow decline of methemoglobinemia in male F344 rats at a dose of 300 mg/kg. Morgan et al. (1985) extended the observations of Bond et al. (1981) on the histopathologic effects of nitrobenzene on the brain by a light and electron microscopic study of male F344 rats receiving single oral doses of 550 mg/kg [^{14}C]-labeled nitrobenzene. Administration of nitrobenzene induced petechial hemorrhages in the brain stem and cerebellum and bilateral symmetric degeneration (malacia) in the cerebellum and cerebellar peduncle. Ultrastructural studies suggested that edematous swelling of a membrane-bounded tissue compartment in the region of the vestibular nuclei and other nuclei lying near the lateral margins of the fourth

ventricle were responsible for the malacia. Hemorrhages were found throughout the brain stem, but there was little evidence of vascular degeneration, and no ultrastructural abnormalities were found in the blood vessel walls. Heinz bodies were observed in the erythrocytes in the hemorrhages, consistent with induction of metHb by nitrobenzene. However, it could not be established whether tissue anoxia due to metHb formation could have contributed to the neurotoxicity of nitrobenzene. Whole body autoradiography indicated that only a small portion of the administered nitrobenzene dose actually penetrated the blood-brain barrier. Radiotracer studies indicated that approximately 0.02% of the total nitrobenzene dose was present in the cerebellum 12 hours after administration. However, no nitrobenzene metabolites could be detected, and the mechanism of nitrobenzene neurotoxicity could not be determined from these studies. Though, quantitatively, the brain appeared not to be a primary target organ of nitrobenzene deposition, a range of marked histopathologic effects of nitrobenzene was identified, including bilateral symmetrical degeneration of the cerebellum and instances of neuronal degeneration.

NTP sponsored a 14-day skin painting toxicological studies with nitrobenzene in F344 rats and B6C3F1 mice (NTP, 1983b). In the study, dose levels ranged from 200–3200 mg/kg, the higher doses (1600 and 3200 mg/kg) inducing death or morbidity before the end of the experiment. Among surviving animals, significantly reduced weight gain (>10%) was observed in all but the low-dose groups. Reticulocyte counts and metHb concentrations were increased significantly, most conspicuously in mice where these effects were seen in the low-dose males. RBCs and Hb concentrations were reduced. Histopathologic changes were evident in brain, liver, spleen, and testis.

Shimkin (1939) demonstrated the ability of nitrobenzene to penetrate the skin and induce toxic effects in female C3H and male A strain mice. In these experiments, nitrobenzene was brushed onto the shaved abdomen of C3H mice, covering less than one-tenth of the body surface. Because of the method of application, the applied dose was unknown. Treatment-related clinical signs, morbidity and mortality, along with associated evidence of incipient methemoglobinemia and other hematological perturbations, were observed. One hour after application, 15/18 female C3H mice were in partial collapse, but all recovered within 24 hours. After a second application three animals died, and after a third application nine more animals died. Approximately 30 minutes after vigorously brushing nitrobenzene over the unshaved abdomens of 10 male A strain mice for 20 seconds, all the mice were in partial collapse and 8/10 died within 3 days. One to 3 hours after application, the skin became dark gray-blue, the blood became chocolate colored and viscous, and the urine was orange with an odor of nitrobenzene. Spectrographic analysis of blood showed a strong absorption band characteristic of metHb. The hematological

data in Shimkin's report emphasized the variability of the cell counts with a normal differential count but a greater than 50% reduction in WBCs (5000 cells/mm^3, reduced from 11,000–14,000 cells/mm^3 in controls). However, while RBC numbers were unaffected, smears indicated hypochromia and hemolysis. Among the necropsy findings, the most significantly affected target organ was the liver, which demonstrated diffuse necrosis, especially in the outer portions of the liver lobules. There was a large amount of dark, brownish pigment in the Kupffer cells; the pigment was more prominent in the necrotic portions of the lobules. Among secondary sites, the kidney showed evidence of enlargement of the glomeruli and tubular epithelium. However, other potential target organs, such as the spleen, lungs, and testis displayed no morphological changes.

4.4.2. Structure-Activity Relationships

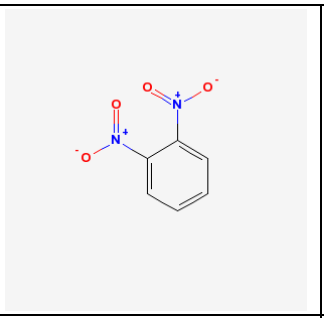
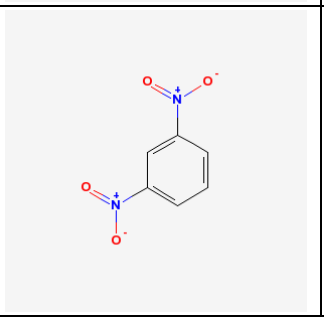
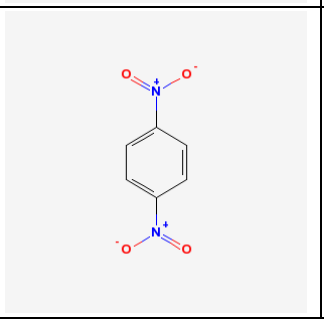
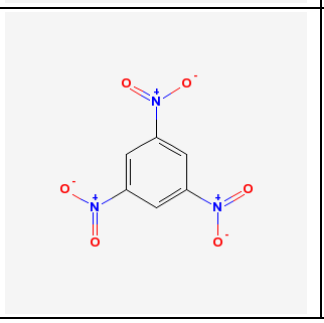
Nitroaromatic compounds include nitrobenzene and four structurally similar compounds that vary based on the number and position of the nitro group (Table 4-37). A large body of toxicological information is available on 1,3-dinitrobenzene and 1,3,5-trinitrobenzene. Toxicity data on these compounds in experimental animals have revealed a similar spectrum of toxicological effects to those seen with nitrobenzene (e.g., metHb formation and splenomegaly) (Tables 4-38 and 4-39) (Salice and Holdsworth, 2001).

For example, the male reproductive toxicity expressed by nitroaromatics is greatly influenced by the structure of the compound. Of the three dinitrobenzene isomers listed in Table 4-37, only 1,3-dinitrobenzene, not 1,2-dinitrobenzene or 1,4-dinitrobenzene, is a potent testicular toxicant that targets the Sertoli cell. However, 1,4-dinitrobenzene, but not 1,2-dinitrobenzene, has a potency similar to that of 1,3-dinitrobenzene in producing cyanosis and splenic enlargement in male Alpk/AP (Wistar derived) rats, indicating that different mechanisms are probably responsible for these two toxic effects (Blackburn et al., 1988). Similarly, the cerebellar neurotoxicity ascribed to1,3-dinitrobenzene and 1,3,5-trinitrobenzene is not observed in animals dosed with 1,4-dinitrobenzene (Chandra et al., 1999; Romero et al., 1995; Morgan et al., 1985).

Table 4-37. Overview of properties and toxicities of nitrobenzenes

IUPAC[a] Name	CASRN	Chemical formula	Structural formula	LOAEL	NOAEL	Critical effect

Table 4-37. Overview of properties and toxicities of nitrobenzenes

1,2-Dinitrobenzene[b]	528-29-0	$C_6H_4N_2O_4$		No data	TLV[c]: 0.15 ppm (as TWA[d]) (skin)	Liver impairment, methemoglobinemia, anemia
1,3-Dinitrobenzene[e,f]	99-65-0	$C_6H_4N_2O_4$		Drinking water: 8 ppm	Drinking water: 3 ppm (0.40 mg/kg-day)	Increased splenic weight
1,4-Dinitrobenzene[g]	100-25-4	$C_6H_4N_2O_4$		No data	TLV: 0.15 ppm (as TWA) (skin)	Liver impairment, methemoglobinemia, anemia
1,3,5-Trinitrobenzene[h,i]	99-65-0	$C_6H_3N_3O_6$		Dietary study: 13.31 mg/kg-day	Dietary study: 2.68 mg/kg-day	Methemoglobinemia and spleen-erythroid cell hyperplasia

[a]IUPAC = International Union for Pure and Applied Chemistry
[b]http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=36593; http://www.inchem.org/documents/icsc/icsc/eics0460.htm; http://www.epa.gov/iris/subst/0633.htm.
[c]TLV = threshold limit value
[d]TWA = time-weighted average
[e]http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=48779; http://www.epa.gov/iris/subst/0318.htm; Cody et al., 1981.
[f]Conversion Factors: Drinking water concentrations converted to dosages by investigators.
[g]http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=15738; http://www.inchem.org/documents/icsc/icsc/eics0692.htm
[h]http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=48734; http://www.epa.gov/iris/subst/0316.htm; Reddy et al., 1996.

[i]Conversion factors and assumptions: based on food consumption data, the authors calculated the intake of trinitrobenzene from dietary concentrations of 0, 5, 60, and 300 ppm as 0, 0.23, 2.68, and 13.31 mg/kg-day (females) and 0, 0.22, 2.64, and 13.44 mg/kg-day (males).

Table 4-38. Summary of toxicological studies with 1,3-dinitrobenzene

Study	Species, strain	Test duration	NOAEL (mg/kg-day)[a]	LOAEL (mg/kg-day)	Effects observed at the LOAEL
Linder et al., 1986; Perreault et al., 1989	Rat (Sprague Dawley)	12-week	0.54	1.1	Reduced spermatid head count
			NA	0.54	Significant reduction in reproductive performance (pups/litter)
Philbert et al., 1987	Rat (F344)	5-day	NA	20	Ataxia in all male rats
Reddy et al., 1994a	Rat (F344)	90-day	0.07	0.35	Methemoglobinemia and an increase in reticulocytes
			0.39	1.73	Reduction in RBCs and in other hematological responses, changes in spleen and testicular histopathology
Reddy et al., 1994b	Rat (F344)	14-day	0.21	0.8	Methemoglobinemia
			0.8	1.98	Splenomegaly
			1.98	5.77	Nephropathy associated with hyaline droplet formation, testicular degeneration
Cody et al., 1981	Rat (Carworth Farms)	8-week	NA	4.72	Splenomegaly, fluctuation in hemoglobin levels, atrophy and histopathologic lesions of the testes
Cody et al., 1981	Rat (Carworth Farms)	16-week	0.48	1.32	Splenomegaly
			1.13	2.64	Depleted spermatogenesis

[a]NA = not applicable.

Table 4-39. Summary of toxicological studies with 1,3,5-trinitrobenzene

Study	Species, strain	Test duration	NOAEL (mg/kg-day)[a]	LOAEL (mg/kg-day)	Effects observed at the LOAEL
Reddy et al., 1996; 2001	Rat (F344)	2 years	2.68	13.31	Methemoglobinemia, spleen erythroid cell hyperplasia, decreased body weight
Reddy et al., 1994a; 1998	Rat (F344)	90 days	NA	3.91	Nephropathy, α_{2u}-globulin-associated hyaline droplet formation in males at all doses
			4.29	22.73	Methemoglobinemia, spleen erythroid cell hyperplasia in high-dose and mid-dose groups (males and females)
Reddy et al., 1994b	Rat (F344)	14 days	NA	4.54	Reduced RBC count and Hct in all female groups
			4.52	16.85	Histopathologic changes to the kidney in males
Kinkead et al., 1994a; 1995	Rat (Sprague-Dawley)	90 days	2.0	9.0	Sperm motility/seminiferous tubular degeneration of the testes
			NA	2.0	Nephropathy, hyaline droplet formation in males at all doses
Kim et al., 1997	Rat (F344)	10, 20, and 30 days	NA	35.5	Nephropathy, α_{2u}-globulin-associated hyaline droplet formation in males at all doses tested
Narayan et al., 1995	Rat (Sprague-Dawley)	90 days	NA	3.0	Increase in tissue concentrations of various neurotransmitters in several brain regions, potentially associated with neurological disorders and histopathologic lesions
Kinkead et al., 1994b	Rat (Sprague-Dawley)	7 weeks	23	51	Testicular degeneration and sperm depletion in males
		10 weeks	4	23	Encephalitis in females
Chandra et al., 1995a	Rat (F344)	10 days	NA	35.5	Hematological deficits and metHb formation
Chandra et al., 1995b	Rat (F344)	10 days	35.5	71	Histopathologic lesions in the brain of males
Chandra et al., 1997	Rat (F344)	10 days	NA	35.5	Testicular degeneration
Cooper and Caldwell, 1995[b]	Rat (Sprague-Dawley)	GDs 6–15	45	90	Developmental deficits among the pups
Reddy et al., 1995	Mouse (*Peromyscus leucopus*)	90 days	67.4	113.5	Testicular degeneration in high-dose males
			23.5	67.4	Erythroid hyperplasia, increase in reticulocyte count in mid- and high-dose males
Reddy et al., 2000	Shrew (*Cryptotis parva*)	14 days	10.75	21.60	Decrease in liver and body weight
			10.68	22.24	Increase in spleen weight of females

[a]NA = not applicable
[b]As cited in Reddy et al., 1997.

4.4.3. Immunotoxicity Studies

Burns et al. (1994) carried out a 14-day gavage study of nitrobenzene in corn oil in which female B6C3F1 mice were administered 0, 30, 100, and 300 mg/kg of the compound. The primary focus of the study was the immunotoxicity of the compound, although some characteristic responses of nitrobenzene's acute toxicity to B6C3F1 mice at these exposure levels were reported. For example, 17 of 200 high-dose mice died during the period of exposure, and others displayed typical signs of toxicologically stressed animals, such as ataxia, lethargy, and circling. Eight distinct investigations of the immunotoxicological effects of nitrobenzene were carried out among the exposed mice, while some nonimmunotoxicological parameters were monitored in all animals. .

Examination of the mice at autopsy 24 hours after the final exposure showed hepatomegaly and splenomegaly in the mid- and high-dose groups, although the overall liver changes were slight. The affected spleens were dark red in color, with mild congestion in the red pulp areas and the appearance of occasional nucleated erythrocytes. Hemosiderin pigment was noted in the red pulp areas, a response thought to be indicative of erythrocyte dysfunction. However, white pulp areas of the spleen appeared to be normal. Compound-related changes in organ weights were noted, including dose-dependent increases in the absolute and relative weights of liver, spleen, and kidney. A number of apparently compound-related effects in hematological responses to nitrobenzene were observed, consistent with the concept of the erythrocyte as a primary target organ of nitrobenzene toxicity. The changes included decreases in erythrocyte number ($7.64 \pm 0.15 \times 10^6$ cells/μL in controls versus $6.94 \pm 0.14 \times 10^6$ cells/μL in mice exposed to 300 mg/kg-day nitrobenzene) but increases in MCV (56 ± 1 fL in controls versus 63.7 ± 1.4 fL in mice receiving 300 mg/kg-day) and MCHb (18.1 ± 0.3 pg in controls versus 20.6 ± 0.6 pg in 300 mg/kg-receiving animals). However, there were no treatment-related changes in Hb concentration or Hct. Although no treatment-related differences in leukocyte differentials were observed after 14 days, there were striking changes in the percentage of circulating reticulocytes as a result of treatment (4.57 ± 0.48% in 300 mg/kg-receiving mice versus 1.03 ± 0.9% in controls).

Burns et al. (1994) also observed some treatment-related changes in clinical chemistry parameters, including a dose-dependent increase in the activity of aspartate aminotransferase (80 ± 9 IU/mL in controls versus 128 ± 16 IU/mL in high-dose mice) and alanine aminotransferase (27 ± 1 IU/mL in controls versus 74 ± 11 IU/mL in high-dose animals).

Other dose-dependent effects of nitrobenzene on clinical chemistry parameters included apparent increases in the levels of bilirubin and albumin but decreases in glucose concentration.

In light of the changes observed in the spleen and hematological parameters, Burns et al. (1994) examined the bone marrow for cell number, status of DNA synthesis, and the number of macrophage and granulocyte-monocyte progenitor cells. DNA synthesis was measured by the incorporation of [^{3}H]-thymidine over a 3-hour incubation period. Progenitor cells were measured by incubating bone marrow cells with 10% colony stimulating factors isolated from either mouse fibroblast L-929 cells or mouse lung-conditioned medium. Colonies were counted after 8 days. The number of nucleated cells/femur was increased dose dependently to a level of 62% above controls, with statistical significance seen in the low-dose group. Overall rates of DNA synthesis also were increased up to 80% above that of controls. As described by the authors, the number of colony-forming unit (granulocyte-monocyte) stem cells was the same as in controls when calculated per 10^5 bone marrow cells. However, the number of cells/femur and the number of colony forming unit (granulocyte-monocyte) stem cells/femur were increased twofold in association with nitrobenzene treatment (Burns et al., 1994).

Burns et al. (1994) determined spleen immunoglobulins G and M (IgG and IgM) antibody responses to T-dependent sheep RBCs in mice exposed to nitrobenzene using a modified hemolytic plaque assay. Animals receiving nitrobenzene were sensitized to sheep RBCs by intravenous injection on day 11 of exposure, and spleen cells were harvested at term. Suspended cells were incubated with guinea pig complement, sheep RBCs, and warm agar. Rabbit anti-mouse IgG-developing serum was added when IgG plaques were evaluated, and cell and plaque counts were obtained after a 3-hour incubation at 37 ^{o}C.

Although there was a dose-dependent increase in spleen weight and spleen cell number 4 days after exposure to nitrobenzene, there was no difference in the splenic IgG responses to sheep erythrocytes as a result of nitrobenzene exposure. By contrast, nitrobenzene exposure caused a dose-dependent decrease in the IgM response to sheep erythrocytes on day 4 (40% and 34% for the mid- and high-dose nitrobenzene groups, respectively). According to the authors, this suppression could be accounted for by the observed compound-induced splenomegaly (Burns et al., 1994). However, treated mice recovered their ability to mount an IgM response within 20 days.

The capacity of spleen cells to undergo a proliferative response to the T cell mitogens (phytohemagglutinin [PHA], concanavalin A [con A], and the B cell mitogen, lipopolysaccharide [LPS]) was investigated. Cells were isolated from excised spleen tissue after 15 days of nitrobenzene exposure and cultured for 3 days in the presence of four concentrations of the above mitogens. The amount of [^{3}H]-thymidine incorporated into the cells over the last 18 hours of the incubation was taken as a measure of spleen cell proliferation. The effects of nitrobenzene on the response to PHA and con A appeared to be dose-related, with a marked suppression of

[^{3}H]-thymidine incorporation following exposure to 100 and 300 mg/kg nitrobenzene (106,152 ± 10,326 cpm/culture in control cultures of spleen cells incubated with 5 μg/mL con A versus 59,602 ± 5189 cpm/culture in cultures of spleen cells from high-dose mice incubated with the same concentration of mitogen). However, there were no effects of nitrobenzene on the response to the B cell mitogen, LPS.

The impact of nitrobenzene exposure on the onset of delayed hypersensitivity of keyhole limpet hemocyanin (KLH) was assessed by administering a subcutaneous injection of 100 μg KLH on days 1 and 8 of nitrobenzene exposure. On the last day of nitrobenzene exposure, mononuclear cells were labeled in vivo by intravenous injection of [^{125}I]-5-iododeoxyuridine (2 μCi) per mouse. On day 15, animals were challenged in the central portion of the left ear with an intradermal injection of 30 μg KLH, and ear biopsies were radioassayed 24 hours later. As expressed by a stimulation index, no effect of nitrobenzene on a delayed hypersensitivity response to KLH was observed. Similarly, in another sequence of observations, there were no differences in serum complement levels between nitrobenzene-exposed and control groups.

Burns et al. (1994) investigated the comparative uptake and organ distribution of injected radiolabeled sheep erythrocytes in control and nitrobenzene-exposed mice. Compared with the vehicle control group, there was a dose-dependent increase in particle uptake into the livers of nitrobenzene-receiving mice (39.4 ± 1.8 in controls versus 55.3 ± 1.7 in high-dose animals). However, this effect was considered to be a consequence of liver enlargement in nitrobenzene-receiving groups.

In other experimental approaches, Burns et al. (1994) monitored the number of cells that could be harvested by lavage from the peritoneal cavity of nitrobenzene-challenged mice, examined the ability of isolated macrophages to take up fluorescent beads (0.85 μm), and determined the effect of nitrobenzene on natural killer cell activity in the spleen. In the latter case, natural killer cell function was assessed by monitoring the capacity of spleen cells to lyse [^{51}Cr]-labeled YAC-1 target cells in vitro. Nitrobenzene exposure caused a dose-dependent decrease in lytic activity at all effector:target cell ratios tested.

The same research report describes a series of experiments to evaluate the effect of nitrobenzene on host resistance to infection with *Plasmodium berghei*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, herpes simplex Type 2 virus, and the metastatic pulmonary tumor, B16F10. Mice treated with nitrobenzene were no more susceptible to *S. pneumoniae* or *P. berghei* than control animals. However, a challenge with 6×10^{3} *L. monocytogenes*/mouse killed 13% of the control mice and 57% of those receiving 300 mg/kg nitrobenzene. Similar differences were observed for different titers in mice exposed to 100-mg/kg nitrobenzene. As pointed out by the authors, host resistance of *L. monocytogenes* is mediated by T lymphocytes,

macrophages, and complement activity. Nitrobenzene exposure did not impair host resistance to herpes simplex virus, as measured by percent mortality or time to death. Host resistance to B16F10 melanoma involves T-lymphocytes and macrophages. Nitrobenzene somewhat impaired host resistence at the highest level, indicating a modest depression of T-cell immunity.

In seeking to explain their results, Burns et al. (1994) considered that most of the effects of nitrobenzene on the immune system could be explained by the increased cellularity of the spleen. However, the perturbation of the bone marrow in mice exposed to nitrobenzene was pronounced, manifested in these studies by significant increases in cells/femur, DNA synthesis, and colony forming units (granulocytes/monocytes)/femur. These results were thought to indicate that the principal target of nitrobenzene toxicity was bone marrow, with consequent hematological and immunotoxicological impacts.

Wulferink et al. (2001) presented findings that nitrosobenzene (but not nitrobenzene, aniline, or *p*-aminophenol) stimulated the production of antigen-specific T-cells in female C57BL/6J mice. The study analyzed primary and secondary popliteal lymph node (PLN) response, an assay that detects the immunostimulatory capacity of low-molecular weight substances. For the primary PLN response, animals received a single subcutaneous injection (50 μL) into the left hind footpad. After 6 days, the PLNs from the treated and untreated sides were removed and cell numbers were counted. Cell counts from nitrobenzene-, aniline-, or *p*-aminophenol-treated animals (0.2 μmol/mouse) were indistinguishable from controls; however, nitrosobenzene caused a statistically significant increase in cell counts at 0.1 and 0.2 μmol/mouse. For the secondary PLN response, animals were primed with a single subcutaneous injection (50 μL) of aniline or nitrosobenzene. Thirteen weeks later (the time period it takes for PLNs to return to normal size and cellularity), a second subcutaneous injection (50 μL) containing a suboptimal dose (a dose too low to stimulate a primary PLN response; 0.005 μmol/mouse of either aniline or nitrosobenzene) was administered to the same footpad. After four days, the PLNs from the treated and untreated sides were removed. Cell counts from animals primed with aniline and challenged with either aniline or nitrosobenzene were consistent with controls. Similarly, the cell counts from animals primed with nitrosobenzene and subsequently challenged with aniline were not significantly different from controls. In contrast, when animals were primed with nitrosobenzene and also challenged with nitrosobenzene, a statistically significant increase in cellularity was observed compared with controls. Hopkins et al. (2005) reported similar findings that dermal application of nitrosobenzene (100 μL; 0.02%, w/v, in 5% dimethyl sulfoxide [DMSO]) for three consecutive days on the nape of the neck of female BALB/c mice caused a statistically significant increase in lymph node cellularity and proliferation 5 days after the initial application.

4.4.4. Neurotoxicity Studies

Signs and symptoms of neurotoxicity following exposure to nitrobenzene have been reported as early as the 1900s. No epidemiological studies have been conducted on occupationally exposed cohorts; however, numerous case reports indicate neurological involvement following accidental or intentional exposure to nitrobenzene. Abbinante et al. (1997) identified dizziness, generalized weakness, and convulsions as the most frequent neurological manifestations from nine individuals intoxicated with nitrobenzene (levels of exposure unknown). Similarly, Stifel (1919) reported 16 cases of nitrobenzene poisoning from shoe dye. Many of the patients complained of headache, nausea, dizziness, and general malaise.

In a more comprehensive report, Ikeda and Kita (1964) presented findings from a woman who was occupationally exposed to nitrobenzene. Seventeen months after starting a new position, the woman's workplace was remodeled and the ventilation became quite poor. After about 6 weeks of working under these conditions, the woman presented with severe headache, nausea, vertigo, and numbness in her legs. After 5 days of bed rest, her condition improved and she returned to work. Nearly 3 months later, the woman presented with similar symptoms. In addition, she experienced hyperalgesia to pin-prick on the backs of her hands and feet, which suggested degenerative changes in the peripheral nerves. She was discharged after 39 days in the hospital with only residual hyperalgesia in the hands and feet.

Adams (1912), as cited in Hamilton (1919), presented observations of a middle-aged woman who was chronically exposed (18-year observation period) to nitrobenzene through its use as an ingredient in cleaning fluid. The symptoms, which progressed very slowly, were those of a multiple neuritis, which finally resulted in contractures and almost complete powerlessness. Interestingly, 1,3-dinitrobenzene, a compound structurally similar to nitrobenzene, has been reported to cause numbness in the distal portions of the limbs in humans (Lazerev and Levina, [1976], as cited in Philbert et al. [1987]).

Obvious shortcomings of the above studies are the lack of quantitative estimates for exposure and effects and the fact that they are primarily anecdotal. However, similar manifestations of toxicity have been reported in nitrobenzene poisonings of experimental animals. Matsumaru and Yoshida (1959) treated male and female rabbits (strain not stated) with nitrobenzene injection via the ear vein or by topical application to the skin of the back. Neurotoxicity was manifest with paralysis of the limbs, elevated sensitivity, and general convulsion. When acute in nature, intoxication was evident mainly as convulsion, whereas chronic intoxication resulted in paralysis. Central nervous system effects were evident with an enormous number of well-defined round vacuoles occurring in the medulla, which was more

marked in those animals in the high-dose intravenous group and those treated dermally for a prolonged term (time period not stated), compared with those in a low-dose (and control) group and and treated for shorter term(s), respectively.

Bond et al. (1981) described a lesion consisting of a bilateral malacic area and reactive gliosis in the cerebellar peduncles. However, this lesion was observed with only one rat (F344[CDF/CrlBR]) 5 days following oral administration of 450 mg/kg nitrobenzene. Marked methemoglobinemia was excluded as the precipitating factor, since administration of sodium nitrate to rats for 3 days resulted in a prolonged methemoglobinemia of severity similar to that produced by nitrobenzene but showed no evidence of toxicity to the brain.

Shimo et al. (1994) treated F344 rats with nitrobenzene at the doses of 0, 5, 25, and 125 mg/kg-day for 28 days via intragastric administration. Absolute brain weights of male rats revealed an increasing trend that became significant in the 25-mg/kg group, and absolute brain weights in female rats followed a similar trend that resulted in significant increases in the 125 mg/kg group. Histopathology revealed moderate to severe spongiform changes and brown pigmentation in the perivascular region of the cerebellum in male and female rats treated with 125 mg/kg. Following a 14-day recovery period, brain weights of treated animals (males and females) were consistent with those of controls; however, moderate to severe spongiotic changes persisted in five of six male rats and four of six female rats, whereas moderate brown pigmentation in the perivascular region was present in three of three male rats and two of four female rats.

Morgan et al. (1985) administered a single oral dose (550 mg/kg) of nitrobenzene to male F344 (CDF/CrlBR) rats. Within 24 hours after dosing, the rats were lethargic and ataxic but responsive to external stimuli (tail pinch). By 36–48 hours, several rats displayed moderate to severe ataxia and loss of righting reflex and no longer responded to external stimuli. Microscopic analysis revealed variable numbers of small hemorrhages scattered throughout the brain stem and cerebellum. Many neurons and areas adjacent to malacia, both lateral and dorsal to the fourth ventricle, showed moderate to severe fine, foamy vacuolation of the perikarya and nuclear condensation. The affected areas exhibited numerous vacuoles, some of which could be identified as distended myelin sheaths of large axons. Swelling of myelin sheaths was also observed in white matter tracts adjacent to areas of malacia.

Burns et al. (1994) treated female B6C3F1 mice with nitrobenzene at 0, 30, 100, or 300 mg/kg for 14 consecutive days. Neurotoxicity was manifest in the 300 mg/kg-bw group only with animals exhibiting marked ataxia, lethargy, and circling. One animal was observed with bobbing head movements. Absolute brain weights for all treatment groups were consistent with controls. Histopathologic changes in the liver of the high-dose group consisted of very mild

hydropic degeneration around focal central veins with elevated levels of serum transaminases and bilirubin.

4.4.5. Genotoxicity Studies

The mutagenicity/genotoxicity of nitrobenzene has been addressed in a number of studies using standard Ames test protocols. For example, in the multi-center survey of compounds that was carried out for the U.S. National Institute of Environmental Health Sciences, nitrobenzene was found to be negative for reverse mutation with or without 9000g microsomal supernatant fraction (S9) in all of the *Salmonella typhimurium* tester strains that were used (Haworth et al., 1983). Similarly, in a survey of nitroaromatic compounds that were evaluated for mutagenicity (without S9) in nine tester strains of *S. typhimurium*, nitrobenzene was negative for reverse mutation at all concentrations in every strain tested (Vance and Levin, 1984). Further, several studies from different laboratories (Dellarco and Prival, 1989; Assmann et al., 1997; Shimizu et al., 1983; Ho et al., 1981; Anderson and Styles, 1978; Chiu et al., 1978; Garner and Nutman, 1977) have reported essentially similar findings for nitrobenzene in this experimental system, irrespective of the presence of an Aroclor-1254-induced S9 liver preparation or added flavin mononucleotide (Dellarco and Prival, 1989).

At contrast with the studies recounted above, two studies by Suzuki et al. (1987, 1983) reported positive findings for a mutagenic action of nitrobenzene in the Ames test with the tester strain TA 98 plus S9 fraction, in the presence of the comutagen norharman (9H-pyrido[3,4-b]indole). None of the compounds was mutagenic without norharman in strains TA98 or TA100. In the presence of S9 and norharman, nitrobenzene induced reverse mutations in TA 98 but not in TA 100. Because norharman-containing controls were negative for reverse mutation in this tester strain, the authors concluded that nitrobenzene could induce reverse mutations in the presence of a comutagen. In a further series of experiments, Suzuki et al. (1987) demonstrated that the nitroreductase-deficient isolate TA 98NR was negative for reverse mutations even in the presence of S9 and norharman. These data are considered to be consistent with the concept that metabolic activation by S9, and norharman, were unrelated to the induction of nitroreductase, but presence of the reductase was required to elicit nitrobenzene mutagenicity.

In general, available data on the mutagenicity of nitrobenzene using the Ames assay demonstrate a lack of significant effects on reverse mutations (Table 4-40). This conclusion may be tempered by the limited range of tests that have been employed for nitrobenzene and the inferential evidence of the compounds' mutagenicity in *S. typhimurium* TA 98 in the presence of the comutagen, norharman (Suzuki et al., 1983). In addition, Clayson and Garner (1976)

speculated that the electrophilic nitrenium ion (NH^{2+}) is the ultimate carcinogen from aromatic amino and nitro compounds and not enough is known about the capability of *S. typhimurium* to create this reactive intermediate from nitrobenzene.

Kligerman et al. (1983) exposed male CDF(F344)/CrlBR rats to doses of 0, 5, 16, or 50 ppm nitrobenzene for 6 hours/day, 5 days/week for 21 days during a 29-day period. The authors assessed the ability of inhaled nitrobenzene to induce cytogenetic damage in the lymphocytes of isolated spleen or peripheral blood. No statistically significant increases in sister chromatid exchanges were observed at any doses tested. Similarly, nitrobenzene did not induce unscheduled DNA synthesis in an in vivo-in vitro hepatocyte DNA repair test (Mirsalis et al., 1982).

In contrast to the above results, nitrobenzene was weakly positive for the induction of chromosome aberrations in cultured human peripheral lymphocytes (Huang et al., 1996, 1995). However, the compound did not induce structural chromosome aberrations in human spermatozoa incubated with 500 μg/mL nitrobenzene for 120 minutes in the absence of S9 fraction (Tateno et al., 1997).

Bonacker et al. (2004) recently demonstrated the induction of micronuclei in V79 hamster lung fibroblast cells following exposure to nitrobenzene. To further delineate the mechanism by which the micronuclei are formed, the authors used primary CREST antibodies that bind to kinetochore proteins at chromosomal centromeres and detect aneugenicity. CREST Syndrome is a disorder of the skin and connective tissue that leads to hardening of the skin's surface; its cause is unknown (Schuler et al., 1997; Miller and Adler, 1990). Following an 18-hour incubation, a doubling of micronuclei was observed at 1, 10, and 100 μM nitrobenzene versus solvent (DMSO) controls. Nitrobenzene (up to 10 μM) was shown to induce mostly kinetochore-positive micronuclei, indicative of an aneugenic effect. To determine the possible effect of nitrobenzene on the cellular spindle apparatus, temperature-dependent assembly (at 37 ^{o}C) and disassembly (at 4 ^{o}C) of tubulin were determined in the presence of nitrobenzene in vitro. A slight inhibitory effect was observed with 1 mM nitrobenzene in the absence of DMSO; however, in the presence of 1% DMSO, nitrobenzene exerted no detectable effect on tubulin assembly up to the solubility limit of about 15 mM. A functional analysis of the tubulin-kinesin motor system revealed that nitrobenzene had a clear dose-dependent affect on the gliding velocity of microtubules with a minimal degree of inhibition above 7.5 μM to complete inhibition at 30 μM.

Li et al. (2003a, b), using the ultra-sensitive method of accelerator mass spectrometry, demonstrated recently that nitrobenzene forms adducts with Hb and with hepatic DNA in male Kunming mice. [^{14}C]-Nitrobenzene was administered intraperitoneally in corn oil at doses of

0.1–100 μg/kg and 10 mg/kg, and animals were sacrificed 2 hours after treatment. The authors found that both Hb and hepatic DNA adducts occurred with similar dose-response relationships within 2 hours of exposure over the whole range of doses. Regressions of log dose versus log adduct per gram Hb or DNA resulted in straight lines with regression coefficients of 0.998 and 0.993, respectively. In addition, a time-course experiment was conducted in which the mice received 4.1 μg/kg nitrobenzene and were sacrificed between 4 and 21 days after dosing. This study revealed a biphasic pattern of adduct elimination, with adduct levels in hepatic DNA attaining peak levels at 4 hours after dosing, then declining with a half-life of 10 hours for the initial 3 days. Thereafter, for up to 21 days, adducts disappeared with a half-life of 6.5 days. Although the findings of Li et al. (2003a, b) appear to point to a genotoxic potential of nitrobenzene, they are disputable. The binding level was extremely low, and any biological significance at such levels of DNA binding is unclear. Also, the DNA adducts were neither characterized nor identified. Further independent confirmation is warranted to elucidate the toxicological meaning of these observations.

More recently, however, Robbiano et al. (2004) reported in vivo and in vitro findings that suggest a genotoxic potential for nitrobenzene. Male Sprague-Dawley rats were administered a single dose of nitrobenzene (300 mg/kg) by gavage and euthanized 20 hours later. A statistically significant increase in DNA damage, measured by the comet assay, and broken or detached chromosomes separated from the spindle apparatus, measured by the micronucleus assay, were observed. The in vitro findings with primary cultures of kidney cells from male Sprague-Dawley rats and human kidney cells obtained from patients with kidney cancer were consistent with the in vivo results. Cells were treated with 0, 0.062, 0.125, 0.25, or 0.5 mM nitrobenzene. This dose range was based on preliminary studies with concentrations that produced a lower than 30% reduction of relative survival. Nitrobenzene caused a statistically significant increase in DNA damage in rat primary kidney cells (0.125 mM–0.5 mM) and human kidney cells (0.062 mM–0.25 mM), following 20-hour incubation with the compound. A statistically significant increase in clastogenic effects was observed in rat primary kidney cells (0.0125–0.5 mM) and human kidney cells (0.250–0.5 mM), following a 48-hour incubation with the compound.

Mattioli et al (2006) provided in vitro and in vivo evidence of a non-genotoxic MOA for nitrobenzene. The authors treated primary human thyroid cells with 1.25, 2.5, or 5 mM nitrobenzene for 20 hours. A dose-dependent increase in DNA fragmentation and unscheduled DNA synthesis was observed; however, the amount of DNA fragmentation at 5 mM nitrobenzene was 8-fold lower than 0.075 mM methyl methenesulfonate, a monofunctional alkylating agent used as a positive control. In the companion in vivo studies, the authors treated rats with 310 mg/kg-bw, po, and examined the degree of DNA fragmentation 16 hours later in

the kidney, liver, and thyroid. The findings showed that the amount of DNA fragmentation was as follows from highest to lowest: liver ≈ kidney > thyroid. Although the results support the ability of nitrobenzene to generate alkali labile sites, as measured by the Comet assay, these findings need to be viewed cautiously with regard to other effects that may be operational at much lower doses. The in vitro and in vivo findings support the notion that a redox couple can be established, with the subsequent generation of reactive oxygen species, and DNA fragmentation. However, liver hypertrophy has been shown to occur at doses as low as 9.38 mg/kg-day in F344 rats (NTP, 1983a), an effect that may alter the hypothalamic-pituitary-thyroid axis by increasing the clearance of thyroxine (T4) (*via* glucuronidation) and triiodothyronine (T3) (*via* sulfation) in rodents. Such an effect may cause a compensatory increase in circulating thyroid-stimulating hormone (TSH) and ultimately follicular cell activation (U.S.EPA, 1998).

In conclusion, results of genotoxicity testing are mixed. Nitrobenzene appears to be at most weakly genotoxic. This determination is based on the almost exclusively negative results in Salmonella assays (Ames tests; the only exception is TA98 in the presence of a comutagen), as well as negative clastogenic findings from in vivo assays of sister chromatid exchange, unscheduled DNA synthesis, and chromosomal aberrations. In vitro chromosome aberration results were mixed, as were the DNA breakage and micronucleus data. For instance, nitrobenzene was weakly positive for the induction of chromosome aberrations in cultured human peripheral lymphocytes but negative in human spermatozoa. Nitrobenzene induced weak DNA fragmentation but no DNA strand breaks. In addition, nitrobenzene did not cause cell transformation in these cell systems. A summary of the genotoxic findings on nitrobenzene is presented in Table 4-40.

Table 4-40. Summary of studies on the direct mutagenicity/genotoxicity of nitrobenzene

Test system	Cell/strain	Result[a] (+/- S9)	Reference	Comments[b]
Bacteria				
S. typhimurium	TA98, TA100, TA1535, TA1537	–/–	Haworth et al., 1983	Reverse mutations
	TA98, TA98NR, TA100, TA100NR, TA97a, TA1535, TA1537, TA1537NR, TA1538	–/ND	Vance and Levin, 1984	
	TA98, TA100, TA1535, TA1538	ND/–	Anderson and Styles, 1978	
	TA98, TA100	–/–	Assmann et al., 1997	
	TA98, TA100	–/ND	Chiu et al., 1978	
	TA98, TA100, TA1535, TA1537, TA1538	–/–	Shimizu et al., 1983	
	TA98	ND/–	Ho et al., 1981	
	TA1538	–/–	Garner and Nutman, 1977	
	TA98, TA100	ND/–	Dellarco and Prival, 1989	
	TA98, TA100 TA98[c] TA100[c] TA98NR[c]	–/– +/– –/– –/–	Suzuki et al., 1983, 1987	Positive in the presence of norharman as comutagen
Mammalian cells in vitro				
Human lymphocytes		+	Huang et al., 1995, 1996	CA
Human spermatozoa		–	Tateno et al., 1997	CA
Hamster lung fibroblasts	V79	+	Bonacker et al., 2004	MN
Human hepatocarcinoma	SMMC-7721	–	Han et al., 2001	DNA damage
Syrian hamster kidney cells	BHK-21 C13	–	Styles, 1978	Cell transformation
Human diploid lung fibroblasts	WI-38	–	Styles, 1978	Cell transformation
Human hepatocytes		–	Butterworth et al., 1989	UDS
Rat hepatocytes		–	Butterworth et al., 1989	UDS
Human thyroid cells		+	Mattiolli et al., 2006	DNA damage and UDS
In vivo tests				
F344 rats	Peripheral blood lymphocytes	–	Kligerman et al., 1983	SCE and CA
F344 rats	Isolated spleen lymphocytes	–	Kligerman et al., 1983	SCE
F344 rats	Hepatocytes	–	Mirsalis et al., 1982	UDS
Kunming mice		+	Li et al., 2003a, b	DNA binding

Table 4-40. Summary of studies on the direct mutagenicity/genotoxicity of nitrobenzene

Test system	Cell/strain	Result[a] (+/- S9)	Reference	Comments[b]
Sprague-Dawley rats	Primary rat kidney cells	+	Robbiano et al., 2004	DNA damage and MN
Male Sprague-Dawley rats		+	Mattiolli et al., 2006	DNA damage and UDS
Human kidney cells	Kidney cell isolates discarded during surgery	+	Robbiano et al., 2004	DNA damage and MN
Male and female B6C3F1 mice	Bone marrow	–	BASF (1995), as cited in IPCS (2003)	MN

[a]ND = no data.
[b]MN = micronuclei.
[c]Presence of norharman

4.4.6. Other Studies in Support of Mode of Action

Han et al. (2001) exposed a human hepatocarcinoma cell line, SMMC-7721, in culture to nitrobenzene. According to the English translation of the Chinese article, they found that concentrations at and above 8 mM caused cell death but no DNA strand breaks. They also observed that typical reactive oxygen scavengers, such as superoxide dismutase, hydrogen peroxidase, or mannitol, provided protection from nitrobenzene-induced cell death. The authors concluded that nitrobenzene causes cellular damage by reactive oxygen species and that nitrobenzene was a nongenotoxic agent.

Hong et al. (2002) studied the nephrotoxic potential of nitrobenzene in vitro using renal cortical slices from male F344 rats. Nitrobenzene was tested at concentrations of 0, 1, 2, 3, 4, or 5 mM for a 2-hour exposure. The authors reported that nitrobenzene was capable of causing a statistically significant change in cellular function, as measured by a decrease in pyruvate-stimulated gluconeogenesis, at 1 mM; however, overt cytotoxicity, as measured by an increase in lactate dehydrogenase release, did not occur at any of the tested concentrations. In contrast to these findings, Mochida et al. (1986) reported that nitrobenzene was more toxic in comparison to two established nephrotoxicants (i.e., 1,2-dichloroethane and carbon disulfide) in two cell lines. The authors exposed a human epidermoid carcinoma cell line, KB, and African green monkey (*Cercopithecus aethiops*) kidney (AGMK) cells with doses of nitrobenzene up to 300 µg/mL for 72 hours. A dose-dependent decrease in cell viability was observed. The concentration of nitrobenzene reducing cell viability to 50% of control values during the 72-hour exposure period (EC_{50}) was calculated to be 42 and 30 µg/mL in KB and AGMK cells, respectively.

4.5. SYNTHESIS AND EVALUATION OF MAJOR NONCANCER EFFECTS AND MODE OF ACTION—ORAL AND INHALATION

The toxicological effects of nitrobenzene in experimental studies are characterized by a broad spectrum of noncancer impacts. In general terms, these include the onset of cyanosis and methemoglobinemia, changes in hematological parameters, histopathologic lesions of key target organs, such as the spleen, liver, adrenal, kidney, and brain, and testicular atrophy with associated functional deficits in the male reproductive system, although species specific differences with respect to these latter endpoints occur depending on the route of exposure. For example, oral administration of nitrobenzene induces methemoglobinemia and histopathological lesions in the liver (bile stasis, fatty degeneration, centrilobular necrosis, and hepatocellular nucleolar enlargement), brain (malacia of the cerebellar peduncle), and testes (necrosis of primary and secondary spermatocytes, multinucleated giant cells) in male F344 rats, but not in male B6C3F1 mice (Bond et al., 1981; Morgan et al., 1985). Unlike oral exposures, however, hepatic, splenic, and testicular lesions were observed in B6C3F1 male mice following short-term inhalation exposure to nitrobenzene (Medinsky and Irons, 1985). In addition, inhalation studies have shown that male and female B6C3F1 mice are more susceptiable to developing histopathologic lesions in the nasal passages and lungs compared to male and female F344 rats (CIIT, 1993). A summary of the mode of action for noncancer effects following oral and inhalation exposures is provided below.

4.5.1. Oral Exposure

The formation of metHb in the blood of human beings and animals appears to be a consistent feature of almost all case-control or experimental studies on the toxicity of nitrobenzene. That this response and potentially associated histopathologic responses such as congestion of the spleen are a primary toxicological effect of nitrobenzene is indicated by their potential to be triggered at lower doses than most of the other responses to the compound. Holder (1999a) hypothesized how interconversion between nitrobenzene and the primary metabolites—nitrosobenzene, phenylhydroxylamine, and aniline—are intimately associated with the oxidation of the Hb prosthetic group to the ferric state (see Figure 3-8). The consequent anemia is caused by depleted oxygen-carrying capacity, globin chains altered by binding to thiol-containing amino acids, and RBC lysis.

The discussion of a case report by Schimelman et al. (1978) pointed out that nitrobenzene is but one of a wide range of toxicants that can induce methemoglobinemia. Toxic methemoglobinemia is likely to occur if the rapid formation of metHb overwhelms the capacity of the protective enzyme systems (i.e., NADH-cytochrome b_5 reductase [major pathway] and NADPH-cytochrome *c* reductase [minor pathway]; see Table 3-5) (Jaffe, 1981). The NADH-

cytochrome b_5 reductase pathway in RBCs may reduce metHb to Hb at a rate of approximately 15% per hour in healthy individuals, assuming no ongoing metHb production (Finch, 1947).

Under normal conditions, the level of metHb in RBCs is kept at less than 1% (Harrison, 1977). However, the presence of excessive amounts of metHb, which is incapable of transporting oxygen in the body, results in tissue hypoxia. There appears to be a progression of incrementally more severe symptoms in humans with increasing metHb concentration. For example, levels below 20% are likely to be asymptomatic, whereas levels of 20–50% are associated with dyspnea, tachycardia, headache, and dizziness. At levels above 60%, coma and death may ensue (Harrison, 1977). This is a life-threatening condition and requires immediate medical attention. Methemoglobin reduces tissue oxygenation by two mechanisms: iron in the ferric rather than the ferrous form is unable to combine with oxygen and consequently the oxygen-carrying capacity of the blood is reduced, and the presence of oxidized iron changes the heme tetramer in such a way as to reduce oxygen release in the tissues (i.e., shifts the oxyHb dissociation curve to the left as in alkalosis) (Ellenhorn et al., 1997).

NTP (1983a) is the single oral study in which experimental animals were exposed to nitrobenzene for a sufficient duration to permit dose-response analysis. In the study, 10 F344 rats/sex/group received 0, 9.38, 18.75, 37.5, 75, and 150 mg/kg-day and 10 B6C3F1 mice/sex/group received 0, 18.75, 37.5, 75, 150, and 300 mg/kg-day by gavage in corn oil for 90 days. There was good consistency in the range of adverse effects attributable to the compound among rats and mice. These included mortality in some animals at the highest doses (150 mg/kg-day in rats and 300 mg/kg-day in mice), dose-dependent increases in absolute and relative weights of the liver and kidney, but a progressive decrease in absolute and relative testis weights. Hematological parameters of F344 rats and mice were markedly affected by nitrobenzene in this study. For example, Hb concentrations, RBC counts, and Hct were dose-dependently reduced in both species, whereas percent reticulocytes and metHb concentration were dose-dependently increased. For the reticulocyte and metHb effects, statistical significance compared to controls was achieved at all dose levels. Histopathologic lesions were observed in the spleen, testis, and brain in both exposed species. In addition, liver lesions were observed in B6C3F1 mice, while kidney effects were observed in F344 rats. The congestion of the spleen (especially in F344 rats) was noteworthy since it may be associated with the presence of metHb in the RBCs of exposed animals.

Among studies where nitrobenzene was administered for shorter durations to laboratory animals via the oral route, Bond et al. (1981) observed a dose-dependent increase in metHb formation in male F344 rats, with the increases becoming significant in the 110 mg/kg and higher dose groups. An increase in metHb in response to orally administered nitrobenzene also

was observed in the OECD-protocol reproductive/developmental toxicity study conducted by Mitsumori et al. (1994). These studies revealed a statistically significant increase in blood metHb at the lowest dose level employed (20 mg/kg). These findings contrast with those of Burns et al. (1994), who, while reporting a number of hematological perturbations in B6C3F1 mice as a result of nitrobenzene exposure (up to 300 mg/kg for 14 days), did not report any compound-related increases in metHb formation. This may be consistent with the observation that mice are more resistant than rats to the metHb-forming properties of nitrobenzene (WHO, 2003).

Closely related to the formation of metHb in nitrobenzene-treated rodents (especially rats) is the range of changes induced in other hematological parameters. These are likely to be part of the same metHb-induced continuum of RBC-related toxicological consequences of nitrobenzene reduction and the uptake of its metabolites by RBCs. The reproductive or developmental toxicity study on nitrobenzene by Mitsumori et al. (1994) identified statistically significant changes compared with controls in a number of hematological parameters, including reductions in RBCs, Hb, and Hct and increases in erythroblast, reticulocyte, and WBC counts as a result of oral administration of nitrobenzene to male and female Sprague-Dawley rats for approximately 41 days (Table 4-30). Burns et al. (1994) documented a similar suite of hematological effects in B6C3F1 mice that were orally exposed to nitrobenzene for 14 days. Increases in reticulocyte counts were especially marked in this species.

The male reproductive system—testis, epididymis, and seminiferous tubules—comprises an important target for nitrobenzene toxicity in rodents. Impairment of this system due to nitrobenzene has become apparent through the formation of histopathologic lesions, the production of sperm with reduced motility and/or viability, and, in some studies, functional deficits such as reduction in fertility. For example, the 90-day oral gavage study in F344 rats and B6C3F1 mice sponsored by the NTP (1983a) showed a dose-dependent atrophy of the testis and the appearance of a range of treatment-related histopathologic lesions. In the single-exposure oral study carried out by Bond et al. (1981) in F344 rats, a number of distinct histopathologic effects in the testes and seminiferous tubules were apparent at a dose level of 300 mg/kg or greater. The lesions were marked by necrosis of spermatogenic cells, the appearance of multinucleated giant cells, and an associated decrease in sperm count. A single dose of 300 mg/kg was also effective in temporarily abolishing spermatogenesis in male F344 rats, in parallel to a marked degeneration of the seminiferous epithelium (Levin et al., 1988). Reestablishment of sperm generation appeared in concert with the partial restoration of normal cellular architecture.

Other short-term oral exposure studies that centered on the effects of nitrobenzene on the male reproductive system include those of Koida et al. (1995) and Matsuura et al. (1995), both of which demonstrated a relative decrease in epididymal weight, reduced sperm motility and viability, histopathologic and morphological abnormalities, and degeneration of the spermatids and pachytene spermatocytes (in Sprague-Dawley rats exposed by gavage to nitrobenzene at doses of 30–60 mg/kg for up to 4 weeks). Kawashima et al. (1995a) observed similar changes in testicular and epididymal responses in male Sprague-Dawley rats exposed orally to 60 mg/kg nitrobenzene for up to 70 days and demonstrated that, for males exposed to nitrobenzene for 21 days or more before mating, there was a reduction in the fertility index of their (unexposed) breeding partners. This was considered to be a consequence of the nitrobenzene-induced production of sperm with low motility and viability.

Notwithstanding the appearance of profound histopathologic effects in the testes and epididymides, Mitsumori et al. (1994) did not observe impaired fertility as a result of exposing Sprague-Dawley rats to up to 100 mg/kg nitrobenzene for 14 days prior to mating, reemphasizing the importance of the spermatogenic cycle to reproductive performance. Taken together, the data of Levin et al. (1988), Kawashima et al. (1995a), and Mitsumori et al. (1994) point to the ability of nitrobenzene to disrupt spermatogenesis by causing the production of sperm with reduced motility and viability. This will result in reduced fertility if the males are mated at the point when the deficient sperm are released.

As set forth in Section 4.3, nitrobenzene has been included as a positive control in studies aimed at refining experimental techniques for evaluating the spermatotoxic effects of potentially harmful chemical agents (Ban et al., 2001; Linder et al., 1992; Allenby et al., 1991, 1990). As reported in a number of meeting abstracts, oral exposure of rats for 14 days resulted in histopathologic changes in the testes and epididymides and in the production of an increased proportion of abnormal sperm (Kito et al., 1999, 1998; Kato et al., 1995). Morphologically normal sperm from rats undergoing these treatments displayed reduced motility.

Other target organs of nitrobenzene toxicity following oral administration to rodents include the liver, kidney, thyroid, and brain, as indicated by changes in relative organ weights and the appearance of histopathologic lesions. For example, the 28-day oral gavage study of Shimo et al. (1994) in F344 rats noted a characteristic brown coloration of the perivascular region of the cerebellum, increased medullary hematopoiesis of the liver, and brown pigmentation of the renal tubular epithelium. The latter symptoms are a likely result of deposition of metHb and/or degradation products.

4.5.2. Inhalation Exposure

In general, long-term studies of the toxicology of nitrobenzene in experimental animals have employed inhalation as the route of administration. As with oral exposures to nitrobenzene, inhalation exposures result in the formation of metHb. However, in contrast to the 2 e- additions that occur in the intestinal lumen of experimental animals following oral exposures to nitrobenzene, metabolism of nitrobenzene from inhalation exposures is expected to occur via 1 e- additions, with the resultant formation of the nitroanion free radical. As depicted by Holder (1999a), the nitroanion free radical can be further reduced in RBCs to nitrosobenzene and phenylhydroxylamine, both of which participate in the formation of metHb. However, the nitroanion free radical may also be oxidized back to the parent compound with the subsequent formation of the superoxide free radical.

The most comprehensive of these studies was a 2-year investigation of the inhalation effects of nitrobenzene in male and female F344 rats, male Sprague-Dawley (CD) rats, and male and female B6C3F1 mice (Cattley et al., 1994; CIIT, 1993). Included in a wide range of cancer and noncancer effects were the dose-dependent increases in metHb that achieved statistical significance in each species and strain under test. For example, in male Sprague-Dawley rats, statistically significant differences in this parameter were observed at all exposure levels after 15 months, compared with controls (interim blood samples). The lowest concentration administered to male Sprague-Dawley rats (1 ppm) is a chronic exposure LOAEL (unadjusted) for metHb formation in this study, which suggests that this strain of rats may form metHb more readily than F344 rats or B6C3F1 mice, for which 1 and 5 ppm, respectively, would be a NOAEL. Of particular importance in this study was the finding of bronchiolization of the alveoli in both male and female B6C3F1 mice. Unlike the systemic effects, this portal-of-entry effect was detectable in $\geq$87% of mice at the lowest dose tested (5 ppm) and nearly 100% of animals at 50 ppm. Bronchialization of the alveoli was not detectable in controls. Pulmonary effects have also been observed in subchronic inhalation studies in both F-344 rats and B6C3F1 mice (CIIT, 1984). In male F-344 rats, 60% of the animals in the 50-ppm group exhibited bronchiolar epithelium hyperplasia, whereas 20% of females were found with this lesion. In B6C3F1 mice, bronchial mucosa hyperplasia was observed in 78% of males and 100% of females at 50 ppm.

Short-term inhalation studies of nitrobenzene toxicity in experimental animals also have resulted in metHb formation (Medinsky and Irons, 1985; CIIT, 1984). The sensitivity of this response supports its possible applicability to setting toxicological standards for the compound.

The 2-year inhalation study of nitrobenzene noted statistically significant reductions in RBCs, Hct, and Hb in those rats exposed to nitrobenzene at the highest dose of 25 ppm (Cattley et al., 1994; CIIT, 1993). This would identify an unadjusted NOAEL of 5 ppm for the onset of

effects on these hematological parameters. Although a statistically significant increase in the incidence of extramedullary hematopoiesis in the spleen was noted in F344 rats exposed to 1 ppm nitrobenzene for 2 years, the extent of the difference from controls was not particularly striking because of the high background incidence in aging rodent spleens (Cattley et al., 1994; CIIT, 1993). Adverse effects on the spleen, however, were more apparent in younger animals exposed to nitrobenzene for 90 days (CIIT, 1984; NTP, 1983a). In both sexes of F344 and CD rats and in B6C3F1 mice, exposure to nitrobenzene at 50 ppm was associated with increases in absolute and relative spleen weights at necropsy, an obvious enlargement of the organ, the appearance of histopathologic lesions characterized by acute sinusoidal congestion, and increased extramedullary hematopoiesis. Other features of altered spleen histopathology included an increase in the number of macrophages infiltrating the red pulp and a proliferation of capsular lesions. Although the effects on the spleen were less severe at lower concentrations, extramedullary hematopoiesis was observed even in the low-concentration (5 ppm) group. An unadjusted LOAEL of 5 ppm would apply to this effect from the data in the study.

Male CD rats exposed to nitrobenzene by inhalation developed histopathologic lesions of the spleen in mid- and high-dose (39 and 112 ppm) groups (DuPont, 1981). Similarly, pregnant female Sprague-Dawley rats exposed via inhalation to 0, 1, 10, or 40 ppm nitrobenzene on GDs 6–15 displayed an increase in the relative spleen weight in the mid- and high-concentration groups (Tyl et al., 1987).

The 2-year and 90-day inhalation studies on the toxicological effects of nitrobenzene in rodents noted a range of histopathologic effects on the reproductive organs (Cattley et al., 1994; CIIT, 1993; CIIT, 1984). For example, in the 2-year study the development of bilateral hypertrophy of the testis in CD rats was considered to be compound-related because of the concentration-related incidence of the lesion among exposed groups and its statistically significant increase, 35/61 at the highest exposure level (25 ppm) versus 11/62 in controls. This suggests that the mid-concentration level of 5 ppm would represent an unadjusted NOAEL for this effect in CD rats. Reductions in testicular weight and associated histopathologic changes also were features of the 90-day study (CIIT, 1984). The effects were noted in F344 rats, CD rats, and B6C3F1 mice at the highest dose of 50 ppm. Bilateral testicular atrophy was observed in 10/10 male CD rats exposed to 50-ppm nitrobenzene, but in only 2/10 animals exposed to 16 ppm. This concentration, therefore, would constitute an unadjusted NOAEL for this effect, based on the data in the 90-day study.

The two-generation reproductive study in Sprague-Dawley rats reported the well-recognized effects of nitrobenzene on the histopathology of the male reproductive system, with reduced fertility resulting from exposed F_0 males mating with exposed F_0 females, exposed F_1

males mating with F_1 females, and "recovered" F_1 males mating with virgin females (Dodd et al., 1987). The authors suggested a NOAEL of 10 ppm for reproductive toxicity in F344 rats.

In contrast to the effects of nitrobenzene on the male reproductive system, nitrobenzene administered to pregnant rats and rabbits displayed few effects on reproductive, developmental, or teratological parameters under the conditions of the studies (BRRC, 1985, 1984; Biodynamics, 1984, 1983).

In the 2-year and 90-day inhalation studies of nitrobenzene (Cattley et al., 1994; CIIT 1993; CIIT, 1984), non-neoplastic lesions of the liver included both morphological and histopathologic effects. For example, in the 2-year study (Cattley et al., 1994; CIIT, 1993), an increase in the incidence of eosinophilic foci in the livers of male F344 rats was observed at the mid-concentration level of 5 ppm, while centrilobular hepatocytomegaly was observed in the males of both strains of rat at the 5 and 25 ppm levels. In addition, in the 90-day study, the formation of histopathologic lesions identified as basophilic hepatocytes was observed in all male B6C3F1 mice exposed to nitrobenzene at the high-concentration level, whereas these lesions were absent from female mice in all dose groups.

4.5.3. Mode of Action Information

As set forth in Section 3.3, plausible schemes have been developed that link nitrobenzene metabolism in the gastrointestinal lumen and tissues with those biochemical, physiological, and toxicological changes observed in target organs (e.g., liver and lung). Phase-I metabolism occurs mostly by intestinal microflora following oral exposure, and, at a lower rate, in the tissues after gastrointestinal absorption or following internalization by any other route of exposure. The extent to which the route of administration determines target organ toxicity is uncertain. It is, however, likely that the metabolites produced by intestinal microflora, such as *o*-, *m*-, and *p*-nitrophenols, *o*-, *m*-, and *p*-aminophenols, and aniline, can undergo further metabolism inside the mammal organism to form a variety of reactive, mostly short-lived intermediates, such as nitrosobenzene, phenylhydroxylamine (Figure 3-3), and the benzene nitrenium ion. These may be formed by the action of microsomal NADPH-cytochrome *c* reductase, by mitochondrial and cytosolic nitroreductases, and by hydroxylases poorly characterized with respect to nitrobenzene. Some of these reactions, such as formation of the nitroanion free radical, are reversed immediately in a nonenzymatic process, leading to futile redox cycling with the regeneration of the parent compound (i.e., nitrobenzene) and the concurrent formation of superoxide anion. A similar type reaction occurs with the production of pulmonary toxicity-that is, redox cycling with the generation of superoxide anion, with paraquat, a prototypical pulmonary toxicant (Parkinson, 2000). Since the activity of nitroreductase type II is the predominant form in the respiratory

system, generation of the nitroanion free radical with subsequent futile cycling, may explain the respiratory effects observed in rats and particularly mice following inhalation exposures to nitrobenzene. In addition, the nitroso derivatives can enter redox processes that result in the formation of reactive oxygen species (nitroanion, nitroxide, and superoxide free radical) (Figure 3-3). Phase-II metabolism appears to involve acetylation at the amino group or conjugation with sulfate, glucuronic acid, and, predominantly, GSH. GSH conjugates may be split to reenter the futile redox cycle. Further support of the protective effects of GSH conjugation came from the studies by Nystrom and Rickert (1987) with three dinitrobenzene isomers (e.g., 1,2-, 1,3-, and 1,4-dinitrobenzene). The authors showed that 1,3-dinitrobenzene was the only isomer that is not conjugated with GSH. The relevance of this finding is that 1,3-dinitrobenzene is the only isomer to cause testicular toxicity. Therefore, they speculated that the testicular toxicity of this compound may be related to the ease of its reduction to a nitroso compound plus the lack of its removal via conjugation. Ellis and Foster (1992) investigated the metabolism of the same three isomers in subcellular fractions from rats of the Alpk:AP (Wister-derived) strain. They found that the soluble fraction from testis homogenate (but not microsomes) contains a powerful nitroreductase that works under aerobic conditions, transforming 1,3-dinitrobenzene to *m*-nitrosonitrobenzene. The authors did not investigate whether this enzyme works on 1,2- or 1,4-dinitrobenzene as well, which might have provided more information on the unique testicular toxicity of the 1,3-isomer. Still, assuming that this enzyme activity is high in testis, as compared with other organs, and that it is able to reduce nitrobenzene to nitrosobenzene, provides a reasonable explanation for the pronounced testicular toxicity of nitrobenzene.

Skeletal malformations following gestational exposure of Sprague-Dawley rats to nitrobenzene were observed only at doses toxic to the mother, thus suggesting strongly that the effect was due to maternal toxicity rather than direct embryotoxicity.

While the details are not understood, there is reasonably strong evidence linking the interconversion of nitrobenzene and its metabolites to the formation of metHb and to the possible binding of nitrosobenzene to important thiol-containing macromolecules such as Hb and GSH. Other intracellular proteins containing cysteine residues also would be expected to undergo such interactions (WHO, 2003; Holder, 1999a). Changes in blood chemistry values and splenic pathology observed after nitrobenzene intoxication are the likely consequences of metHb formation, Hb destruction, and the deposition of degradation products in these tissues. Splenic toxicity is likely related to erythrocyte toxicity, because a primary function of the spleen is to scavenge senescent or damaged RBCs. Splenic injury may arise from the deposition of massive amounts of iron or other RBC breakdown products, with an added potential for reactive metabolites of nitrobenzene to take part in additional intracellular reactions.

The six-step/one-electron transfer per step reduction sequence that has been proposed for intracellular metabolism of nitrobenzene (Figure 3-7) may result in reactive intermediates that can react with cells or tissues where this sequence is operative, leading to gross and microscopic changes. As demonstrated by Ohkuma and Kawanishi (1999), reactive oxygen species formed in the metabolic processing of nitrobenzene and its derivatives can cause damage to DNA. While this would imply a genotoxic mechanism of action for the carcinogenic potential of nitrobenzene, the bulk of experimental evidence from genotoxicity assays has provided negative results for the chemical. Reactive oxygen species, in general, have the potential to initiate, promote, and/or accelerate the progression of nonneoplastic or neoplastic changes in cells (Dreher and Junod, 1996; Feig et al., 1994; Guyton and Kensler, 1993; Kensler et al., 1989). According to one study conducted in hepatocarcinoma cells in culture, the most likely toxic outcome of reactive oxygen species is cell death. The precise link between the action of reactive oxygen species and various forms of cellular damage is as yet unknown.

4.6. WEIGHT-OF-EVIDENCE EVALUATION AND CANCER CHARACTERIZATION—SYNTHESIS OF HUMAN, ANIMAL, AND OTHER SUPPORTING EVIDENCE, CONCLUSIONS ABOUT HUMAN CARCINOGENICITY, AND LIKELY MODE OF ACTION

4.6.1. Summary of Overall Weight-of-Evidence

Applying the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), nitrobenzene is classified as *likely to be carcinogenic to humans* exposed via any route of exposure. However, this designation lies on the low end of the range for this descriptor.

Nitrobenzene has been shown to be a carcinogen in rats and mice (see Table 4-19). Adenomas and/or carcinomas with a pronounced dose-response relationship were found in livers of male F344 and male CD rats and in thyroids of male F344 rats. Less pronounced dose-related trends were observed for endometrial polyps in female F344 rats, cancers of the lung and thyroid in male B6C3F1 mice, and cancers in the mammary gland in female B6C3F1 mice. In all cases the incidence at the highest dose was elevated significantly compared to controls. Several studies have suggested that the carcinogenic action of nitrobenzene follows from the production of reactive oxygen species (see Figure 3-7) (Cattley et al., 1994; CIIT, 1993). While there are no human carcinogenicity data on nitrobenzene, the cancer characterization is based on evidence of the compound's tumorigenicity in a single well-conducted study in two animal species (Cattley et al., 1994; CIIT, 1993). Furthermore, the 2005 cancer guidelines (U.S. EPA, 2005) state that when tumors occur at a site other than the point of initial contact, the descriptor generally applies to all exposure routes that have not been adequately tested at sufficient doses. An exception occurs when there is convincing information, e.g., toxicokinetic data that absorption does not occur by another route. Thus, nitrobenzene is *likely to be carcinogenic to humans by any route of exposure*. This decision is based on the observations that nitrobenzene is absorbed via all routes and reductive and oxidative metabolites of nitrobenzene are produced following inhalation, oral, or dermal exposures. The carcinogenic action of nitrobenzene may be related to these intermediates or the oxygen free radicals they may produce.

4.6.2. Synthesis of Human, Animal, and Other Supporting Evidence

The carcinogenicity of nitrobenzene has been evaluated in male and female mice (B6C3F1), male rats of two strains (F344/N and Sprague-Dawley), and female rats of one strain (F344/N). When administered to mice and rats by inhalation, nitrobenzene caused significantly increased incidences of tumors at multiple tissue sites in both species. Exposure to nitrobenzene caused lung and thyroid tumors in male B6C3F1 mice and mammary gland tumors in female

B6C3F1 mice (Cattley et al., 1994; CIIT, 1993). Exposure to nitrobenzene caused liver tumors in male rats of the F344/N and Sprague-Dawley strains, kidney tumors in male F344/N rats, and endometrial polyps in female F344/N rats. In addition, significantly positive increases in the incidences of liver tumors in female mice and female F344/N rats and thyroid tumors in male F344/N rats were observed with increasing nitrobenzene exposure levels (Cattley et al., 1994; CIIT, 1993). A summary of the carcinogenicity results is presented in Table 4-41.

Table 4-41. Neoplasms in F344 and CD rats and B6C3F1 mice exposed to nitrobenzene via inhalation for 2 years

Species/strain/site	Incidence of neoplasms[a]			
	Concentration of nitrobenzene (ppm)			
Rats	**0**	**1**	**5**	**25**
F344 rats (male) Liver: combined adenomas/carcinomas	1/43	4/50	5/47	16/46
Kidney: combined adenomas/carcinomas	0/43	0/50	0/47	6/46
Thyroid: combined adenomas/carcinomas	1/43	1/50	5/47	8/46
F344 rats (female) Endometrial polyps	9/48	15/50	14/50	19/49
CD rats (male) Liver: combined adenomas/carcinomas	0/23	0/23	1/25	5/23
	Concentration of nitrobenzene (ppm)			
Mice	**0**	**5**	**25**	**50**
B6C3F1 mice (male) Lung: combined adenomas/carcinomas	8/42	16/44	20/45	21/48
Thyroid: combined adenomas/carcinomas	0/41	4/44	1/45	6/46
B6C3F1 mice (female) Mammary gland: combined adenomas/carcinomas	0/48	ND[b]	ND	5/60

[a]All tumor incidences in this table displayed statistically significant ($p<0.05$), dose-related trends in the Cochran-Armitage test.

[b]ND = no data.

Sources: Cattley et al., 1994; CIIT, 1993.

While no evidence exists to directly address the issue of the carcinogenicity of nitrobenzene in humans, the *likely* weight-of-evidence descriptor is chosen because the compound was shown to be carcinogenic in a 2-year inhalation experiment that resulted in the dose-related formation of tumors at multiple tissue sites in both species of animals employed in the study (Cattley et al., 1994; CIIT, 1993). In this study the strongest individual carcinogenic response to nitrobenzene was the dose-dependent increase in the incidence of hepatocellular tumors in male F344 rats, for which the incidence and trend data showed statistically significant effects in the formation of both adenomas and carcinomas. These data constitute sufficient evidence of carcinogenicity, and hepatocellular tumors may be considered to be the primary carcinogenic effect of the compound. This overall conclusion is strengthened by the nitrobenzene-induced formation of hepatocellular adenomas and carcinomas in male CD rats, though these tumors were predominantly benign. Neoplastic effects were observed also in other organs, such as the endometrium in female F344 rats, thyroid and kidney in male F344 rats, lung and thyroid in male B6C3F1 mice, and mammary gland in female B6C3F1 mice. It should be noted that although the thyroid and kidney tumors observed in male rats and thyroid tumors in male mice are suggestive of rodent specific MOAs, the experimental data do not satisfy the criteria set forth in the U.S. EPA's guidance on "Assessment of Thyroid Follicular Cell Tumors" and "Alpha2u-globulin: association with chemical induced renal toxicity and neoplasia in the male rat" to make this determination (U.S. EPA 1998c, 1991b). Other evidence that supports the classification of nitrobenzene as a likely human carcinogen is the known carcinogenicity of aniline, a metabolite of nitrobenzene (IRIS, 1994). Recent studies by Bonacker et al. (2004) pointed to an aneugenic potential of nitrobenzene. Studies by Li et al. (2003a, b) showed that nitrobenzene is capable of binding to hepatic DNA.

No information is available on the carcinogenic effects of nitrobenzene via the oral route. However, the available information from subchronic oral studies suggests that the compound could be carcinogenic via the oral route, also. This conclusion is based on the ready absorption of the compound at the intestinal absorption barrier and the fact that, in the 2-year inhalation study, tumors were formed in tissues remote from the site of absorption. These findings suggest that nitrobenzene or its metabolites can cause tumor formation at multiple sites following passage into the general circulation. Such a capability would be expected to apply to nitrobenzene when administered orally. However, the issue of the carcinogenicity of nitrobenzene by the oral route constitutes a significant data gap.

4.6.3. Mode of Action Information

Based on the studies discussed in section 4.4.5., evidence for the genotoxicity of nitrobenzene is mixed. Nitrobenzene appears to be at most weakly genotoxic. This determination is based on the almost exclusively negative results in Salmonella assays (Ames tests; the only exception is TA98 in the presence of a comutagen), as well as negative clastogenic findings from in vivo assays of sister chromatid exchange, unscheduled DNA synthesis, and chromosomal aberrations. In vitro chromosome aberration results were mixed, as were the DNA breakage and micronucleus data. For instance, nitrobenzene was weakly positive for the induction of chromosome aberrations in cultured human peripheral lymphocytes but negative in human spermatozoa. Nitrobenzene induced weak DNA fragmentation but no DNA strand breaks. In addition, nitrobenzene did not cause cell transformation in these cell systems.

The six-step/one-electron transfer per step reduction sequence that has been proposed for intracellular metabolism of nitrobenzene suggests that nitrobenzene may act as a promoter, since the reactive intermediates generated during nitrobenzene metabolism may have the potential to initiate, promote, and/or accelerate the progression of nonneoplastic or neoplastic changes in cells (Figure 3-7) (Dreher and Junod, 1996; Feig et al., 1994; Guyton and Kensler, 1993; Kensler et al., 1989). Ohkuma and Kawanishi (1999) induced DNA damage in vitro using calf thymus DNA; nitrosobenzene, a primary metabolite of nitrobenzene (5–20 μM); Cu^{2+} ions (20 μM); and NADH in a nonenzymatic reaction. Other metal ions, such as Fe^{2+}, Fe^{3+}, or Mn^{2+}, were ineffective. Bathocuproine, an agent that binds Cu^{+}, chelating agents, or catalase, an enzyme that destroys H_2O_2, prevented DNA damage, suggesting that adduct formation proceeded via an oxidative process requiring the presence of both Cu^{+} and H_2O_2. Superoxide anion or free radical scavengers did not suppress DNA damage. The authors found that NADH plus Cu^{2+} caused damage mostly to thymidine and cytosine residues, whereas the •OH radical attacked DNA in a nonspecific fashion. Therefore, they suggested that Cu^{2+} binds in a site-specific manner to DNA, where it is reduced to Cu^{+} by NADH plus nitrosobenzene, with the release of H_2O_2. The latter then forms a DNA-Cu^{+}-H_2O_2 complex that releases •OH and attacks the nucleotide at which it was formed. The authors stated that the concentration of NADH used was well within the physiological range, but they did not elaborate on physiological Cu^{2+} concentrations. Still, it is conceivable that a mechanism like this could play a role in organ-specific carcinogenesis by nitrobenzene.

Further insight as to the carcinogenic mode of action for nitrobenzene comes from Bonacker et al. (2004). These researchers provided evidence that the mechanism by which nitrobenzene affects cell replication involves damage to tubulin assembly and the spindle apparatus.

The thyroid and kidney tumors observed in experimental animals may be suggestive of rodent-specific MOAs; however, as discussed below, experimental data required by the EPA's guidance for excluding these tumors are lacking (U.S. EPA, 1991; U.S. EPA, 1998c). Tumors of the rodent thyroid may develop by the following MOA. A sustained increase in conjugating enzymes alters the hypothalamic-pituitary-thyroid axis by increasing the clearance of thyroxine (T_4) (*via* glucuronidation) and triiodothyronine (T_3) (*via* sulfation) in rodents, which causes a compensatory increase in circulating thyroid-stimulating hormone (TSH) and ultimately follicular cell activation (U.S. EPA, 1998c). Since the levels of T_3 and T_4 are tightly regulated in humans, chemicals that cause tumors of the thyroid via this MOA are not relevant to humans. However, the EPA requires that specific data be available to support this MOA, such as studies determining the effects of a chemical on circulating blood levels of TSH, T_4, and T_3. Since these data are not available for nitrobenzene, the thyroid tumors are considered relevant for assessing carcinogenic risk to humans by default (U.S. EPA, 1998c). Similarly, tubule tumors of the rodent kidney are known to occur by the following MOA. After chronic exposure to some chemicals, α_{2u}-globulin nephropathy may result from sustained target cytotoxicity and necrosis that leads to increased cell proliferation followed by promotion of spontaneously initiated cells. EPA has determined that the risks of kidney damage posed to humans from chemicals that cause toxicity to rodents *via* this MOA are not relevant for assessing human risk. In order to support this MOA, the EPA requires that the following criteria are met: an increase in the number and size of hyaline (protein) droplets in kidney proximal tubule cells of treated male rats; immunohistochemical evidence of α_{2u}-globulin accumulating protein in the hyaline drops; and histopathological evidence of kidney lesions associated with α_{2u}-globulin nephropathology (U.S. EPA, 1991b). Data establishing these criteria are not available for nitrobenzene. Because of the absence of experimental data that meet the data requirements for excluding tumors of the thyroid and kidney, the MOA framework from the *Guidelines for Carcinogen Risk Assessment* has not been applied and tumors of the thyroid and kidney are deemed relevant to humans by default (U.S. EPA, 2005).

4.7. SUSCEPTIBLE POPULATIONS AND LIFE STAGES

4.7.1. Possible Childhood Susceptibility

Fetal Hb is more easily oxidized to metHb than adult Hb (Seger, 1992; Goldstein and Rickert, 1984). The switch ("hemoglobin switching") in the globin chain composition from fetal to adult Hb (i.e. $\alpha_2\gamma_2$ to $\alpha_2\beta_2$) is nearly complete by 30 weeks postnatal age (Nienhuis and Stamatoyannopoulos, 1978; Wood, 1976). Therefore, the time period of heightened

susceptibility to methemoglobinemia due to the globin chain composition of Hb spans from about 6 weeks postconceptual age to about 30 weeks postnatal age (Miller, 2002). However, the susceptibility of infants and young children persists past this period due to reduced levels of NAD(P)H, the cofactors for NADPH-cytochrome *c* reductase and NADH-cytochrome b_5 reductase (Seger, 1992). Wentworth et al. (1999) suggested that newborns are susceptible because the activity of NADH-cytochrome b_5 reductase in the RBCs of children is only about 60% that of adults, slowing the reduction of metHb to Hb. Finally, the blood of newborns is low in glucose-6-phosphate dehydrogenase activity, an enzyme that is crucial for replenishing NADPH reducing equivalents (see Table 3-5) (Goldstein et al., 1969). Although the available developmental studies with nitrobenzene were relatively negative, metHb levels were not examined in the offspring (BRRC, 1985, 1984; Biodynamics, 1984, 1983). Hence, uncertainty exists as to the susceptibility of the test species' hemoglobin to oxidation compared to that of developing humans.

As indicated by Pinkerton and Joad (2000), approximately 80% of the human alveoli develop after birth, and continue to develop through early adulthood. This time period for the developing respiratory system may predispose infants and children to adverse pulmonary effects from nitrobenzene.

4.7.2. Possible Gender Differences

Nitrobenzene has been shown to cause endometrial polyps in female F344/N rats and mammary tumors in female B6C3F1 mice. It is not known whether these findings reflect gender-specificity or whether estrogen-responsive tissues (e.g., endometrium and mammary gland) are targets due to a disturbance of estrogen homeostasis.

In male rats (F344/N and CD) and mice (B6C3F1), nitrobenzene exposure via the inhalation and oral routes has been shown to cause testicular atrophy, including a dramatic decrease in sperm count with ensuing loss of fertility. This suggests that nitrobenzene is a male-specific reproductive toxicant.

4.7.3. Other

A review by Harrison (1977) stressed the fundamental difference between hereditary and chemically induced forms of methemoglobinemia. There are at least two inherited diseases that affect an organism's susceptibility to metHb formation (Goldstein et al., 1969). First, genetic deficiency of NADPH-cytochrome *c* reductase, the enzyme that restores to Hb the small amount of metHb always being formed in RBCs, imparts a comparatively higher susceptibility to affected populations upon nitrobenzene exposure. In addition, there is glucose-6-phosphate

dehydrogenase (G6PD) deficiency (see above), more commonly known because it imparts intolerance to the antimalarial primaquine. Because the gene for the enzyme is located on the X-chromosome, females are usually heterozygotes and thus not affected by the deficiency. A high frequency of variants of G6PD deficiency is found in African, Mediterranean, and Asiatic populations (Porter et al., 1964). Within the United States, about 13% of African-Americans are affected with the condition. Second, chemically induced methemoglobinemia can occur from much lower levels of exposure in patients with comorbidities, such as anemia, cardiovascular disease, lung disease, sepsis, or the presence of abnormal Hb species (e.g., carboxyhemoglobin, sulfhemoglobin, or sickle cell Hb) (Goldfrank et al., 1998).

5. DOSE-RESPONSE ASSESSMENTS

5.1. ORAL REFERENCE DOSE

The RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. It can be derived from a NOAEL, LOAEL, or benchmark dose, with uncertainty factors generally applied to reflect limitations of the data used.

5.1.1. Choice of Principal Study and Critical Effect with Rationale and Justification

The 90-day gavage study (10 animals/dose/sex) conducted by NTP (1983a) is the most suitable study for deriving an RfD for nitrobenzene. Several other studies have been conducted that are not considered suitable for the derivation of an RfD (e.g., reproductive toxicity studies using single administration, single dose [Levin et al., 1988]; single administration, multiple dose [Bond et al., 1981]; multiple administration up to 4 weeks only [Koida et al., 1995; Matsuura et al., 1995]; or administration of a single dose for up to 70 days [Kawashima et al., 1995a, b]). In addition, the studies by Koida et al. (1995) and Matsuura et al. (1995) were presented in abstract form only and were not published in peer-reviewed journals, so they were not considered further. Also, since Kawashima et al. (1995a, b) administered a single dose, no dose-response relationship could be determined. Moreover, single dose and short duration studies are not appropriate when determining an RfD that applies to a lifetime exposure because they cover too small a fraction of the normal life of the laboratory animal. Mitsumori et al. (1994) conducted a reproductive toxicity study of nitrobenzene in male and female Sprague-Dawley rats (10 animals/dose/sex), using 0, 20, 60, and 100 mg/kg for up to 54 days. Because of the experimental protocol used, total nitrobenzene exposure time for most animals was only 40–41 days. Some effects were observed at the lowest dose (cf. Tables 4-30, 4-32), which was, however, more than twice the lowest dose used in the NTP (1983a) study. As detailed in Section 4.3, the reproductive/developmental toxicity studies suggest that nitrobenzene is not teratogenic but acts as a male reproductive toxicant at comparatively high doses.

Burns et al. (1994) assessed the immunotoxic potential of nitrobenzene for select immunologic and host resistance responses over a 14-day treatment period. The doses used, 30–300 mg/kg, were higher than in the NTP (1983a) study and essentially confirmed the toxic effect of nitrobenzene on the spleen and hematology parameters. However, toxic effects on the immune system were mild.

The NTP (1983a) study included both sexes and two species, the F344 rat and the B6C3F1 mouse; five dose groups plus controls (0, 9.38, 18.75, 37.5, 75, and 150 mg/kg-day for rats and 0, 18.75, 37.5, 75, 150, and 300 mg/kg-day for mice); and 10 animals/sex/dose group. In rats, there were seven survivors among the highest dose females but only one survivor among the highest dose males. In mice, there were no deaths among the highest dose females but three deaths among the highest dose males. The study reported multiple potentially toxic endpoints, including changes in absolute and relative organ weights, changes in hematological parameters, and histopathologic outcomes. The nitrobenzene-induced pathological changes were much less pronounced in mice than in rats. Since the mice were treated with higher doses and generally more resistant to nitrobenzene toxicity, the mouse data were not considered further for RfD evaluation. The similarity of endpoints in both species, however, had considerable bearing on the choice of critical effect(s).

Organ weights affected by subchronic nitrobenzene exposure included liver and kidney (increase) in both sexes, and testis (decrease) in male F344 rats (Tables 4-3 and 4-4). The statistically significant increases in liver and kidney weights were generally not supported by other tissue-specific findings, such as histopathology. Therefore, changes in liver weight were not considered further. Moreover, kidney weight increases were not considered for risk evaluation because of the lack of confirmatory tests (e.g., histopathology, urinary GGTP, etc) and the absence of kidney effects in nitrobenzene-exposed humans.

There is evidence that nitrobenzene is a male reproductive toxicant (see Section 4.3). However, a significant effect on testis weight in males was generally seen only at the two highest doses in rats (75 and 150 mg/kg-bw), accompanied by an up to 90% lethality (NTP, 1983a). Similarly, a significant effect on testis weight was only observed with the highest dose in male mice (300 mg/kg-bw) with an accompanying 30% mortality. Because of the high doses required to demonstrate testicular toxicity and the lack of this response in the available human exposure or poisoning data, this endpoint was not used in the RfD assessment for nitrobenzene, as more relevant endpoints were identified at lower levels of exposure.

A number of dose-dependent hematological changes were observed in both species in the NTP (1983a) study, including hematology-related histopathologic splenic congestion and increased reticulocyte count. It was assumed that these changes reflected primary or secondary effects of the nitrobenzene-induced methemoglobinemia (cf. Tables 4-5, 4-6, 4-11, and 4-12). Because methemoglobinemia and splenic congestion have been observed with most human poisonings and animal studies, these outcomes were considered co-critical effects of nitrobenzene exposure, along with increased reticulocyte count.

5.1.2. Method of Analysis—Benchmark Dose Modeling

The BMD software (BMDS) (U.S. EPA, 1999) was used to estimate a point of departure (POD) for deriving an RfD for nitrobenzene from data on effects, based on a benchmark response (BMR) of one SD for data presented in continuous form or of a 10% extra risk (ER) for dichotomous data. This software calculates two core values for each data set, a central estimate of exposure (BMD) and a 95% lower bound on dose (BMDL). Table 5-1 lists the results for low-range BMD/BMDL values for metHb, splenic congestion, and reticulocyte count. A full compilation of all BMD/BMDL values for other critical effects, is presented in Appendix B-1, Table B-1.1. Data for splenic congestion were presented in dichotomous form; therefore, the BMR was chosen as 10% ER above control value, as stipulated in *Benchmark Dose Technical Guidance Document [external review draft]* (U.S. EPA, 2000c). Data for metHb concentration and reticulocyte count were presented in continuous form; therefore, the BMR was chosen as 1 SD above the control mean value (U.S. EPA, 2000c).

Table 5-1. Summary of noncancer BMD modeling results in the F344 rat

Endpoint	BMR	Sex	Model used[a]	*p*-Value	BMD (mg/kg-day)	BMDL (mg/kg-day)
MetHb	1 SD	M	2nd degree polynomial	0.36	3.08	2.17
		F	NF[c]	--	--	--
Reticulocyte count	1 SD	M	2nd degree polynomial[b]	0.21	7.49	5.80
		F	3rd degree polynomial	0.46	2.37	1.77
Splenic congestion	10% ER	M	various[d]	0.48	2.73	1.81
		F	various[d]	≤1.00	7.07	2.70

[a]Values shown are those with highest *p* value (≥0.1) and/or lowest Akaike Information Criterion (AIC) score.
[b]Highest dose not included in BMD modeling.
[c]Data cannot be fitted with BMDS.
[d]Value is the average of all models with lowest AIC scores within 0.5.
Source: NTP, 1983a

Since the increase in metHb, reticulocyte count, and splenic congestion are the biological consequences of the oxidation of the iron in hemoglobin, these effects are considered co-critical effects. Accordingly, the average value of the benchmark dose calculations is used to derive the point of departure for RfD development:

(1) Splenic congestion (average BMDL for males and females): (1.8 + 2.7)/2 = 2.3 mg/kg-day
(2) Reticulocyte count (average BMDL for males and females): (5.8 + 1.8)/2 = 3.8 mg/kg-day
(3) MetHb levels (BMDL for males): 2.2 mg/kg-day
(4) Point of departure: (2.3 + 3.8 + 2.2)/3 = 2.8 mg/kg-day

The BMD modeling for splenic congestion and reticulocyte count in male and female F344 rats, and metHb levels in male F344 rats is provided in Appendix B-1.3. In the NTP (1983a) study, the animals were gavaged 7 days/week; thus, no adjustment for intermittent exposure was required.

5.1.3. RfD Calculation

The human RfD for nitrobenzene was calculated as follows:

$$\text{BMDL} \div \text{UF} = \text{RfD}$$
$$2.8 \text{ mg/kg-day} \div 1000 = 3 \times 10^{-3} \text{ mg/kg-day}$$

where UF = uncertainty factor.

The UF of 1000 is composed of four parts:

- An intraspecies uncertainty factor of 10 was applied to account for human variability and protect potentially sensitive humans (e.g., G6PD deficiency) and lifestages (e.g., children). The default value was selected in the absence of information indicating the degree to which humans might vary in susceptibility to nitrobenzene toxicity.
- An interspecies uncertainty factor of 10 was applied for extrapolation from animals to humans. No suitable data on the toxicity of nitrobenzene to humans exposed by the oral route were identified. Insufficient information is currently available to assess rat-to-human differences in nitrobenzene toxicokinetics or toxicodynamics.
- An UF to account for the extrapolation from a LOAEL to a NOAEL was not applied because BMD modeling was used to determine the point of departure for derivation of the RfC.

- A subchronic to chronic uncertainty factor of 3 was applied to account for less-than-lifetime exposure in the principal study. A chronic oral study is not available. However, the severity of hematological effects (e.g., metHb, reticulocyte count, and splenic congestion) observed by the inhalation route of exposure did not increase between subchronic (CIIT, 1984) and chronic (CIIT, 1993) exposure durations. In addition, the hematological endpoints identified in the subchronic oral study and the inhalation studies appear to result from similar metabolic processes involving cycling of nitrosobenzene and phenylhydroxylamine in red blood cells. However, the metabolic activation of nitrobenzene proceeds by different pathways for oral (*e.g.*, 3 step, 2e- per step) and inhalation (*e.g.*, 6 step, 1e- per step) exposures. For example, oral exposure leads to the formation of the intermediate nitrosobenzene, whereas inhalation exposure leads to nitroanion radical formation. The route-specific differences in metabolism may lead to other toxic endpoints over long term exposure conditions that were not observed in the subchronic oral study. A subchronic to chronic UF of 3 is appropriate to account for the uncertainty associated with additional adverse effects, other than the abovementioned hematological endpoints, that might occur in a chronic oral study.

- A database deficiency uncertainty factor of 3 was applied. The database of oral studies includes the principal study (NTP, 1983b), a 90-day gavage study in two species and both sexes; high quality reproductive/developmental studies (Mitsumori et al., 1994; Morrissey et. al., 1988; Bond et al., 1981); structure-activity relationship studies with dinitro- and trinitrobenzene; and a multidose immunological study in mice (Burns et al., 1994). Due to the lack of an oral multigeneration reproductive toxicity study and evidence of male reproductive toxicity, a factor of 3 is warranted. There is a two-generation reproductive toxicity study (Dodd et al., 1987) via inhalation exposure but there are known differences in metabolism between oral and inhalation exposures that may produce uncertainty in the potential for transgenerational effects from longer term oral exposures.

5.1.4. Previous Oral Assessment

The previous IRIS assessment based the RfD for nitrobenzene of 5×10^{-4} mg/kg-day on a 90-day inhalation study in F344 rats and B6C3F1 mice (CIIT, 1984). Critical endpoints included

methemoglobinemia and histopathologic lesions to the adrenal gland, kidney, and liver. A route-to-route extrapolation was performed, and the LOAEL–NOAEL approach was used to derive the RfD. A POD of 25 mg/m^3 (LOAEL) was identified and converted to an equivalent oral dose of 4.6 mg/kg-day using default assumptions about the mouse breathing rate and body weight. A combined UF of 10,000 was applied, resulting in an RfD of 5×10^{-4} mg/kg-day. The slightly older study (NTP, 1983a) is preferred because it is a GLP study with nitrobenzene administered via the oral route of exposure.

5.2. INHALATION REFERENCE CONCENTRATION

5.2.1. Choice of Principal Study and Critical Effect, with Rationale and Justification

There are no studies in humans that investigate outcomes of long-term inhalation exposure to nitrobenzene combined with quantitative measures of exposure. However, there are animal studies that examine inhalation effects of nitrobenzene in rats and mice with short-term exposure (Medinsky and Irons, 1985; DuPont, 1981), subchronic exposure (CIIT, 1984), and 2-year chronic exposure (Cattley et al., 1994; CIIT, 1993). Noncancer effects of inhalation exposure to nitrobenzene were generally similar to those observed following oral exposure (methemoglobinemia, altered hematology with signs of hemolytic anemia, damage to the male reproductive system, changes in relative organ weights, and pigment deposition in organs). In chronically-exposed animals, the most prominent portal-of-entry effects were bronchiolization of the alveoli and olfactory degeneration in both male and female B6C3F1 mice (CIIT, 1993). Pulmonary effects have also been observed in subchronic inhalation studies in both F-344 rats and B6C3F1 mice (CIIT, 1984). The DuPont (1981), and Medinsky and Irons (1985) studies were not considered as principal studies for an RfC evaluation because both studies had short exposure times (14 days) and comparatively high levels of exposure (10–125 ppm nitrobenzene).

A 90-day subchronic study was conducted using both sexes of F344 and CD rats as well as B6C3F1 mice (CIIT, 1984). Exposure concentrations were 0, 5, 16, or 50 ppm, 6 hours/day, 5 days/week. The treatments had no effect on body weights, but spleen weights were increased and testis weights were decreased in rats. In rats signs of hemolytic anemia, and methemoglobinemia were consistently observed in both species (Table 4-18). Pulmonary effects were also observed in F-344 rats and B6C3F1 mice. In male F-344 rats, 60% of the animals in the 50-ppm group exhibited bronchiolar epithelial hyperplasia, whereas 20% of females were found with this lesion. In B6C3F1 mice, bronchial mucosal hyperplasia was observed in 78% of males and 100% of females at 50 ppm.

The 2-year study, also conducted by CIIT (Cattley et al., 1994; CIIT, 1993), is the most suitable study for an RfC evaluation because of the chronic exposure duration and large group

sizes (70 animals/sex/group). The study used B6C3F1 mice and F344 rats of both sexes and male CD rats. Rats were exposed to 0, 1, 5, or 25-ppm nitrobenzene and mice to 0, 5, 25, or 50-ppm nitrobenzene, for 6 hours/day, 5 days/week (for details see Section 4.2.2.2). Animals were sacrificed at 24 months of exposure when blood analyses and complete necropsies were performed. Ten rats/sex/strain/group were terminated 15 months into the study to provide samples for an interim evaluation of hematological parameters. Cattley et al. (1994) identified the following target tissues: thyroid, spleen, nose, and liver in all strains and species; kidney in rats only; and respiratory tissues in mice only. Testis and epididymis were target tissues in male CD rats.

A statistically significant difference in the incidence of centrilobular hepatocytomegaly was observed in a concentration-dependent fashion in both strains of male rats but not at all in female rats. The incidence of renal tubular hyperplasia in male F344 rats showed a significant positive trend and was statistically significantly different from the controls at the highest dose tested. Chronic nephropathy and tubular hyperplasia were observed in both males and females. Bilateral testicular atrophy was reported with effects appearing in the high-concentration group only in both male CD and F344 rats. Bilateral hypospermia was observed in high-concentration male CD rats.

At interim sacrifice, a statistically significant increase in methemoglobin was observed at all concentrations with male CD rats, and only at the highest concentration with male and female F-344 rats. At terminal sacrifice, a statistically significant increase in methemoglobin was observed with both sexes of mice at the highest concentrations tested. An approximate 2-fold increase in methemoglobin was observed with male and female B6C3F1 mice, female F344 rats, and male CD rats, whereas an approximate 1.5-fold increase was observed with male F344 rats (Cattley et al., 1994; CIIT, 1993). Hematocrit and hemoglobin were reduced only in female mice, highly significantly at the 5-ppm concentration, and less, albeit still significantly, reduced at 25 ppm but not at 50 ppm. Since this effect occurred only in female mice and did not exhibit concentration dependency, it was considered not treatment-related because of the lack of a dose response.

Exposure-related degeneration and loss of olfactory epithelium were observed in both males and females with the females being more sensitive than the males. At the highest concentration tested (50 ppm), the incidence was 62% in males 69% in females. Bronchiolization of the alveoli was observed at all concentrations in both sexes with 94% incidence in males and 100% incidence in females at the highest concentration tested. Follicular cell hyperplasia of the thyroid was observed in both sexes of mice with males being more sensitive than females. At the highest concentration, this response was reported in 19% of the

males. Exposure-related hepatocellular changes (e.g., centrilobular hepatocytomegaly) were observed in males with incidence up to 89% at the highest concentration, and occurred in 11% of females only at the highest concentration. Hypercellularity of the bone marrow, an effect secondary to hemolytic anemia, was recorded for males in a concentration-dependent fashion with low incidence; in females, only animals exposed at the highest concentrations were examined for this effect, and the response was even lower than in males. There was also evidence for testicular toxicity in males, but only the high-concentration animals were examined.

The most consistent histopathologic findings in mice were degeneration and loss of the olfactory epithelium and bronchiolization of the alveoli. Degeneration and loss of the olfactory epithelium occurred in a concentration-dependent manner with high incidences (≥62%) in both males and females, with females being more sensitive than males (Table 5-2). In females, all three treatment groups displayed loss or degeneration of olfactory epithelium to variable degrees. Olfactory degeneration was nearly absent in male mice in the low-concentration group. In males exposed to the highest concentration, one side of the septum was affected more frequently than the other.

Table 5-2. Incidence of olfactory degeneration in mice following chronic nitrobenzene inhalation

		Exposure level (ppm)			
	Sex	**0**	**5**	**25**	**50**
Incidence	M [a]	1/67	1/66	32/65 [b]	41/66 [b]
	F [a]	0/52	19/60 [b]	47/63 [b]	42/61 [b]

[a] Significant positive trend by Armitage-Cochran test, $p<0.05$.
[b] Significantly different from controls, Fisher Exact test, $p<0.05$.
Source: Cattley et al., 1994; CIIT, 1993.

However, bronchiolization of the alveoli was the most prominent endpoint and occurred with high incidence (≥94%) in both males and females in the 50-ppm groups (Table 5-3). The lesions were characterized by a pronounced change in the alveolar epithelium in the region of the terminal bronchioles from a simple squamous to a tall columnar epithelium resembling that of the terminal bronchioles. The change was concentration-related in severity. In the low – concentration dosed animals, bronchiolization was located almost entirely in the region of the terminal bronchioles. In the mid- and high-concentration animals, the lesions were more florid and involved a large proportion of the lung parenchyma. Bronchiolization was chosen as the critical effect for the derivation of the RfC.

Table 5-3. Incidence of bronchiolization of the alveoli in mice following chronic nitrobenzene inhalation

	Sex	Exposure level (ppm)			
		0	5	25	50
Incidence	M	0/68	58/67[a]	58/65[a]	62/66[a]
	F	0/53	55/60[a]	63/64[a]	62/62[a]

[a] Significantly different from controls, Fisher Exact test, $p<0.05$.
Source: Cattley et al., 1994; CIIT, 1993.

Bronchiolization of the alveoli is a histologically distinct lesion which may indicate a variety of pathological conditions, including inflammation, chemical irritation, and exposure to carcinogens (Nettesheim and Szakal, 1972). It is possible that this effect is a precursor to tumor formation, as 36% of male mice and 9% of female mice developed bronchio-alveolar adenomas/carcinomas at the lowest concentration tested versus 19% and 0% of controls, respectively.

Although bronchiolization was chosen as the critical effect, the dose-responses for hematological endpoints, the critical effects used to derive the RfD, and olfactory degeneration were modeled and RfCs were derived from these endpoints as a comparison. The results of the RfC derivation based on metHb levels and olfactory degeneration are provided in Appendix B-2.

5.2.2. Method of Analysis — LOAEL/NOAEL Approach

5.2.2.1. Bronchiolization — Mouse, Chronic

Bronchiolization of the alveoli was present in ≥87% of all treated male and female mice at the lowest concentration (5 ppm) tested. This effect was not present in any of the control animals. At the middle (25 ppm) and high (50 ppm) concentrations, bronchiolization of the alveoli was found in 89% and 94% of males and 98% and 100% of females, respectively. Because of the absence of a concentration response, this type of data is not amenable to benchmark concentration modeling. Moreover, since the data for bronchiolization of the alveoli are presented in dichotomous form, the ≥87% response at the lowest concentration tested is nearly 9-times higher than a benchmark response of 10% extra risk above control values. Therefore, the LOAEL/NOAEL approach was used.

5.2.3. Evaluation of Human Equivalent Concentrations

Because the RfC is a metric that addresses continuous human exposure for a lifetime, adjustments need to be made to animal data obtained from intermittent and/or less-than-lifetime exposure scenarios, as supported in the *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b). The first step is adjustment of the intermittent inhalation exposure to continuous exposure, based on the assumption that the product of exposure concentration and exposure time is constant (U.S. EPA, 2002). In the chronic studies (Cattley et al., 1994; CIIT, 1993), animals were exposed for 6 hours/day for 5 days/week. Therefore, the POD (adjusted LOAEL; $LOAEL_{ADJ}$) for inhalation of nitrobenzene is as follows:

$LOAEL_{ADJ}$ = LOAEL × daily exposure/24 hours × exposure time/lifetime

$LOAEL_{ADJ}$ = 5 ppm × 6/24 × 5/7 = 0.893 ppm

Furthermore, because the RfC is expressed in mg/m^3, the above ppm value needs to be converted to mg/m^3 using the conversion factor for nitrobenzene of 1 ppm = 5.04 mg/m^3. Thus, the POD value is:

$LOAEL_{ADJ}$ = 0.893 × 5.04 = 4.5 mg/m^3

5.2.3.1. Human Equivalent Concentration

EPA guidance for RfC evaluation provides procedures for determining a human equivalent concentration (HEC) from the duration-adjusted POD [here: $LOAEL_{ADJ}$] obtained from animal data (U.S. EPA, 1994b). The approach considers the physicochemical characteristics of the gas or vapor in question as well as the toxicological specifics of the target tissue (respiratory vs. systemic and, in the former case, extrathoracic, thoracic, tracheobronchial, or pulmonary). The effect considered, bronchiolization, is a pulmonary effect. Nitrobenzene qualifies as a category 2 gas: moderately water soluble, reactive in respiratory tissue, and toxicologically active at remote sites (U.S. EPA, 1994b). For Category 2 gases, HEC values are calculated using methods for category 1 gases for portal-of- entry effects and category 3 methods for systemic effects (U.S.EPA, 1994b). Since bronchiolization of the alveoli is a portal-of-entry effect, the method for Category 1 gases was used to derive HEC.

Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry (U.S. EPA, 1994b) suggest that HECs be estimated by applying to the duration-adjusted exposure level [here: the $LOAEL_{ADJ}$], a factor that is specific for the affected region of the respiratory tract and the breathing characteristic of the species to be compared. This factor, the regional gas dose ratio (RGDR), as detailed in the RfC guidance (U.S. EPA, 1994b) is determined for the pulmonary (PU) region as follows:[6]

$$RGDR_{PU} = (MV_a/S_{a,PU}) \div (MV_h/S_{h,PU}) \qquad (5\text{-}1)$$

where:

MV_a = minute volume for mice = 0.06 m^3/day
MV_h = minute volume for humans = 20 m^3/day
$S_{a,PU}$ = default pulmonary surface area for mice = 0.05 m^2
$S_{h,PU}$ = default pulmonary surface area for humans = 54 m^2

The minute volume, MV_a, for female B6C3F1 mice in chronic studies was calculated as:

$$\text{Ln } V_E = b_0 + b_1 \times \ln BW \qquad (5\text{-}2)$$

where:

V_E = minute volume
b_0 = intercept from algorithm to calculate the default minute volume in mice = 0.326
b_1 = coefficient from algorithm to calculate the default minute volume in mice = 1.050
BW = default body weight for female B6C3F1 mice in chronic studies = 0.0353 kg

Hence:

$$\ln V_E = 0.326 + 1.05 \times \ln 0.0353 = 0.326 + 1.05 \times -3.34$$
$$\ln V_E = -3.19$$
$$V_E = 0.0414 \text{ L/min} = 0.06 \text{ m}^3\text{/day}.$$

For humans, V_E = 20 m^3/day.

Substituting these values into equation 5-1, the RGDR is calculated as:

[6] The equation for portal category 1 gases for portal of entry effects in the pulmonary region is more complicated, but the additional factors extrathoracic and tracheobronchial regions are very close to 1.

$$RGDR_{PU} = (0.06\ m^3/day)/(0.05\ m^2) \div (20\ m^3/day)/(54\ m^2) = 3.24$$

Finally, the $LOAEL_{HEC}$ is derived as follows:

$$LOAEL_{HEC} = LOAEL_{ADJ} \times RGDR \qquad (5\text{-}3)$$

$$LOAEL_{HEC} = 4.4982 \times 3.24 = 14.57\ mg/m^3$$

5.2.4. Calculation of the RfC — Application of Uncertainty Factors

The RfC for bronchiolization as the critical effect is calculated from the $LOAEL_{HEC}$ by application of UFs as follows:

$$RfC = LOAEL_{HEC} \div UF \qquad (5\text{-}4)$$
$$RfC = 14.57 \div 300 = 0.04856\ mg/m^3 = 5 \times 10^{-2}\ mg/m^3$$

The UF of 300 is composed of four parts:

- An intraspecies uncertainty factor of 10 was applied to account for human variability and to protect potentially sensitive humans and lifestages (e.g., children). The default value was selected in the absence of information indicating the degree to which humans might vary in susceptibility to nitrobenzene toxicity.

An uncertainty factor of 3 was applied to account for uncertainty in extrapolating from laboratory animals to humans. This value is adopted by convention where an adjustment from an animal-specific $LOAEL_{ADJ}$ to a $LOAEL_{HEC}$ already has been incorporated. Application of a full uncertainty factor of 10 would depend on two areas of uncertainty (i.e., toxicokinetic and toxicodynamic uncertainties). In this assessment, the toxicokinetic component is mostly addressed by the determination of a HEC as described in the RfC methodology (U.S. EPA, 1994). The toxicodynamic uncertainty is also accounted for to a certain degree by the use of the applied dosimetry method.

- A LOAEL-to-NOAEL extrapolation was performed because the critical effect was pronounced at the lowest concentration tested. An uncertainty factor of 10 was applied because animals in the lowest concentration group exhibited a ≥87% response versus 0% in controls.

- A subchronic-to-chronic uncertainty factor for extrapolation to lifetime exposure was not applied since the data used originated from a two-year (lifetime) chronic study.

An uncertainty factor of 1 was applied to account for database deficiencies. The inhalation database is considered complete. The database includes the following studies: a two-year (lifetime) chronic inhalation study with an interim (15-month sacrifice), two-generation reproductive and developmental inhalation studies, a subchronic (10-week) inhalation neurotoxicity study, and 90-day inhalation study

5.2.5. Previous Inhalation Assessment

An inhalation risk assessment was not provided in the previous IRIS evaluation of nitrobenzene.

5.3. CANCER ASSESSMENT

No studies exist on the carcinogenicity of nitrobenzene in humans. In animals, there is no cancer bioassay available following oral administration of nitrobenzene, but there is a single chronic inhalation cancer bioassay. In this study, both sexes of F344 rats and B6C3F1 mice, along with male CD rats, were exposed to nitrobenzene for 2 years via inhalation (CIIT, 1993; published as Cattley et al., 1994). Data from this study are used as the basis of a cancer assessment for nitrobenzene, as described in the following sections. For a detailed discussion of the CIIT study design and results, see Section 4.2.2.2.

5.3.1. Choice of Principal Study and Target Organ, with Rationale and Justification

A two-year inhalation cancer bioassay (CIIT, 1993; published as Cattley et al., 1994) was used for development of an inhalation unit risk for nitrobenzene. Table 5-6 presents an overview of the tumor incidence data from this study.

Cattley et al. (1994) reported that nitrobenzene caused an increased incidence of neoplasms in the respiratory tract and in follicular cells of the thyroid in male B6C3F1 mice, as well as an elevated incidence of liver and mammary gland neoplasias in female B6C3F1 mice. A slightly elevated incidence of thyroid neoplasias, without strong evidence of a dose response, was also observed in female B6C3F1 mice. Significant dose-related trends (at $p<0.05$ in the Cochran-Armitage Test) were observed for lung adenomas or carcinomas and thyroid follicular

cell adenomas in male B6C3F1 mice, and for hepatocellular adenomas in female B6C3F1 mice. Significantly elevated incidences of mammary gland adenocarcinomas also occurred at the highest concentration (50 ppm) in female B6C3F1 mice; however, the female mice at the two lower concentrations (5 and 25 ppm) were not evaluated histopathologically for this tumor type, so the existence of a dose response could not be determined.

Table 5-6. Selected cancer incidences in B6C3F1 mice, F344 rats, and CD rats following 2-year inhalation exposure to nitrobenzene

Species, sex, strain	Target organ, tumor type [a]	Nitrobenzene concentration (ppm)			
		0	5	25	50
Mouse, male, B6C3F1	Lung, bronchio-alveolar adenoma or carcinoma	8/42 (19.0%)	16/44 (36.4%)	20/45 (44.4%)	21/48 (43.8%)
	Thyroid, follicular cell adenoma	0/41 (0%)	4/44 (9.1%)	1/45 (2.2%)	6/46 (13.0%)
Mouse, female, B6C3F1	Liver, hepatocellular adenoma	4/31 (12.9%)	4/38 (10.5%)	5/46 (10.9%)	11/34 (32.4%)
	Mammary, adenocarcinoma	0/30 (0%)	Not evaluated	Not evaluated	2/34 (5.8%)
	Thyroid, follicular cell adenoma	1/30 (3.3%)	0/37 (0%)	2/45 (4.4%)	2/34 (5.9%)
		Nitrobenzene concentration (ppm)			
		0	1	5	25
Rat, female, F344	Liver, hepatocellular adenoma or carcinoma	0/49 (0%)	2/50 (4.0%)	0/59 (0%)	3/49 (6.1%)
Rat, male, F344		1/43 (2.3%)	4/50 (8.0%)	5/47 (10.6%)	16/46 (34.8%)
Rat, male, CD		0/23 (0%)	0/23 (0%)	1/25 (4.0%)	5/23 (21.7%)
Rat, female, F344	Uterus, endometrial stromal polyp	9/48 (18.8%)	15/50 (30.0%)	14/50 (28.0%)	19/49 (38.8%)
Rat, male, F344	Kidney, tubular adenoma or carcinoma	0/43 (0%)	0/50 (0%)	0/47 (0%)	6/46 (13.0%
	Thyroid, follicular cell adenoma or carcinoma	1/43 (2.3%)	1/50 (2.0%)	5/47 (10.6%)	8/46 (17.4%)

[a] All incidences shown have significant dose response trends at $p<0.05$ (Cochran-Armitage Test).
Source: CIIT, 1993.

Mammary gland neoplasia data for female B6C3F1 mice were not used for quantitative dose-response assessment because only the controls and highest dosed animals were evaluated for this tumor type. Adenomas and carcinomas of the thyroid in male B6C3F1 mice were considered for quantitative assessment, even though they did not exhibit a monotonic dose response and had a rather low incidence. Lung adenomas and carcinomas combined in male B6C3F1 mice were significantly elevated at all doses relative to concurrent controls, and were also considered for use in a quantitative dose-response assessment.

Significant trends were reported for hepatocellular adenomas or carcinomas in male and female F344 rats and male CD rats, endometrial stromal polyps in female F344 rats, and kidney and thyroid follicular cell adenomas or carcinomas in male F344 rats. However, kidney tubular adenomas or carcinomas in male F344 rats were observed only at the highest dose, and only one carcinoma was detected. Moreover, no corresponding renal neoplasias occurred in female F344 rats or male CD rats in the same study.

The incidence data for uterine endometrial stromal polyps in female F344 rats, a common benign lesion in this rat strain (NTP historical controls = 11.6%), displayed a high incidence in controls (18.8%), but there was still some evidence of a dose response. However, these data were not modeled because of the lower overall response in female F344 rats versus the male F344 rats for other tumor types. There was also a high incidence of testicular interstitial cell tumors in male F344 and CD rats (data not shown). However, because the same incidence was observed in controls and no dose response was seen, this neoplasm was not considered to be a treatment-related response.

Hepatocellular adenomas and carcinomas were consistently seen in both rat strains (i.e., F344 and CD) and also in both sexes of the F344 strain. The incidence of these neoplasms in male CD rats was lower than in male F344 rats. The clearest dose response for this endpoint occurred in male F344 rats; therefore, this data set and the datasets for kidney and thyroid adenomas or carcinomas in male F344 rats were chosen for cancer dose-response assessment. In addition, thyroid and lung adenomas and carcinomas in male B6C3F1 mice were also considered for cancer dose-response assessment.

5.3.2. Benchmark Concentration Modeling

Because there are no biologically-based dose-response models suitable for the mice and rat tumor data identified above, these data were modeled using the multistage model, as implemented by BMDS 1.3.2 (U.S. EPA, 2001). This model has the form:

$$P(d) = 1 - exp[-(q_0 + q_1 d + q_2 d^2 + ... + q_k d^k)],$$

where P(d) represents the lifetime risk (probability) of cancer at dose d (i.e., human equivalent exposure in this case), and $q_i \geq 0$ (for i = 0, 1, ..., k) are parameters estimated in fitting the model. Benchmark concentration modeling results are shown in Appendix B-3.

All three tumor sites (and types) for male rats listed in Table 5-6 were modeled separately. Adenoma and carcinoma incidences within each site were combined by counting animals with either of these responses. This practice was performed under the assumption that

adenomas and carcinomas represent stages along a continuum of carcinogenic effects resulting from the same mechanism, as recommended by the EPA cancer guidelines (U.S. EPA, 2005).

For male F344 rats, BMRs consistent with the lowest tumor incidences observed were selected (U.S. EPA, 2005); a 5% increase in tumor incidence for liver and thyroid adenomas or carcinomas, and a 10% increase in tumor incidence for kidney adenomas or carcinomas. Table 5-7 shows the estimated BMCs, BMCLs, and chi-square p-values derived for the three tumor sites and types modeled.

The most suitable endpoint for use as a point of departure (POD) for derivation of the inhalation unit risk is liver tumors in male F344 rats (NTP historical controls = 2.6%; Cattley, et. al. [1994] = 2.3%). This tumor site was selected because the BMCL based on liver tumors was lower that the BMCL based on thyroid and kidney tumors, and furthermore, was calculated from a model which showed good fit to the data. Therefore, 2.2 ppm was chosen as the POD for evaluation of hepatocellular cancer, a systemic cancer effect.

Benchmark concentration (BMC) modeling was attempted using the male mice thyroid tumor data, but these data were not suitable for BMC modeling (chi-square *p*-value = 0.05; see Appendix B-3.2, Part V). The male mice lung tumor data yielded a chi-square *p*-value = 0.18, and thus did not exhibit significant lack of fit. The BMC_5 and its corresponding 95% lower bound ($BMCL_5$) were 7.5 and 4.1 ppm, respectively (Appendix B-3.2, Part IV). Given the better model fit and lower benchmark concentrations yielded by the F344 rat data, as well as the multiple tumor sites observed in the F344 rat, only the rat data were pursued in developing potency estimates.

Table 5-7. Estimated BMCs and BMCLs based on tumor incidence data in male F344 rats exposed to nitrobenzene via inhalation*

Target organ, tumor type		BMC (ppm)	BMCL (ppm)	Chi-square p-value for lack of fit
Kidney	Tubular adenoma	24.4	18.0	1.0
	Tubular carcinoma	42.3	27.5	1.0
	Tubular adenoma and carcinoma (combined)	22.8	16.8[a]	1.0
Thyroid	Follicular cell adenoma	18.0	8.7	0.34
	Follicular cell carcinoma	10.2	5.2	0.81
	Follicular cell adenoma and carcinoma (combined)	6.6	3.8[b]	0.37
Liver	Hepatocellular adenoma	7.0	3.0	0.44
	Hepatocellular carcinoma	13.5	6.3	0.60

	Hepatocellular adenoma and carcinoma (combined)	3.3	2.2[b]	0.63

[a]$BMCL_{10}$; [b]$BMCL_{05}$; *See Appendix B-3.4 for individual and combined modeling results.

5.3.3. Inhalation Dose Adjustments, Inhalation Unit Risk, and Extrapolation Methods

The current EPA *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005) stipulate that the method used to characterize and quantify cancer risk from a chemical is determined by what is known about the mode-of-action of the carcinogen and the shape of the cancer dose-response curve at low dose. The dose response is assumed to be linear in the lowest dose range, when evidence supports a genotoxic mode-of-action because of DNA reactivity, or if another mode-of-action that is anticipated to be linear is applicable. An assumption of nonlinearity is appropriate when the mode-of-action theoretically has a threshold (e.g., when the carcinogenic action is secondary to another toxic effect that itself has a threshold). If the mode-of-action of carcinogenicity is not adequately understood, a linear dose-response relationship at low doses cannot be discounted, and the linear extrapolation is used. (U.S. EPA, 2005).

The available evidence suggests that nitrobenzene is not, or is at most weakly, mutagenic (see Section 4.4.4). In addition, nitrobenzene has been shown to undergo redox cycling (see Section 3.3) and cause oxidative stress (see Section 4.4.4). This process can cause DNA damage and is also thought to be cytotoxic. However, as described in Section 4.6.3, the data available on the role of redox cycling and oxidative stress generated during the metabolism of nitrobenzene are not complete enough to substantiate these phenomena as the mode of carcinogenic action. Accordingly, the linear approach is used for the derivation of carcinogenic potency.

The inhalation unit risk (IUR) for liver adenomas and carcinomas combined was estimated, along with IURs for liver carcinomas and adenomas separately for comparison.

In order to derive an IUR based on hepatocellular adenomas and carcinomas combined, the BMCL value for liver tumors from inhalation exposure to nitrobenzene reported in Table 5-7 was converted to mg/m^3 (1 ppm = 5.04 mg/m^3 under 0.15 mm Hg at 25 ^oC) and adjusted for lifetime exposure as follows:

$$\text{BMCL (adjusted)} = \text{BMCL} \times 5.04\ mg/m^3 \times 6/24\ \text{hours} \times 5/7\ \text{days}$$

The critical cancer effect from nitrobenzene inhalation (e.g., liver cancer) is a systemic effect, and hence adjustment of the Human Equivalent Concentration (HEC) requires the nitrobenzene air:blood partition coefficients for humans and rats (EPA, 1994b). In the absence of such data, the ratio of animal to human air:blood partition coefficients is assumed to be unity.

The estimated central tendency unit potency is derived by converting the BMC to a BMC_{HEC}, and then dividing the BMR (e.g., BMR = 5% or 0.05) by the BMC_{HEC}. Estimates of the central tendency unit potencies based on liver adenomas, liver carcinomas, and liver adenomas and carcinomas combined in male F344 rats are shown in Table 5-8.

Table 5-8. Estimates of Central Tendency Unit Potencies for Nitrobenzene Based on Liver Tumors in Male F344 Rats.

Target organ/tumor type		BMC (ppm)	BMC_{HEC}[a] (mg/m^3)	Estimated central tendency unit potency[b] $(ug/m^3)^{-1}$
Liver	Hepatocellular adenoma	7.0	6.3	8.0E-06
	Hepatocellular carcinoma	13.5	12.2	4.1E-06
	Hepatocellular adenoma or carcinoma (combined)	3.3	3.0	1.7E-05

[a]HEC = BMC x 5.04 mg/m^3 x 5/7 x 6/24; assumes ratio of animal to human air:blood partition coefficients is 1.
[b]Central tendency unit potency = BMR (0.05) / BMC_{HEC}

Estimated IURs are calculated by dividing the BMR (e.g., BMR=5% or 0.05) by the $BMCL_{HEC}$. Estimates of the IURs based on liver adenomas, liver carcinomas, and liver adenomas and carciomas combined in male F344 rats are shown in Table 5-9.

Table 5-9. Estimates of IURs for Nitrobenzene Based on Liver Tumors in Male F344 rats.

Target organ/tumor type		BMCL (ppm)	$BMCL_{HEC}$[a] (mg/m^3)	Estimated IUR[b,c] $(ug/m^3)^{-1}$
Liver	Hepatocellular adenoma	2.5	2.2	2×10^{-5}
	Hepatocellular carcinoma	6.3	5.7	9×10^{-6}
	Hepatocellular adenoma or carcinoma (combined)	2.2	2.0	3×10^{-5}

[a]$BMCL_{HEC}$ = BMCL x 5.04 mg/m^3 x 5/7 x 6/24; assumes ratio of animal to human air:blood partition coefficients is 1.
[b]IUR = BMR (0.05) / $BMCL_{HEC}$.
[c]These IURs should not be used at continuous exposure concentrations above 2.0×10^3 $\mu g/m^3$ because above this concentration, the observed dose response is no longer linear.

Finally, estimated nitrobenzene air concentrations, based on combined adenomas and carcinomas, corresponding to specific lifetime cancer risks are as follows:

Concentration at 10^{-4} risk: 4×10^0 $\mu g/m^3$

Concentration at 10^{-5} risk: 4×10^{-1} $\mu g/m^3$

Concentration at 10^{-6} risk: 4×10^{-2} μg/m^3

With a multiplicity of tumors, as is the case for nitrobenzene, the concern is that a potency or risk estimate based solely on one tumor site (e.g., hepatocellular adenomas or carcinomas) may underestimate the overall cancer risk associated with exposure to this chemical. An alternative approach was also considered in which a summed IUR for tumors of the liver, kidney, and thyroid was developed (See Appendix B-3.5). The cumulative IUR, after rounding to one significant figure is the same as the IUR based on combined liver adenomas and carcinomas, 3×10^{-5} (μg/m^3)$^{-1}$.

6. MAJOR CONCLUSIONS IN THE CHARACTERIZATION OF HAZARD AND DOSE RESPONSE

6.1. HUMAN HAZARD POTENTIAL

6.1.1. Exposure Pathways

At room temperature nitrobenzene is a liquid with a vapor pressure high enough to allow human exposure to occur via inhalation. It is also able to penetrate human skin, both as liquid and as vapor. Most serious poisonings with nitrobenzene appear to have happened in domestic settings via either accidental or intentional ingestion or by dermal and inhalation exposure from its use in pesticides. Nitrobenzene is also used in significant amounts as an intermediate in chemical syntheses and as a solvent in products such as paint, printing ink, and shoe polish or as a scenting agent in soap. There are no epidemiological studies of the health effects of nitrobenzene in humans.

6.1.2. Toxicokinetics

The lipophilicity of nitrobenzene and the composition of membranes in the human body are the main determinants for systemic absorption. Reports from accidental poisonings (Myslak et al., 1971), studies in human volunteers (Piotrowski, 1967; Salmowa et al., 1963), and occupational studies (Ikeda and Kita, 1964) indicate that nitrobenzene is absorbed well from the human gastrointestinal tract as well as from the lungs. In addition, Feldmann and Maibach (1970) demonstrated that nitrobenzene is absorbed through the skin. Although their data pointed to a rather insignificant amount penetrating the skin, poisoning cases in children seem to indicate that at least young humans are at risk from dermal exposure to nitrobenzene. Beauchamp et al. (1982) calculated that, in adults, about equal parts of a dose originating from

exposure to nitrobenzene vapor are due to inhalation and dermal absorption, respectively. Animal experiments have supported the findings in humans.

Although nitrobenzene is rather lipophilic, it does not display a high affinity for fatty tissues. The only study on the distribution of nitrobenzene in animals, (Albrecht and Neumann, 1985), showed highest levels after an oral dose to female Wistar rats was present in the blood 1 or 7 days after administration, followed by kidney, with lower levels in liver and lung. The tendency of nitrobenzene to associate with blood has been confirmed by Goldstein and Rickert (1984). The main targets are RBCs, which are chemically modified by binding with nitrobenzene metabolites, and the spleen.

Nitrobenzene is metabolized via reduction of the nitro group to aniline and/or by hydroxylation of the aromatic ring to phenolic compounds. Reduction of the nitro group appears to be the dominant process. Two processes have been described for the reduction of the nitro group with CYP450 flavin enzymes as catalysts and NAD(P)H as the cofactor: an aerobic three-step, two-electrons/step process in intestinal microflora that operates at a high metabolic rate, and an anaerobic six-step, one-electron/step process in mammalian cells that is much less effective because it is inhibited by normal tissue levels of oxygen. RBCs command a set of enzymes that force nitrobenzene into a futile redox cycle between the nitrobenzene metabolite, nitrosobenzene, and phenylhydroxylamine (Holder, 1999b). This pathway also can result in the formation of glutathione conjugates. Redox cycling of nitrobenzene is thought to contribute to the development of methemoglobinemia and to DNA damage caused by reactive oxygen species (Levin and Dent, 1982).

Nitrobenzene is eliminated in humans and animals mostly via urine, independent of the route of exposure. *Ortho*-, *meta*-, and *para*-variants of both nitrophenol and aminophenol have been identified in the urine of nitrobenzene-exposed experimental animals (Parke, 1956; Robinson et al., 1951) and humans (Myslak et al., 1971; Feldmann and Maibach, 1970; Piotrowski, 1967). Experiments with specific pathogen-free animals suggest that more than half of the urinary nitrobenzene metabolites are formed by intestinal microflora (Reddy et al., 1976). Fecal and exhalatory elimination also have been observed in rats and mice, with about 1/6 of a dose of [^{14}C]-labeled nitrobenzene excreted via feces and about 1/40 exhaled in air (Rickert et al., 1983; Levin and Dent, 1982). Elimination of nitrobenzene from the human or rodent organism is not a rapid process. In rats, it took about 3 days to eliminate 80% of a 22.5 mg/kg dose of nitrobenzene (Rickert et al., 1983). In some of the human poisoning cases, it took about a week to overcome the clinical signs of methemoglobinemia.

6.1.3. Characterization of Noncancer Effects

The database of studies of nitrobenzene effects in animals is considerably more robust than that of studies in humans. Case reports dealing with acute poisonings via ingestion or dermal exposure indicate that the hallmark effect of nitrobenzene exposure in humans is methemoglobinemia. This condition can be treated with blood transfusions or with reducing agents, such as vitamin C and methylene blue, that return the iron in methemoglobin from iron (III) to its normal, oxygen-carrying iron(II) state. Severe cases have been known to have a fatal outcome, particularly in children. The splenic pathology can be traced to the role that the spleen plays in scavenging RBCs damaged by nitrobenzene metabolites.

There is a considerably more detailed database for nitrobenzene effects in animals. In animals, methemoglobinemia and other signs of acute toxicity can be observed, including signs of neurotoxicity, likely due to a lack of oxygen and possibly due to a general solvent effect. A 90-day oral gavage study (NTP, 1983a) found dose-dependent increases in liver, kidney, and spleen weights (both absolute and relative) in both sexes of mice and rats and a decrease in testis weight in male F344 rats. By the end of the study, animals surviving the highest dose displayed substantial levels of methemoglobinemia (>12%) and considerable blood pathology (decreased Hb, Hct, and RBC count, but increased reticulocyte count), all compatible with hemolytic anemia caused by metHb formation. Histopathologic evaluation revealed congestion and lymphoid depletion of the spleen, pigment (hemosiderin) deposition in the kidney and brain, and testicular atrophy in males. The only effect observed at low doses was splenic congestion. The splenic pathology, too, can be traced to metHb formation and subsequent RBC hemolysis. Generally, similar pathology was observed in male and female B6C3F1 mice in the oral subchronic NTP study (1983a) and in a 28-day gavage study in F344 rats (Shimo et al., 1994). In that study, some of the animals were allowed a 14-day recovery period; while most of the pathology observed tended to return to normal within 2 weeks, testicular atrophy in male rats treated with the highest dose, 125 mg/kg-day, showed little tendency for improvement.

Several studies were conducted with inhalation exposure of experimental animals including 14-day studies (Medinsky and Irons, 1985; DuPont, 1981), a 90-day subchronic study (CIIT, 1984), and a 2-year chronic study (CIIT, 1993; published as Cattley et al., 1994). The chronic study (CIIT, 1993) was conducted in compliance with GLP and contemporary requirements for chronic studies. Both the 90-day and the 2-year studies were carried out using both sexes of F344 rats and B6C3F1 mice; in addition, the 90-day study included both sexes of CD rats, while the chronic study included only male CD rats. Several of the same target tissues as in the oral study were identified following inhalation exposure, with the

addition, at lower exposures, of the degeneration of the olfactory epithelium of the nasal turbinates and bronchiolization of the alveoli in mice. Other pathologies following 90-day or 2-year inhalation exposure to nitrobenzene common to both species were changes in target organ weights, blood pathology, and methemoglobinemia.

NTP (1983b) also conducted a 90-day dermal study with nitrobenzene in F344 rats and B6C3F1 mice of both sexes. Again, the pathologic effects were very similar to those observed in the gavage study (NTP, 1983a); but, in addition, congestion of the lung was observed at higher doses (≥100 mg/kg-day) as was uterine atrophy in female rats at the highest dose, 800 mg/kg-day.

In summary, the major effects of mid- to long-term exposure to nitrobenzene, independent of the route of exposure, appear to be increases in liver, kidney, and spleen weights and methemoglobinemia with subsequent hemolytic anemia and splenic congestion. Administration of nitrobenzene via inhalation additionally elicited olfactory degeneration and bronchiolization of the alveoli as effects specific for this route of exposure. The olfactory degeneration occurred in a concentration dependent manner, and bronchiolization of the alveoli occurred in $\underline{\geq}$ 86% of male and female mice at the lowest concentration tested. Effects on the male reproductive system, which are also potentially critical effects, are discussed in the following section.

6.1.4. Reproductive Effects and Risks to Children

As young children are more susceptible to methemoglobinemia, a toxic effect of nitrobenzene, than adults, they may be more susceptible to this aspect of nitrobenzene toxicity. There are several reasons for this. First, newborns still have fetal Hb, which is more susceptible to metHb formation than adult Hb (Goldstein et al., 1969). Next, the activity of NADH-cytochrome b_5 reductase, an enzyme required for the conversion of ferric iron to ferrous iron in Hb, is not fully developed in infants and very young children (Wentworth et al., 1999) and neither is glucose-6-phosphatase dehydrogenase activity, an enzyme required to replenish NADPH (Goldstein et al., 1969). Additionally, the observation of more accidental fatal poisonings in children exposed dermally indicates a potential greater sensitivity to dermal nitrobenzene exposures.

There is no information available concerning potential reproductive toxicity of nitrobenzene in humans. In rodents, however, nitrobenzene is a moderately effective male reproductive toxicant. A single 300 mg/kg dose of nitrobenzene to male F344 rats caused sperm production to decrease 20 days after administration, eventually dropping to zero by

50 days (Levin et al., 1988). By 100 days after treatment, sperm production had returned to 78% of control levels. This time course reflects the normal spermatogenic cycle of rats. In another experiment, the same dose was found to cause lesions to seminiferous tubules and marked necrosis of spermatogenic cells (Bond et al., 1981), as well as decreases in sperm mobility and viability and morphologically abnormal sperm (Koida et al., 1995; Matsuura et al., 1995). Dosing with 60 mg/kg-day nitrobenzene for 7–70 days had no effect on the copulatory behavior of male Sprague-Dawley rats, but their fertility decreased dramatically with exposure times longer than 14 days. By 4 weeks of dosing, the males were effectively sterile (Kawashima et al., 1995a, b).

In a reproductive toxicity study (Mitsumori et al., 1994) with 20, 60, and 100 mg/kg-day nitrobenzene administered orally to Sprague-Dawley rats for 14 days preceding mating, no effect on fertility or the offspring was observed (dosing was continued throughout pregnancy and the first 4 days of lactation). There was substantial mortality among the high-dose females while survivors and their offspring showed no signs of pathology. In a two-generation study where Sprague-Dawley rats were exposed to 1, 10, and 40 ppm nitrobenzene via inhalation, starting 10 weeks before mating, a strong, dose-dependent reduction in fertility was observed that was more marked in the F_1 generation than in the F_0 generation (Dodd et al., 1987; BRRC, 1985).

In a study with inhalation exposure of pregnant Sprague-Dawley rats to 1, 10, or 40 ppm nitrobenzene on GDs 6–15, no effects on number of implantations, resorptions, or stillbirths were observed (Tyl et al., 1987). There were no typical signs of teratogenicity in the offspring, although some effects on ossification were observed. However, the authors were uncertain whether those observations were compound-related. Several other inhalation reproductive/developmental studies in New Zealand rabbits (Biodynamics Inc., 1984, 1983) and in CD rats (Tyl et al., 1987) also produced no indication of a teratogenic action of nitrobenzene. In summary, there is strong evidence for nitrobenzene to act as a male reproductive toxicant, although at higher exposures than those eliciting other effects, but there is no indication that nitrobenzene affects female fertility or acts as a developmental toxicant.

6.1.5. Noncancer Mode of Toxic Action

Nitrobenzene elicits an array of toxic effects, and for any of these to occur, it appears that metabolic activation or conversion of the parent compound may be involved. A prominent critical effect identified here is methemoglobinemia. This effect requires metabolism, which is mostly carried out by intestinal microflora (Reddy et al., 1976). The active metabolite appears to be nitrosobenzene, which is taken up into RBCs, where it binds

with high affinity to Hb (Holder, 1999a; Kiese, 1966). The exact mechanism is not completely understood, but it is likely that redox cycling of nitrosobenzene via phenylhydroxylamine results in oxidation of Fe^{2+} to Fe^{3+} in Hb and thus formation of metHb. This leads to destruction of the RBC, with resulting hemolysis, anemia, and splenic congestion.

There is, as yet, no hypothesis concerning the development of olfactory degeneration, bronchiolization of the alveoli, or the potential for immunotoxicity from nitrobenzene. Humans are facultative nose breathers, while rodents are obligatory nose breathers. Olfactory degeneration observed following long-term nitrobenzene inhalation in rodents may therefore not be relevant for humans, but supportive or refuting evidence is not available. However, bronchiolization of the alveoli is of relevance to both facultative and obligatory nose breathers. It has been proposed that metabolism of nitrobenzene involves the formation of reactive oxygen species (Han et al., 2001) that can be the cause of damage to point-of-entry tissues, provided they command suitable activities of metabolizing enzymes.

The male reproductive toxicity of nitrobenzene affects the Sertoli cells (Allenby et al., 1990). Shinoda et al. (1998) demonstrated that the loss of germ cells following nitrobenzene exposure was due to apoptosis, and they speculated that factor(s) released from Sertoli cells might be responsible. Another potent testicular toxicant, mono-(2-ethylhexyl) phthalate, caused apoptosis in germ cells via the Fas/Jun/AP-1 system, but nitrobenzene-induced testicular toxicity did not proceed via this pathway (Richburg and Nañez, 2003). The action of reactive oxygen species cannot be excluded as a causative factor here. In summary, the noncancer mode of action of nitrobenzene requires metabolism of the parent compound and may involve reactive oxygen species but otherwise is not well elucidated.

6.1.6. Characterization of the Human Carcinogenic Potential

Under the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), nitrobenzene is *likely to be carcinogenic to humans*. This descriptor is based on the fact that 2-year inhalation of nitrobenzene caused cancers in two species of laboratory animals, rats and mice, in both sexes, in two strains of rats (F344 and male CD), and in multiple sites. There are no studies that document the carcinogenicity of nitrobenzene in humans. The weight-of-evidence warrants calling nitrobenzene a "likely" human carcinogen; however, this designation lies on the low end of the range for this descriptor.

There are no nitrobenzene exposure data or studies in humans from which to assess a potential mechanism of action for cancer. Nitrobenzene has caused neoplasia in a 2-year chronic inhalation study (Cattley et al., 1994; CIIT, 1993) in a dose-related fashion in the livers of male F344 rats and the lungs of male B6C3F1 mice. Increased incidences of neoplasia with

statistically significant, positive dose trends were also observed as kidney and thyroid adenomas and carcinomas in male F344 rats, endometrial polyps in female F344 rats, hepatocellular adenomas and carcinomas in male CD rats, and kidney neoplasia in male B6C3F1 mice. Although the probable human carcinogen, aniline, is a metabolite of nitrobenzene, there is no evidence that it is causative agent (IRIS, 1994).

Based on the results of genotoxicity tests, nitrobenzene appears to be at most weakly genotoxic. This determination is based on the almost exclusively negative results in Salmonella assays (Ames tests; the only exception is TA98 in the presence of a comutagen), as well as negative clastogenic findings from in vivo assays of sister chromatid exchange, unscheduled DNA synthesis, and chromosomal aberrations. Cytolethality with subsequent regenerative hyperplasia, a promotion-type, nongenotoxic mode of action, has not been described in connection with nitrobenzene. Ohkuma and Kawanishi (1999) have provided evidence that nitrobenzene may cause oxidative DNA damage, and Li et al. (2003a, b) have shown that nitrobenzene can produce DNA adducts. It is not known whether there are any specific, qualitative, or quantitative differences in nitrobenzene metabolism between rodents and humans, and there is no reason to assume that a cancer mode of action exists in animals that might not be relevant to humans. Therefore, a final conclusion on whether nitrobenzene acts in a genotoxic or epigenetic way, and whether a threshold might apply cannot be drawn at this time. This is reflected in the use of a linear approach as a default in extrapolating the carcinogenic potential of nitrobenzene.

6.2. DOSE RESPONSE

A few studies have been conducted with nitrobenzene in human research subjects. However, they were of short duration, used nontoxic doses, and only examined clinical signs. All dose-response assessments are therefore based on animal data obtained from chronic or subchronic studies.

6.2.1. Oral RfD

The only study in which nitrobenzene was administered orally for an extended period of time, 90 days (NTP, 1983a), was conducted in a well-controlled fashion in accordance with GLP guidelines valid at that time. The NTP (1983a) study included both sexes and two species, the F344 rat and the B6C3F1 mouse; 10 animals per sex and dose group; and five dose groups plus controls (0, 9.38, 18.75, 37.5, 75, and 150 mg/kg-day for rats and 0, 18.75, 37.5, 75, 150, and 300 mg/kg-day for mice). The study reported an abundance of toxic endpoints, including changes in absolute and relative organ weights, changes in hematological parameters, and histopathologic outcomes. Methemoglobinemia, splenic congestion, and reticulocyte count in male F344 rats were considered as potential critical effects. Dose-response data were evaluated using BMDS (version 1.3.2), with 10% ER as the BMR for splenic congestion, and with 1SD as the BMR for reticulocyte count and metHb levels. The corresponding POD was 2.8 mg/kg-day. After application of a UF of 1000, the oral RfD was identified as 3×10^{-3} mg/kg-day.

The composite UF consists of an interspecies uncertainty factor of 10 for extrapolation from animals to humans, an intraspecies uncertainty factor of 10 to adjust for sensitive subpopulations (most importantly small children), a subchronic-to-chronic uncertainty factor of 3 to correct for the less-than-lifetime exposure duration of the principal study, and a database deficiency uncertainty factorof 3 to account for lack of an oral multigeneration reproductive study.

The overall confidence in the RfD is medium. The critical effect on which the RfD is based is well supported by several other oral gavage studies over time periods of up to 70 days (Kawashima et al., 1995a, b). Nitrobenzene also displayed toxicity in reproductive and immunological studies, but at doses higher than those used in the principal study. On the basis of these considerations, confidence in the principal study is high. Confidence in the database is medium because there is no 2-year oral study, no NOAEL in the 90-day gavage study, and no multigeneration reproductive/developmental oral study. The medium confidence rating is driven by significant deficits in the database.

6.2.2. Inhalation RfC

A few studies have been conducted with nitrobenzene in human research subjects that were of short duration with nontoxic doses, and their target was not pathological evaluation. There are four animal studies available dealing with inhalation toxicity of nitrobenzene, ranging in duration from acute to chronic. A 90-day subchronic study was conducted using F344 and CD rats as well as B6C3F1 mice of both sexes (CIIT, 1984). Exposure concentrations were 0, 5, 16, and 50 ppm, 6 hours/day, 5 days/week. This study identified a variety of hematological endpoints, above all methemoglobinemia, with several other outcomes secondary to hemolytic anemia. The 2-year study, also conducted by CIIT (Cattley et al., 1994; CIIT, 1993), used B6C3F1 mice and F344 rats of both sexes and male CD rats. Rats were exposed to 0, 1, 5, and 25-ppm nitrobenzene and mice to 0, 5, 25, and 50 ppm nitrobenzene for 6 hours/day, 5 days/week (except holidays). This study identified a range of noncancer endpoints, of which bronchiolization of the alveoli was the most sensitive endpoint in both male and female mice. Bronchiolization of the alveoli was chosen as the critical effect for deriving the RfC, over methemoglobinemia, because the severity of bronchiolization of the alveoli increased with concentration, compared to the lack of a clear concentration-dependent response for methemoglobinemia at final sacrifice.

The effect selected for RfC evaluation is bronchiolization of the alveoli in female B6C3F1 mice as a portal of entry effect, using data from the chronic study. The $LOAEL_{ADJ}$ for bronchiolization was 0.87, or 4.6 mg/m^3. A composite UF of 300 was applied to this value, resulting in an RfC of 5×10^{-2} mg/m^3. The combined UF was composed of a reduced interspecies uncertainty factor of 3 to adjust for animal to human extrapolation, since an HEC had been used in its evaluation. An intraspecies uncertainty factor of 10 was applied to adjust for sensitive human populations, and a database deficiency uncertainty factor of 1 was used. Since a LOAEL-to-NOAEL extrapolation was used, an uncertainty factor of 10 was applied to account for the high response in animals at the lowest concentration tested.

The overall confidence in the RfC evaluation is medium. Confidence in the principal study is high because it was a 2-year bioassay with sufficient number of animals and conducted in accordance with good laboratory practices, and it is reasonable to assume that the endpoint is relevant to humans.

6.2.3. Oral Cancer Risk

The lack of available data precludes an assessment of a potential cancer risk for humans following oral exposure to nitrobenzene. Since a PBPK model for nitrobenzene is not available,

a quantitative comparison of the IRIS drinking water unit risk for aniline with the levels of aniline produced from metabolism of inhaled nitrobenzene cannot be made (IRIS, 1994).

6.2.4. Inhalation Cancer Risk

The mode of carcinogenic action of nitrobenzene cannot be classified as either genotoxic or nongenotoxic. Nitrobenzene was inactive in all bacterial mutagenicity assays and gave equivocal results in both in vivo and in vitro mammalian assay systems. There is limited experimental evidence that nitrobenzene can form DNA adducts or cause oxidative DNA damage, but no evidence was seen that would support a threshold mechanism such as cytotoxicity followed by regenerative hyperplasia. According to the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), the default approach in such a case is to use a no-threshold linear dose extrapolation approach. Nitrobenzene caused cancers in multiple organs, two species (rat and mouse), both sexes, and two different strains of rats in a 2-year inhalation study (Cattley et al., 1994; CIIT, 1993). Nitrobenzene caused lung adenomas and carcinomas in male B6C3F1 mice only, providing minimal evidence for point-of-entry carcinogenesis.

Male F344 rats appeared to be the most sensitive animal and presented with tumors of the liver, kidney and thyroid.

Liver tumors were deemed the most relevant tumor types for deriving the IUR. The recommended upper bound estimate on human extra cancer risk from continuous lifetime inhalation exposure to nitrobenzene was calculated at 3×10^{-5} $(\mu g/m^3)^{-1}$, an estimate that reflects the exposure-response relationships for liver cancer.

Confidence in this assessment is low to medium, since there is no data evidencing cancer in humans. Although there are tumors in multiple species and sexes, the tumors are generally not found in the same organs.

7. REFERENCES

Abbinante, A; Zerpa, R; Pasqualatto, D. (1997) Intoxication due to ingestion of bitter almond oil contaminated with nitrobenzene: clinical experience. Toxicologist 36:43.

Ajmani, A; Prakash, SK; Jain, SK; et al. (1986) Acquired methaemoglobinaemia following nitrobenzene poisoning. J Assoc Physicians India 34:891-892.

Albrecht, W; Neumann, HG. (1985) Biomonitoring of aniline and nitrobenzene. Hemoglobin binding in rats and analysis of adducts. Arch Toxicol 57:1-5.

Alcorn, CJ; Simpson, RJ; Leahy, D; et al. (1991) In vitro studies of intestinal drug absorption. Determination of partition and distribution coefficients with brush border membrane vesicles. Biochem Pharmacol 42:2259-2264.

Allenby, G; Sharpe, RM; Foster, PM. (1990) Changes in Sertoli cell function in vitro induced by nitrobenzene. Fundam Appl Toxicol 14:364-375.

Allenby, G; Foster, PM; Sharpe, RM. (1991) Evaluation of changes in the secretion of immunoactive inhibin by adult rat seminiferous tubules in vitro as an indicator of early toxicant action on spermatogenesis. Fundam Appl Toxicol 16:710-724.

Anderson, D; Styles, JA. (1978) The bacterial mutation test. Six tests for carcinogenicity. Br J Cancer 37:924-930.

Ask, K; Décologne, N; Asare, N; et al. (2004) Distribution of nitroreductase activity toward nilutamide in rat. Toxicol Appl Pharmacol 201:1-9.

Assmann, N; Emmrich, M; Kampf, G; et al. (1997) Genotoxic activity of important nitrobenzenes and nitroanilines in the Ames test and their structure-activity relationship. Mutat Res 395:139-144.

ATSDR (Agency for Toxic Substances and Disease Registry). (1990) Toxicological profile for nitrobenzene. Public Health Service, U.S.Department of Health and Human Services, Atlanta, GA. Available from <http://www.atsdr.cdc.gov/toxprofiles/tp140.html >.

Bairoch, A. (2000) The enzyme database in 2000. Nucleic Acids Res 28:304-305.

Ban, Y; Naya, M; Nishimura, T; et al. (2001) Collaborative study on rat sperm motion analysis using CellSoft Series 4000 semen analyzer. J Toxicol Sci 26:9-24.

Beauchamp, RO, Jr; Irons, RD; Rickert, DE; et al. (1982) A critical review of the literature on nitrobenzene toxicity. Crit Rev Toxicol 11:33-84.

Beutler, E. (1991) Glucose-6-phosphate dehydrogenase deficiency. N Engl J Med 324:169-174.

Biodynamics Inc. (1983) Range-finding study to evaluate the toxicity of nitrobenzene in the pregnant rabbit—draft final report with cover letter dated 092683. company of publication, place of publication, if available; report number. Submitted under TSCA Section 4A; EPA Document No. 40+8324347; NTIS No. OTS0509345.

Biodynamics Inc. (1984) An inhalation teratology study in rabbits with nitrobenzene (final report). company of publication, place of publication, if available; report number. Submitted under TSCA Section 4; EPA Document No. 40-8424492; NTIS No. OTS0510651.

Blackburn, DM; Gray, AJ; Lloyd, SC; et al. (1988) A comparison of the effects of three isomers of dinitrobenzene on the testes in the rat. Toxicol Appl Pharmacol 92:54-64.

Bonacker, D; Stoiber, T; Bohm, KJ; et al. (2004) Chromosomal genotoxicity of nitrobenzene and benzonitrile. Arch Toxicol 78:49-57.

Bond, JA; Chism, JP; Rickert, DE; et al. (1981) Induction of hepatic and testicular lesions in Fischer 344 rats by single oral doses of nitrobenzene. Fundam Appl Toxicol 1:389-394.

Bradberry, SM; Aw, TC; Williams, NR; et al. (2001) Occupational methaemoglobinaemia. Occup Environ Med 58:611-615.

BRRC (Bushy Run Research Center). (1984) Teratogenicity evaluation of inhaled nitrobenzene in the CD rat (final report). company of publication, place of publication, if available; report number. Submitted under TSCA Section 4; EPA Document No. 40-8424493; NTIS No. OTS0510652.

BRRC. (1985) Potential effects of nitrobenzene inhalation on reproductive performance and fertility in rats. Company of publication, place of publication, if available; report number. Submitted under TSCA Section 4; EPA Document No. 40-8524494; NTIS No. OTS0510653.

Bryant, C; DeLuca, M. (1991) Purification and characterization of an oxygen-insensitive NAD(P)H nitroreductase from *Enterobacter cloacae*. J Biol Chem 266:4119-4125.

Burns, LA; Bradley, SG; White, KL, Jr; et al. (1994) Immunotoxicity of nitrobenzene in female B6C3F1 mice. Drug Chem Toxicol 17:271-315.

Butterworth, RF. (2003) Hepatic encephalopathy. Alcohol Res Health 27:240-246.

Butterworth, BE; Smith-Oliver, T; Earle, L; et al. (1989) Use of primary cultures of human hepatocytes in toxicology studies. Cancer Res 49:1075-1084.

Cattley, RC; Everitt, JI; Gross, EA; et al. (1994) Carcinogenicity and toxicity of inhaled nitrobenzene in B6C3F1 mice and F344 and CD rats. Fundam Appl Toxicol 22:328-340.

Cave, DA; Foster, PM. (1990) Modulation of *m*-dinitrobenzene and *m*-nitrosonitrobenzene toxicity in rat Sertoli-germ cell cocultures. Fundam Appl Toxicol 14:199-207.

Chandra, AM; Qualls, CW, Jr.; Reddy, G; et al. (1995a) Hematological effects of 1,3,5-trinitrobenzene (TNB) in rats in vivo and in vitro. J Toxicol Environ Health 46:57-72.

Chandra, AM; Qualls, CW, Jr.; Reddy, G. (1995b) 1,3,5-Trinitrobenzene-induced encephalopathy in male Fischer-344 rats. Toxicol Pathol 23:527-532.

Chandra, AM; Qualls, CW, Jr.; Campbell, GA; et al. (1997) Testicular effects of 1,3,5-trinitrobenzene (TNB). II. Immunolocalization of germ cells using proliferating cell nuclear antigen (PCNA) as an endogenous marker. J Toxicol Environ Health 50:379-387.

Chandra, AM; Campbell, GA; Reddy, G; et al. (1999) Neurotoxicity of 1,3,5-trinitrobenzene (TNB): immunohistochemical study of cerebrovascular permeability. Vet Pathol 36:212-220.

Chiu, CW; Lee, LH; Wang, CY; et al. (1978) Mutagenicity of some commercially available nitro compounds for *Salmonella typhimurium*. Mutat Res 58:11-22.

Chongtham, DS; Phurailatpam, J; Singh, MM; et al. (1997) Methaemoglobinaemia in nitrobenzene poisoning. J Postgrad Med 43:73-74.

Chongtham, DS; Phurailatpam, J; Singh, MM; et al. (1999) Methaemoglobinaemia in nitrobenzene poisoning—a case report. J Indian Med Assoc 97:469-470.

CIIT (Chemical Industry Institute of Toxicology). (1984) Ninety day inhalation toxicity study of nitrobenzene in F344 rats, CD rats, and B6C3F1 mice. Company of publication, Research Triangle Park, NC; report number. Submitted under TSCA Section 8D; EPA Document No. 878214291; NTIS No. OTS0206507.

CIIT. (1993) Initial submission: a chronic inhalation toxicity study of nitrobenzene in B6C3F1 mice, Fischer 344 rats and Sprague-Dawley (CD) rats. Company of publication, place of publication, if available; report number. EPA Document No. FYI-OTS-0794-0970; NTIS No. OTS0000970.

Clark, MR; Shohet, SB. (1985) Red cell senescence. Clin Haematol 14:223-257.

Clayson, DB; Garner, RC. (1976) Carcinogenic aromatic amines and related compounds. In: Searle, CE, ed. Chemical carcinogens. American Chemical Society monograph 173. Washington, DC: American Chemical Society; pp. 366-461.

Cody, TE; Witherup, S; Hastings, L; et al. (1981) 1,3-Dinitrobenzene: Toxic effect in vivo and in vitro. J. Toxicol. Environ. Health. 7(5): 829-847.

Dellarco, VL; Prival, MJ. (1989) Mutagenicity of nitro compounds in *Salmonella typhimurium* in the presence of flavin mononucleotide in a preincubation assay. Environ Mol Mutagen 13:116-127.

DiSanto, AR; Wagner, JG. (1972) Pharmacokinetics of highly ionized drugs. II. Methylene blue—absorption, metabolism, and excretion in man and dog after oral administration. J Pharm Sci 61:1086-1090.

Dodd, DE; Fowler, EH; Snellings, WM; et al. (1987) Reproduction and fertility evaluations in CD rats following nitrobenzene inhalation. Fundam Appl Toxicol 8:493-505.

Dreher, D; Junod, AF. (1996) Role of oxygen free radicals in cancer development. Eur J Cancer 32A:30-38.

DuPont. (1981) Inhalation median lethal concentration (LC50) with cover letter. Company of publication, place of publication, if available; report number. Submitted under TSCA Section 8D; EPA Document No. 878220423; NTIS No. OTS0215040.

Ellenhorn, MJ; Schoenwald, S; Ordog, G, eds. (1997) Antidotes. In: Ellenhorn's medical toxicology: diagnosis and treatment of human poisoning. 2nd edition. Baltimore, MD: Williams & Wilkins; pp. 89-105.

Ellis, MK; Foster, PM. (1992) The metabolism of 1,3-dinitrobenzene by rat testicular subcellular fractions. Toxicol Lett 62:201-208.

Eyer, P. (1979) Reactions of nitrosobenzene with reduced glutathione. Chem Biol Interact 24:227-239.

Eyer, P; Ascherl, M. (1987) Reactions of para-substituted nitrosobenzenes with human hemoglobin. Biol Chem Hoppe Seyler 368:285-294.

Facchini, V; Griffiths, LA. (1981) The involvement of the gastro-intestinal microflora in nitro-compound-induced methaemoglobinaemia in rats and its relationship to nitrogroup reduction. Biochem Pharmacol 30:931-935.

Feig, DI; Reid, TM; Loeb, LA. (1994) Reactive oxygen species in tumorigenesis. Cancer Res 54:1890s-1894s.

Feldmann, RJ; Maibach, HI. (1970) Absorption of some organic compounds through the skin in man. J Invest Dermatol 54:399-404.

Finch, C. (1947) Treatment of intracellular methemoglobinemia. Bull N Engl Med Center 6:241-245.

Garner, RC; Nutman, CA. (1977) Testing of some azo dyes and their reduction products for mutagenicity using *Salmonella typhimurium* TA 1538. Mutat Res 44:9-19.

Goldfrank, L; Flomenbaum, N; Lewin, N; et al., eds. (1998) Goldfrank's Toxicologic Emergencies. 6th edition. Stamford, CT: Appleton & Lange.

Goldstein, RS; Rickert, DE. (1984) Macromolecular covalent binding of [14C]nitrobenzene in the erythrocyte and spleen of rats and mice. Chem Biol Interact 50:27-37.

Goldstein, A; Aronow, L; Kalman, SM. (1969) Principles of drug action: the basis of pharmacology. New York, NY: Harper and Row Publishers; pp. 274-452.

Gupta, G; Poddar, B; Salaria, M; et al. (2000) Acute nitrobenzene poisoning. Indian Pediatr 37:1147-1148.

Gutteridge, JM. (1995) Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin Chem 41:1819-1828.

Guyton, KZ; Kensler, TW. (1993) Oxidative mechanisms in carcinogenesis. Br Med Bull 49:523-544.

Hamilton, A. (1919) Industrial poisoning by compounds of the aromatic series. J Ind Hyg 1:200-212.

Han, C; Wang, Q; Wu, P. (2001) [A study on mechanism for cytotoxicity of nitrobenzene to hepatocarcinoma cell line]. Zhonghua Yu Fang Yi Xue Za Zhi 35:48-50.

Harada, N; Omura, T. (1980) Participation of cytochrome P-450 in the reduction of nitro compounds by rat liver microsomes. J Biochem 87:1539-1554.

Harrison, MR. (1977) Toxic methaemoglobinaemia. A case of acute nitrobenzene and aniline poisoning treated by exchange transfusion. Anaesthesia 32:270-272.

Haworth, S; Lawlor, T; Mortelmans, K; et al. (1983) Salmonella mutagenicity test results for 250 chemicals. Environ Mutagen 5(Suppl 1):1-142.

Ho, CH; Clark, BR; Guerin, MR; et al. (1981) Analytical and biological analysis of test materials from the synthetic fuel technologies. Mutat Res 85:335-345.

Holder, JW. (1999a) Nitrobenzene potential human cancer risk based on animal studies. Toxicol Ind Health 15:458-463.

Holder, JW. (1999b) Nitrobenzene carcinogenicity in animals and human hazard evaluation. Toxicol Ind Health 15:445-457.

Hong, SK; Anestis, DK; Ball, JG; et al. (2002) In vitro nephrotoxicity induced by chloronitrobenzenes in renal cortical slices from Fischer 344 rats. Toxicol Lett 129:133-141.

Hopkins, JE; Naisbitt, DJ; Humphreys, N; et al. (2005) Exposure of mice to the nitroso metabolite of sulfamethoxazole stimulates interleukin 5 production by CD4(+) T-cells. Toxicology 206:221-231.

HSDB (Hazardous Substances Data Bank). (2003) Nitrobenzene. National Library of Medicine, National Institutes of Health, U.S. Department of Health and Human Services, Bethesda, MD. Available from: <http://toxnet.nlm.nih.gov>.

Huang, Q; Wang, L; Han, S. (1995) The genotoxicity of substituted nitrobenzenes and the quantitative structure-activity relationship studies. Chemosphere 30:915-923.

Huang, QG; Kong, LR; Liu, YB; et al. (1996) Relationships between molecular structure and chromosomal aberrations in in vitro human lymphocytes induced by substituted nitrobenzenes. Bull Environ Contam Toxicol 57:349-353.

Ikeda, M; Kita, A. (1964) Excretion of *p*-nitrophenol and *p*-aminophenol in the urine of a patient exposed to nitrobenzene. Br J Ind Med 21:210-213.

IPCS (2003) Nitrobenzene (EHC 230, 2003), http://www.inchem.org/documents/ehc/ehc/ehc230.htm

IRIS (1994) Aniline (CASRN 62-53-3), IRIS, Environmental Protection Agency, http://www.epa.gov/iris/subst/0350.htm

IUBMB (International Union for Biochemistry and Molecular Biology), (2005a) EC 1.6.99.1-NADPH dehydrogenase. Available from: <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/6/99/1.html>.

IUBMB. (2005b) EC 1.6.99.3-NADH dehydrogenase. Available from: <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/6/99/3.html >.

IUBMB. (2005c) EC 1.6.5.3-NADH dehydrogenase (ubiquinone). Available from: <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/6/5/3.html>.

IUBMB. (2005d) EC 1.15.1.1-Superoxide dismutase. Available from: <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/15/1/1.html>.

IUBMB. (2005e) EC 1.11.1.9-Glutathione peroxidase. Available from: <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/11/1/9.html>.

IUBMB. (2005f) EC 1.11.1.6-Catalase. Available from: <http://www.chem.qmul.ac.uk /iubmb/enzyme/EC1/11/1/6.html>.

IUBMB. (2005g) EC 2.5.1.18-Glutathione transferase. Available from: <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC2/5/1/18.html>.

IUBMB. (2005h) EC 1.8.1.7-Glutathione-disulfide reductase. Available from: <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/8/1/7.html>.

IUBMB. (2005i) EC 1.6.2.4-NADPH—hemoprotein reductase. Available from: <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/6/2/4.html>.

IUBMB. (2005j) EC 1.6.2.2-Cytochrome-b5 reductase. Available from: <http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/6/2/2.html>.

Jaffe, ER. (1981) Methemoglobin pathophysiology. Prog Clin Biol Res 51:133-151.

Jensen-Taubman, SM; Steinberg, SM; Linnoila, RI. (1998) Bronchiolization of the alveoli in lung cancer: pathology, patterns of differentiation and oncogene expression. Int J Cancer 75: 489-496.

Kato, M; Kimura, H; Hayashi, H; et al. (1995) Sperm viability in rats treated with nitrobenzene and alpha-chlorohydrin. Teratology 52:44B.

Kawaguchi, T; Kawachi, M; Morikawa, M; et al. (2004) Key parameters of sperm motion in relation to male fertility in rats given alpha-chlorohydrin or nitrobenzene. J Toxicol Sci 29:217-231.

Kawashima, K; Usami, M; Sakemi, K; et al. (1995a) Studies on the establishment of appropriate spermatogenic endpoints for male fertility disturbance in rodent induced by drugs and chemicals. I. Nitrobenzene. J Toxicol Sci 20:15-22.

Kawashima, K; Momma, J; Takagi, A; et al. (1995b) Examination of sperm motility defects by nitrobenzene with an image processor. Teratology 52:37B.

Kawashima, K; Momma, J; Kitajima, S; et al. (1996) Sperm test using CASA (HTM-IVOS): II. Nitrobenzene and its analog. Teratology 54:41A.

KEGG. (2005) C06813. Available from: http://www.genome.ad.jp/dbget-bin/www_bget?cpd:C06813

Kensler, TW; Egner, PA; Taffe, BG; et al. (1989) Role of free radicals in tumor promotion and progression. Prog Clin Biol Res 298:233-248.

Kiese, M. (1966) The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. Pharmacol Rev 18:1091-1161.

Kim, S; Qualls, CW, Jr.; Reddy, G; et al. (1997) 1,3,5-Trinitrobenzene-induced alpha-2u-globulin nephropathy. Toxicol Pathol 25:195-201.

Kinkead, ER; Wolfe, RE; Flemming, CD; et al. (1994a) Reproductive toxicity screen of 1,3,5-trinitrobenzene administered in the diet of Sprague-Dawley rats--final report for the period September 1993 through June 1994. Available from: National Technical Information Service, Springfield, VA; AD-A298 912.

Kinkead, ER; Wolfe, RE; Salins, SA; et al. (1994b) Range-finding study for a reproductive assessment of 1,3,5-trinitrobenzene administered in the diet of Sprague-Dawley rats. Available from: National Technical Information Service, Springfield, VA; AD-A299 032.

Kinkead, ER; Wolfe, RE; Fleming, CD; et al. (1995) Reproductive toxicity screen of 1,3,5-trinitrobenzene administered in the diet of Sprague-Dawley rats. Toxicol Ind Health 11(3):309-323.

Kito, Y; Hamamatsu, Y; Naya, M. (1998) Effects of nitrobenzene on sperm motility and fertility in rats (2). Teratology 57:29A.

Kito, Y; Hamamatsu, Y; Naya, M. (1999) Application of Crj:CD(SD)IGS rats to reproductive and developmental toxicity study: effects of nitrobenzene on sperm examination. Teratology 59:39A-40A.

Kligerman, AD; Erexson, GL; Wilmer, JL; et al. (1983) Analysis of cytogenetic damage in rat lymphocytes following in vivo exposure to nitrobenzene. Toxicol Lett 18:219-226.

Koida, M; Nakagawa, T; Irimura, K; et al. (1995) Effects on the sperm and testis of rats treated with nitrobenzene: age and administration period differences. Teratology 52:39B.

Kumar, A; Chawla, R; Ahuja, S; et al. (1990) Nitrobenzene poisoning and spurious pulse oximetry. Anaesthesia 45:949-951.

Lazerev, NV; Levina, EN. (1976) *o*-, *m*-, *p*-DNB. In: Harmful substances in industry II. Leningrad, Russia: Khiimya Press; pp. 724-727. (cited in Philbert et al., 1987)

Levin, SJ. (1927) Shoe-dye poisoning—relation to methemoglobin formation. JAMA 89:2178-2180.

Levin, AA; Dent, JG. (1982) Comparison of the metabolism of nitrobenzene by hepatic microsomes and cecal microflora from Fischer 344 rats in vitro and the relative importance of each in vivo. Drug Metab Dispos 10:450-454.

Levin, AA; Bosakowski, T; Earle, LL; et al. (1988) The reversibility of nitrobenzene-induced testicular toxicity: continuous monitoring of sperm output from vasocystotomized rats. Toxicology 53:219-230.

Lewis, RJ, Sr, ed. (1992) Sax's dangerous properties of industrial materials. 8th edition. New York, NY: Van Nostrand Reinhold.

Li, H; Cheng, Y; Wang, H; et al. (2003a) Inhibition of nitrobenzene-induced DNA and hemoglobin adductions by dietary constituents. Appl Radiat Isot 58:291-298.

Li, H; Wang, H; Sun, H; et al. (2003b) Binding of nitrobenzene to hepatic DNA and hemoglobin at low doses in mice. Toxicol Lett 139:25-32.

Linder, RE; Hess, RA; Strader, LF. (1986) Testicular toxicity and infertility in male rats treated with 1,3-dinitrobenzene. J Toxicol Environ Health 19(4):477-489.

Linder, RE; Strader, LF; Slott, VL; et al. (1992) Endpoints of spermatotoxicity in the rat after short duration exposures to fourteen reproductive toxicants. Reprod Toxicol 6:491-505.

Mallouh, AA; Sarette, WO. (1993) Methemoglobinemia induced by topical hair oil. Ann Saudi Med 13:78-80.

Maples, KR; Eyer, P; Mason, RP. (1990) Aniline-, phenylhydroxylamine-, nitrosobenzene-, and nitrobenzene-induced hemoglobin thiyl free radical formation in vivo and in vitro. Mol Pharmacol 37:311-318.

Mason, RP; Holtzman, JL. (1975a) The mechanism of microsomal and mitochondrial nitroreductase. Electron spin resonance evidence for nitroaromatic free radical intermediates. Biochemistry 14:1626-1632.

Mason, RP; Holtzman, JL. (1975b) The role of catalytic superoxide formation in the O2 inhibition of nitroreductase. Biochem Biophys Res Commun 67:1267-1274.

Matsumaru, H; Yoshida, T. (1959) Experimental studies of nitrobenzol poisoning. Kyushu J Med Sci 10:259-264.

Matsuura, I; Hoshino, N; Wako, Y; et al. (1995) Sperm parameter studies on three testicular toxicants in rats. Teratology 52:39B.

Mattioli, F; Martelli, A; Gosmar, M; et al. (2006) DNA fragmentation and DNA repair synthesis induced in rat and human thyroid cells by chemicals carcinogenic to the rat thyroid. Mutat Res 609: 146-153.

McConnell, EE; Solleveld, HA; Swenberg, JA; Boorman, GA. (1986) Guidelines for combining neoplasms for evaluation of rodent carcinogenesis studies. JNCI 76: 283-289.

McLaren, TT; Foster, PM; Sharpe, RM. (1993a) Identification of stage-specific changes in protein secretion by isolated seminiferous tubules from the rat following exposure to either *m*-dinitrobenzene or nitrobenzene. Fundam Appl Toxicol 21:384-392.

McLaren, TT; Foster, PM; Sharpe, RM. (1993b) Effect of age on seminiferous tubule protein secretion and the adverse effects of testicular toxicants in the rat. Int J Androl 16:370-379.

Medinsky, MA; Irons, RD. (1985) Sex, strain, and species differences in the response of rodents to nitrobenzene vapors. In: Ricker, DE, ed. Toxicity of nitroaromatic compounds. New York, NY: Hemisphere Publishing Corporation; pp 35-51.

Miller, JL. (2002) Hemoglobin switching and modulation: genes, cells, and signals. Curr Opin Hematol 9:87-92.

Miller, BM; Adler, ID. (1990) Application of antikinetochore antibody staining (CREST staining) to micronuclei in erythrocytes induced in vivo. Mutagenesis 5:411-415.

Mirsalis, JC; Tyson, CK; Butterworth, BE. (1982) Detection of genotoxic carcinogens in the in vivo-in vitro hepatocyte DNA repair assay. Environ Mutagen 4:553-562.

Mitsumori, K; Kodama, Y; Uchida, O; et al. (1994) Confirmation study, using nitrobenzene, of the Combined Repeat Dose and Reproductive/Developmental Toxicity Test protocol proposed by the Organization for Economic Cooperation and Development (OECD). J Toxicol Sci 19:141-149.

Mochida, K; Ito, Y; Saito, K; et al. (1986) Cytotoxic effects of 1,2-dichloroethane, nitrobenzene, and carbon disulfide on human KB and monkey AGMK cells. J Pharm Sci 75:1190-1191.

Morgan, KT; Gross, EA; Lyght, O; et al. (1985) Morphologic and biochemical studies of a nitrobenzene-induced encephalopathy in rats. Neurotoxicology 6:105-116.

Morrissey, RE; Schwetz, BA; Lamb, JC, IV; et al. (1988) Evaluation of rodent sperm, vaginal cytology, and reproductive organ weight data from National Toxicology Program 13-week studies. Fundam Appl Toxicol 11: 343-358.

Myslak, Z; Piotrowski, JK; Musialowicz, E. (1971) Acute nitrobenzene poisoning. A case report with data on urinary excretion of *p*-nitrophenol and *p*-aminophenol. Arch Toxikol 28:208-213.

Narayan, L; Caldwell, DJ; Miller, CR. (1995) Alteration in neurotransmitters and their metabolite levels in 1,3,5-trinitrobenzene-treated Sprague-Dawley rats. AL/OET-TR-1995-0133. U.S. Air Force Armstrong Laboratory, Wright-Patterson AFB, OH.

Nettesheim, P; Szakal, M.S. (1972) Morphogenesis of alveolar bronchiolization. Lab Invest 26: 210-219.

Nienhuis, AW; Stamatoyannopoulos, G. (1978) Hemoglobin switching. Cell 15:307-315.

NRC (National Research Council). (1983) Risk assessment in the federal government: managing the process. Washington, DC: National Academy Press.

NTP (National Toxicology Program). (1983a) Report on the subchronic toxicity via gavage of nitrobenzene (C60082) in Fischer 344 rats and B6C3F1 mice [unpublished]. Prepared by the EG&G Mason Research Institute, Worcester, MA, for the National Toxicology Program, National Institute of Environmental Health Services, Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC; MRI-NTP 08-83-19.

NTP. (1983b) Report on the subchronic dermal toxicity of nitrobenzene (C60082) in Fischer 344 rats and B6C3F1 mice [unpublished]. Prepared by the EG&G Mason Research Institute, Worcester, MA, for the National Toxicology Program, National Institute of Environmental Health Services, Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC; MRI-NTP 06-83-13.

NTP. (2004) 11th Report on Carcinogens. Available from: http://ntp.niehs.nih.gov/ntp/roc/eleventh/profiles/s121zntb.pdf

Nystrom, DD; Rickert, DE. (1987) Metabolism and excretion of dinitrobenzenes by male Fischer 344 rats. Drug Metab Dispos 15:821-825.

Ohkuma, Y; Kawanishi, S. (1999) Oxidative DNA damage by a metabolite of carcinogenic and reproductive toxic nitrobenzene in the presence of NADH and Cu(II). Biochem Biophys Res Commun 257:555-560.

Parke, DV. (1956) Studies in detoxication. 68. The metabolism of [14C]nitrobenzene in the rabbit and guinea pig. Biochem J 62:339-346.

Parkinson, A. (2000) Chapter 6 Biotransformation of Xenobiotics, In: Casarett & Doull's Toxicology The Basic Science of Poisons, 6th Edition (Ed. Curtis D. Klaassen), pp. 133-224.

Percy, MJ; McFerran, NV; Lappin, TR. (2005) Disorders of oxidised haemoglobin. Blood Rev 19: 61-68.

Perreault, SD; Linder, RE; Strader, LF; et al. (1989) The value of multiple endpoint data in male reproductive toxicology: revelations in the rat. Prog Clin Biol Res 302:179-192.

Philbert, MA; Gray, AJ; Connors, TA. (1987) Preliminary investigations into the involvement of the intestinal microflora in CNS toxicity induced by 1,3-dinitrobenzene in male F-344 rats. Toxicol Lett 38(3):307-314.

Pinching, AJ; Doving, KB. (1974) Selective degeneration in the rat olfactory bulb following exposure to different odours. Brain Res 82:195-204.

Pinkerton, KE; Joad, JP. (2000) The mammalian respiratory system and critical windows of exposure for children's health. Environ Health Perspect 108 Suppl. 3: 457-462.

Piotrowski, J. (1967) Further investigations on the evaluation of exposure to nitrobenzene. Br J Ind Med 24:60-65.

Porter, IH; Schulze, J; McKusick, VA. (1962) Genetical linkage between the loci for glucose-6-phosphate dehydrogenase deficiency and colour-blindness in American Negroes. Ann Hum Genet 26: 107-122.

Reddy, BG; Pohl, LR; Krishna, G. (1976) The requirement of the gut flora in nitrobenzene-induced methemoglobinemia in rats. Biochem Pharmacol 25:1119-1122.

Reddy, TV; Daniel, FB; Robinson, M; et al. (1994a) Subchronic toxicity studies on 1,3,5-trinitrobenzene, 1,3-dinitrobenzene and tetryl in rats: Subchronic toxicity evaluation of 1,3,5-trinitrobenzene in Fischer 344 rats. ADA283663. Prepared by the U.S. Environmental Monitoring Systems Laboratory, Cincinnati, OH, for the U.S. Army Medical Research and Development Command, Ft. Detrick, Frederick, MD.

Reddy, TV; Daniel, FB; Robinson, M; et al. (1994b) Subchronic toxicity studies on 1,3,5-trinitrobenzene, 1,3-dinitrobenzene and tetryl in rats: 14-day toxicity evaluation of 1,3,5-trinitrobenzene in Fischer 344 rats. ADA283664. Prepared by the U.S. Environmental Monitoring Systems Laboratory, Cincinnati, OH, for the U.S. Army Medical Research and Development Command, Ft. Detrick, Frederick, MD.

Reddy, TV; J. Torsell, FB; Daniel, GR; et al. (1995) Ninety-day Toxicity Evaluation of 1,3,5-Trinitrobenzene (TNB) in Peromyscus leucopus. Second Society of Environmental Toxicology and Chemistry World Congress. November 5-9, 1995, Vancouver, British Columbia, Canada, (Abstract), p. 189.

Reddy, TV; Daniel, FB; Olson, GR; et al. (1996) Chronic toxicity studies of 1,3,5-trinitrobenzene in Fischer 344 rats. Final report. Prepared by the U.S. EPA Environmental Monitoring Systems Laboratory, Cincinnati, OH, for the U.S. Army Medical Research and Development Command, Fort Detrick, MD. NTIS No. AD-A315 216/2.

Reddy, G; Reddy, TV; Choudhury, H; et al. (1997) Assessment of Environmental Hazards of 1,3,5-trinitrobenzene. J. Toxicol. Environ. Health 52: 447-460.

Reddy, TV; Olson, GR; Wiechman, B; et al. (1998) Subchronic toxicity of 1,3,5-trinitrobenzene in Fischer 344 rats. International Journal of Toxicology 17:393-411.

Reddy, G; Reddy, TV; Daniel, FB; et al. (2000) Fourteen-day toxicity evaluation of 1,3,5-trinitrobenzene (TNB) in shrew (Cryptotis parva). Abstract, Twenty-first Meeting of the American College of Toxicology. November 12-15, 2000, San Diego, CA.

Reddy, TV; Olson, GR; Wiechman, B; et al. (2001) Chronic toxicity of 1,3,5-trinitrobenzene in Fischer 344 rats. Int. J. Toxicol. 20: 59-67.

Rice-Evans, C. (1990) Iron-mediated oxidative stress and erythrocytes. In: Harris, JR., ed. Blood cell biochemistry. New York, NY: Plenum Press; pp. 429-453.

Richburg, JH; Boekelheide, K. (1996) Mono-(2-ethylhexyl) phthalate rapidly alters both Sertoli cell vimentin filaments and germ cell apoptosis in young rat testes. Toxicol Appl Pharmacol 137:42-50.

Richburg, JH; Nañez, A. (2003) Fas- or FasL-deficient mice display an increased sensitivity to nitrobenzene-induced testicular germ cell apoptosis. Toxicol Lett 139:1-10.

Rickert, DE. (1987) Metabolism of nitroaromatic compounds. Drug Metab Rev 18:23-53.

Rickert, DE; Bond, JA; Long, RM; et al. (1983) Metabolism and excretion of nitrobenzene by rats and mice. Toxicol Appl Pharmacol 67:206-214.

Robbiano, L; Baroni, D; Carrozzino, R; et al. (2004) DNA damage and micronuclei induced in rat and human kidney cells by six chemicals carcinogenic to the rat kidney. Toxicology 204:187-195.

Robinson, D; Smith, JN; Williams, RT. (1951) Studies in detoxication. 40. The metabolism of nitrobenzene in the rabbit; *o*-, *m*- and *p*-nitrophenols, *o*-, *m*- and *p*-aminophenols and 4-nitrocatechol as metabolites of nitrobenzene. Biochem J 50:228-235.

Romero, IA; Lister, T; Richards, HK; et al. (1995) Early metabolic changes during m-dinitrobenzene neurotoxicity and the possible role of oxidative stress. Free Radic Biol Med 18:311-319.

Salice, CJ; Holdsworth, G. (2001) Wildlife toxicity assessment for 1,3,5-trinitrobenzene (1,3,5-TNB). U.S. Army Center for Health Promotion and Preventative Medicine (USACHPPM) Project Number 39-EJ1138-01B, Aberdeen Proving Ground, MD.

Salmowa, J; Piotrowski, J; Neuhorn, U. (1963) Evaluation of exposure to nitrobenzene. Absorption of nitrobenzene vapour through lungs and excretion of *p*-nitrophenol in urine. Br J Ind Med 20:41-46.

Schimelman, MA; Soler, JM; Muller, HA. (1978) Methemoglobinemia: nitrobenzene ingestion. JACEP 7:406-408.

Schuler, M; Rupa, DS; Eastmond, DA. (1997) A critical evaluation of centromeric labeling to distinguish micronuclei induced by chromosomal loss and breakage in vitro. Mutat Res 392:81-95.

Sealy, RC; Swartz, HM; Olive, PL. (1978) Electron spin resonance-spin trapping. Detection of superoxide formation during aerobic microsomal reduction of nitro-compounds. Biochem Biophys Res Commun 82:680-684.

Seger, DL. (1992) Methemoglobin-forming chemicals. In: Sullivan, JB; Krieger, GR, eds. Hazardous materials toxicology: clinical principles of environmental health. Baltimore, MD: Williams & Wilkins; pp. 800-806.

Shimizu, M; Yasui, Y; Matsumoto, N. (1983) Structural specificity of aromatic compounds with special reference to mutagenic activity in *Salmonella typhimurium*—a series of chloro- or fluoro-nitrobenzene derivatives. Mutat Res 116:217-238.

Shimkin, MB. (1939) Acute toxicity of mononitrobenzene in mice. Proc Soc Exp Biol Med 42:844-846.

Shimo, T; Onodera, H; Matsushima, Y; et al. (1994) [A 28-day repeated dose toxicity study of nitrobenzene in F344 rats]. Eisei Shikenjo Hokoku 112:71-81.

Shinoda, K; Mitsumori, K; Yasuhara, K; et al. (1998) Involvement of apoptosis in the rat germ cell degeneration induced by nitrobenzene. Arch Toxicol 72:296-302.

Stevens, A. (1928) Cyanosis in infants from nitrobenzene. JAMA 90:116.

Stevenson, A; Forbes, RP. (1942) Nitrobenzene poisoning: report of a case due to exterminator spray. J Pediat 21:224-228.

Stifel, RE. (1919) Methemoglobinemia due to poisoning by shoe dye. JAMA 72:395-396.

Styles, JA. (1978) Mammalian cell transformation in vitro. Six tests for carcinogenicity. Br J Cancer 37:931-936.

Suzuki, J; Koyama, T; Suzuki, S. (1983) Mutagenicities of mono-nitrobenzene derivatives in the presence of norharman. Mutat Res 120:105-110.

Suzuki, J; Takahashi, N; Kobayashi, Y; et al. (1987) Dependence on salmonella-typhimurium enzymes of mutagenicities of nitrobenzene and its derivatives in the presence of rat-liver S9 and norharman. Mutat Res 178:187-194.

Takahashi, T; Tanaka, M; Brannan, CI; et al. (1994) Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell 76:969-976.

Tateno, H; Iijima, S; Asaka, A; et al. (1997) Evaluation of clastogenicity of chemical agents using in vitro assay with human spermatozoa. Mutat Res 379:S89.

Tyl, RW; France, KA; Fisher, LC; et al. (1987) Development toxicity evaluation of inhaled nitrobenzene in CD rats. Fundam Appl Toxicol 8:482-492.

U.S. EPA (Environmental Protection Agency). (1986a) Guidelines for the health risk assessment of chemical mixtures. Federal Register 51(185):34014-34025.

U.S. EPA. (1986b) Guidelines for mutagenicity risk assessment. Federal Register 51(185):34006-34012.

U.S. EPA. (1988) Recommendations for and documentation of biological values for use in risk assessment. EPA 600/6-87/008. Available from: National Technical Information Service, Springfield, VA; PB88-179874/AS.

U.S. EPA. (1991a) Guidelines for developmental toxicity risk assessment. Federal Register 56(234):63798-63826.

U.S.EPA. (1991b) Alpha2u-globulin: association with chemical induced renal toxicity and neoplasia in the male rat. Risk Assessment Forum, Washington, DC; EPA/625/3-91/019F.

U.S. EPA. (1994a) Interim policy for particle size and limit concentration issues in inhalation toxicity studies. Federal Register 59(206):53799.

U.S. EPA. (1994b) Methods for derivation of inhalation reference concentrations and application of inhalation dosimetry. EPA/600/8-90/066F. Available from: National Technical Information Service, Springfield, VA; PB2000-500023, and <http://www.epa.gov/iris/backgr-d.htm>.

U.S. EPA. (1995) Use of the benchmark dose approach in health risk assessment. EPA/630/R-94/007. Available from: National Technical Information Service, Springfield, VA; PB95-213765, and <http://www.epa.gov/iris/backgr-d.htm>.

U.S. EPA. (1996) Guidelines for reproductive toxicity risk assessment. Federal Register 61(212):56274-56322.

U.S. EPA. (1998a) Guidelines for neurotoxicity risk assessment. Federal Register 63(93):26926-26954.

U.S. EPA. (1998b) Science policy council handbook: peer review. Prepared by the Office of Science Policy, Office of Research and Development, Washington, DC; EPA 100-B-98-001. Available from: National Technical Information Service, Springfield, VA; PB98-140726, and <http://www.epa.gov/iris/backgr-d.htm>.

U.S. EPA. (1998c) Assessment of Thyroid Follicular Cell Tumors. U.S. Environmental Protection Agency, Washington, DC, EPA/630/R-97/002.

U.S. EPA. (1999) Benchmark dose software (BMDS) version 1.3.2. Available from: <http://www.epa.gov/ncea/bmds.htm> (last modified May 23, 2003).

U.S. EPA. (2000a) Science policy council handbook: peer review. 2nd edition. Prepared by the Office of Science Policy, Office of Research and Development, Washington, DC; EPA 100-B-00-001. Available from: <http://www.epa.gov/iris/backgr-d.htm>.

U.S. EPA. (2000b) Science policy council handbook: risk characterization. Prepared by the Office of Science Policy, Office of Research and Development, Washington, DC; EPA 100-B-00-002. Available from: <http://www.epa.gov/iris/backgr-d.htm>.

U.S. EPA (2000c) Benchmark dose technical guidance document [external review draft]. Risk Assessment Forum, Washington, DC; EPA/630/R-00/001. Available from: <http://www.epa.gov/iris/backgr-d.htm>.

U.S. EPA. (2000d) Supplemental guidance for conducting health risk assessments of chemical mixtures. Risk Assessment Forum, Washington, DC; EPA/630/R-00/002. Available from: <http://www.epa.gov/iris/backgr-d.htm>.

U.S. EPA. (2002) A review of the reference dose and reference concentration processes. Risk Assessment Forum, Washington, DC; EPA/630/P-02/0002F. Available from: <http://www.epa.gov/iris/backgr-d.htm>.

U.S. EPA. (2005) Guidelines for carcinogen risk assessment [review draft]. Risk Assessment Forum, Washington, DC; EPA/630/P-03/001B. Available from: <http://www.epa.gov/iris/backgr-d.htm>.

Vance, WA; Levin, DE. (1984) Structural features of nitroaromatics that determine mutagenic activity in *Salmonella typhimurium*. Environ Mutagen 6:797-811.

Watanabe-Fukunaga, R; Brannan, CI; Copeland, NG; et al. (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 356:314-317.

Wentworth, P; Roy, M; Wilson, B; et al. (1999) Toxic methemoglobinemia in a 2-year-old child. Lab Med 30:311-315.

Westerman, MP; Pierce, LE; Jensen, WN. (1963) Erythrocyte lipids: a comparison of normal young and normal old populations. J Lab Clin Med 62:394-400.

WHO (World Health Organization). (2003) Nitrobenzene. Environmental health criteria 230. World Health Organization, Geneva, Switzerland.

Wood, WG. (1976) Haemoglobin synthesis during human fetal development. Br Med Bull 32:282-287.

Wulferink, M; Gonzalez, J; Goebel, C; et al. (2001) T cells ignore aniline, a prohapten, but respond to its reactive metabolites generated by phagocytes: possible implications for the pathogenesis of toxic oil syndrome. Chem Res Toxicol 14:389-397.

Zeitoun, MM. (1959) Nitrobenzene poisoning in infants due to inunction with false bitter almond oil. J Trop Pediatr 5:73-75.

Zeligs, M. (1929) Aniline and nitrobenzene poisoning in infants. Arch Pediat 46:50