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TOXICOLOGICAL REVIEW

OF

NITROBENZENE

(CAS No. 98-95-3)

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

January 2009

U.S. Environmental Protection Agency
Washington, DC

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LIST OF ACRONYMS

ADAF	age-dependent adjustment factor
AGMK	African green monkey kidney
AIC	Akaike Information Criterion
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ATSDR	Agency for Toxic Substances and Disease Registry
BBMV	brush border membrane vesicle
BMC	benchmark concentration
BMCL	95% lower bound on the BMC
BMD	benchmark dose
BMDL	95% lower bound on the BMD
BMDS	benchmark dose software
BMR	benchmark response
BRRC	Bushy Run Research Center
BUN	blood urea nitrogen
CASRN	Chemical Abstracts Service Registry Number
CIIT	Chemical Industry Institute of Toxicology
con A	concanavalin A
CREST	calcinosis, Raynaud's phenomenon, esophageal motility disorders, sclerodactyly, and telangiectasia
DMPO	5,5-dimethyl-1-pyrroline-1-oxide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EC	Enzyme Commission (only in combination with numbers [e.g., EC 1.6.99.1])
EC_{50}	median effective concentration
EPA	Environmental Protection Agency
ESR	electron spin resonance
FasL	Fas ligand
G6PD	glucose-6-phosphate dehydrogenase
GD	gestation day
GLP	good laboratory practice
GSH	reduced glutathione
Hb	hemoglobin
Hct	hematocrit
HEC	human equivalent concentration
IgG	immunoglobulin G
IgM	immunoglobulin M
i.p.	intraperitoneal
IPCS	International Programme on Chemical Safety
IRIS	Integrated Risk Information System
IUBMB	International Union of Biochemistry and Molecular Biology
IUR	inhalation unit risk
KLH	keyhole limpet hemocyanin
LOAEL	lowest-observed-adverse-effect level
LPS	lipopolysaccharide
MCHb	mean corpuscular hemoglobin
MCV	mean corpuscular volume

metHb	methemoglobin
MOA	mode of action
NAD(H)	(reduced) nicotine adenine dinucleotide
NADP(H)	(reduced) nicotinamide adenine dinucleotide phosphate
NLM	National Library of Medicine
NOAEL	no-observed-adverse-effect level
NRC	National Research Council
NTP	National Toxicology Program
OECD	Organization for Economic Cooperation and Development
oxyHb	oxyhemoglobin
PBPK	physiologically based pharmacokinetic
PHA	phytohemagglutinin
PLN	popliteal lymph node
PND	postnatal day
POD	point of departure
RBC	red blood cell
RfC	reference concentration
RfD	reference dose
RGDR	regional gas dose ratio
S9	9000 × g microsomal supernatant fraction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T3	triiodothyronine
T4	thyroxine
TSH	thyroid-stimulating hormone
UF	uncertainty factor
WBC	white blood cell

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.

The intent of Section 6, *Major Conclusions in the Characterization of Hazard and Dose Response*, is to present the major conclusions reached in the derivation of the reference dose, reference concentration and cancer assessment, where applicable, and to characterize the overall confidence in the quantitative and qualitative aspects of hazard and dose response by addressing the quality of data and related uncertainties. 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 (phone), (202) 566-1749 (fax), or hotline.iris@epa.gov (email address).

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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 oral reference dose (RfD) and inhalation reference concentration (RfC) values for chronic and other exposure durations, and a carcinogenicity assessment.

The RfD and RfC, if derived, provide quantitative information for use in risk assessments for health effects known or assumed to be produced through a nonlinear (presumed threshold) mode of action. The RfD (expressed in units of mg/kg-day) is defined as an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily 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 (expressed in units of mg/m^3) is analogous to the oral RfD, but provides a continuous inhalation exposure estimate. The inhalation RfC considers toxic effects for both the respiratory system (portal of entry) and for effects peripheral to the respiratory system (extrarespiratory or systemic effects). Reference values are generally derived for chronic exposures (up to a lifetime), but may also be derived for acute (≤24 hours), short-term (>24 hours up to 30 days), and subchronic (>30 days up to 10% of lifetime) exposure durations, all of which are derived based on an assumption of continuous exposure throughout the duration specified. Unless specified otherwise, the RfD and RfC are derived for chronic exposure duration.

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 may be derived. 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 may be derived from the application of a low-dose extrapolation procedure. If derived, the oral slope factor is a plausible upper bound on the estimate of risk per mg/kg-day of oral exposure. Similarly, an inhalation unit risk is a plausible upper bound on the estimate of risk per $\mu g/m^3$ air breathed.

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 (1983). EPA Guidelines and Risk Assessment Forum Technical Panel Reports that may have been used in the development of this assessment include the following: *Guidelines for the Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 1986a), *Guidelines for Mutagenicity Risk Assessment* (U.S. EPA, 1986b), *Recommendations for and Documentation of Biological Values for Use in Risk Assessment* (U.S. EPA, 1988), *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991a), *Interim Policy for Particle Size and Limit*

Concentration Issues in Inhalation Toxicity (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), *Guidelines for Reproductive Toxicity Risk Assessment* (U.S. EPA, 1996), *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998a), *Science Policy Council Handbook*: *Risk Characterization* (U.S. EPA, 2000a), *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2000b), *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 2000c), *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002), *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (U.S. EPA, 2005b), *Science Policy Council Handbook: Peer Review* (U.S. EPA, 2006a), and *A Framework for Assessing Health Risks of Environmental Exposures to Children* (U.S. EPA, 2006b).

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 March 2008.

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.

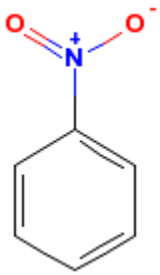

Figure 2-1. Chemical structure of nitrobenzene.

Pertinent physical and chemical properties of nitrobenzene are listed as follows (National Library of Medicine's [NLM] Hazardous Substances Data Bank, 2003; World Health Organization [IPCS], 2003; Agency for Toxic Substances and Disease Registry [ATSDR], 1990):

Chemical formula	$C_6H_5NO_2$
Molecular weight	123.11
Melting point	5.7°C
Boiling point	210.8°C
Density	1.2 g/mL (at 20°C)
Water solubility	1,900 mg/L (at 20°C)
Log K_{OW}	1.87
Log K_{OC}	1.56
Vapor pressure	0.15 mm Hg at 25°C (20 Pa at 20°C)
Henry's law constant	1.31×10^{-5} atm-m^3/mol
Conversion factor	1 ppm = 5.04 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 (IPCS, 2003). Other chemical products of nitrobenzene include benzidine, quinoline, and azobenzene (NLM, 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 (IPCS, 2003), about one-third of which was produced in the U.S. 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 (IPCS, 2003).

The majority (97–98%) of the nitrobenzene produced is retained in closed manufacturing systems. Losses to wastewater have been estimated at <2% of production (ATSDR, 1990); releases to air, land, and water via industrial processes also occur. EPA's Toxics Release Inventory reported total on- and off-site releases of nitrobenzene in 2002 as 320,836 pounds, the majority of which (~75%) were on-site disposal to Class I underground injection wells (U.S. EPA, 2004). The chemical industry reported ~89% of the nitrobenzene releases in 2002 with the largest reported in Louisiana (226,526 pounds), followed by Texas (45,480 pounds) and Nevada (31,669 pounds). Since 1988, trends data have shown annual declines in total on- and off-site releases of nitrobenzene, comprised of decreases in surface water discharges, underground injection, and releases to land; total air emissions have varied between 25,529 and 81,297 pounds, with no evident upward or downward trend.

Nitrobenzene exposure is predominantly occupational via the inhalation and/or dermal routes. For members of the general population, both inhalation (ambient air) and ingestion (drinking water) exposures are possible and are likely to be highest for those individuals living near industrial/manufacturing sources or hazardous waste sites. Potential exposure through the use of consumer products is also possible, but data are lacking to quantify these exposures.

Little or no information is available on nitrobenzene in foods. Nitrobenzene has been measured in fish samples in Japan (4 of 147 samples) at levels ranging from 11–26 μg/kg (IPCS, 2003). In a recent British study, no nitrobenzene was detected (<2 μg/kg) in 49 honey samples collected from hives fumigated with "Frow mixture" containing petroleum-derived substances in addition to nitrobenzene to treat hives against parasitic mites (Castle et al., 2004).

Nitrobenzene may be absorbed through the skin as either a liquid or a vapor and may be an additional primary route of exposure, besides inhalation, for workers in industries in which nitrobenzene is used. The general population may be exposed dermally to nitrobenzene through the use of consumer products containing nitrobenzene, but information about these potential exposures is lacking (IPCS, 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 (BBMVs) from the small intestines of Sprague-Dawley rats (in vitro). Absorption assays with isolated BBMVs 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 IPCS (2003) has 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 high levels 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 animals, the author found that nearly 70% of the radioactivity had been eliminated from the body. This included 1% of the radioactivity expired as CO_2, 0.6% expired as nitrobenzene (up to 30 hours), 58% excreted as metabolites in the urine (up to 4–5 days), and 9% eliminated in the 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 (metHb) resulting from nitrobenzene exposure. Germ-free rats do not develop methemoglobinemia 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 hemoglobin (Hb) in the blood was converted to metHb within 1–2 hours. When the same dose was administered to germ-free or antibiotic-pretreated rats, there was no measurable metHb

formation, even when measured up to 7 hours after treatment. The nitroreductase activities of various tissues (liver, kidney, gut wall) were not appreciably 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 metHb 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 nearly two-thirds of orally administered 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 by Myslak et al. (1971) discussed above. 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 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/hour 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 with 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 (percent dose per hour) over the 120-hour period was as follows: 0.022%/hour: 0–12 hours; 0.022%/hour: 12–24 hours; 0.013%/hour: 24–48 hours; 0.013%/hour: 48–72 hours; 0.011%/hour: 72–96 hours; and 0.006%/hour: 96–120 hours. Continued excretion of [^{14}C]-label at the 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 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 (1,030 ± 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 (1,024 ± 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 by 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 by 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 [^{14}C]-nitrobenzene in the erythrocytes and spleen of rats and mice. SDS-PAGE of RBC lysates from rats showed that most radioactivity coeluted with Hb. The radioactivity bound to spleen homogenates coeluted with 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.

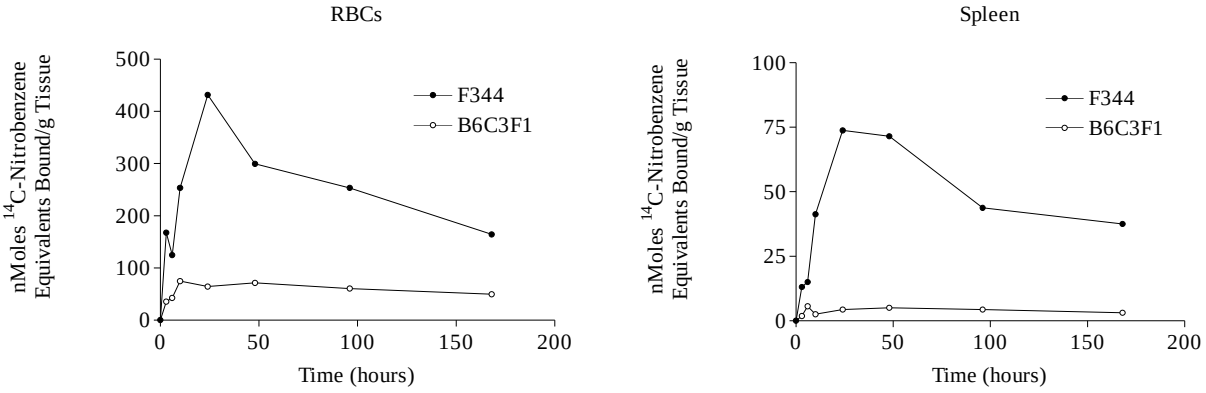

Figure 3-1. Time course of covalently bound [^{14}C]-nitrobenzene in RBCs and spleen of rats and mice.

Note: 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 of three to four

[1] MetHb (a greenish-brown to black pigment) may be formed from Hb, which is made of four globin polypeptide chains, each of which has a single heme group (iron-containing porphyrin) capable of reversibly binding one oxygen molecule. The ability of Hb to bind and transport oxygen depends on the heme valance (oxidation) state such that the ferrous iron (Fe^{+2}) in Hb may readily bind oxygen, while formation of metHb, due to loss of an electron from the heme iron (becoming Fe^{+3} or ferric iron), causes heme to lose its ability to combine reversibly with and transport oxygen (Smith, 1996). 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 et al., 1997). Additional information on metHb and methemoglobinemia, including clinical effects resulting from different metHb levels, may be found in footnote 3 and in section 4.5.1.

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

Source: Adapted from Goldstein and Rickert (1984).

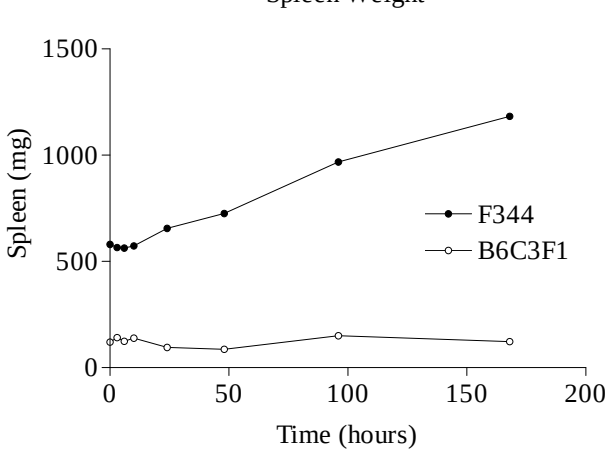

Figure 3-2. Time-related changes in spleen weight in rats and mice following nitrobenzene treatment.

Note: 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).

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.[2] 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.

[2] The metabolic competency of lung towards nitrobenzene has not been explored; however, as summarized in sections 3.3.1 and 3.3.2, studies in whole tissues, including lung, are available on nitroreductases towards the nitroaromatic drug nilutamide.

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).

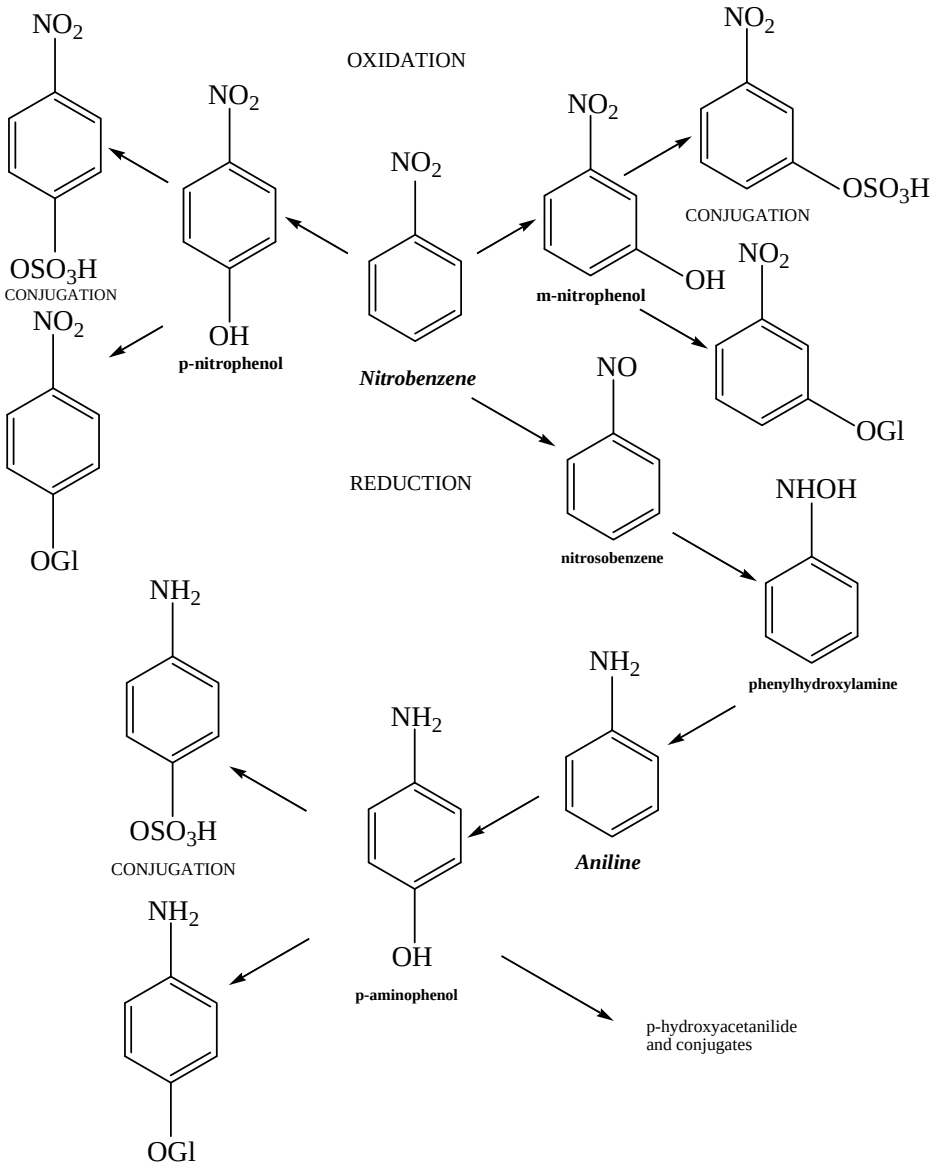

Figure 3-3. Outline of the metabolism of nitrobenzene: a substrate for oxidation and reduction reactions.

Sources: Adapted from IPCS (2003); Rickert (1987).

As illustrated by Holder (1999), intermediates in the latter process include a nitro anion free radical, nitrosobenzene, an hydronitroxide free radical, phenylhydroxylamine, 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 nitro anion 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, 1999; 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[3] 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).

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[3] Methemoglobinemia, which may be defined as a metHb concentration exceeding 2–3% of total Hb (Lee and Ferguson, 2007; Smith, 1996; Harrison, 1977), arises when the rate of metHb formation exceeds the rate of reduction of oxidized heme iron, and it can develop by three distinct mechanisms: genetic mutation resulting in the presence of abnormal Hb, a deficiency of metHb reductase enzyme, and toxin-induced oxidation of Hb. Small amounts of metHb are continually produced due to autoxidation of Hb during the normal respiratory function of loading and unloading of oxygen by erythrocytes. A variety of xenobiotics, including nitrobenzene and aromatic amines, can cause methemoglobinemia by accelerating the oxidation of Hb to metHb, which loses its ability to combine reversibly with oxygen (also see footnote 1) (Percy et al., 2005; Smith, 1996, Harrison, 1977).

In normal erythrocytes, maintenance of metHb at low levels (<1% of total Hb) is achieved by the steady reduction of metHb mainly by the NADH-dependent cytochrome b5 metHb reductase (also known as NADH-diaphorase). Chronic congenital methemoglobinemia is a rare condition that may be caused by an inherited deficiency in this enzyme, resulting in metHb levels of 15–30%, which makes these individuals particularly susceptible to metHb-generating chemicals. Also, low levels of functional enzyme render premature babies susceptible to metHb-forming chemicals. Normal erythrocytes also have another minor pathway, known as NADPH-diaphorase (or flavin reductase), which can be enhanced by administration of methylene blue (MB) as an antidote for chemically induced methemoglobinemia, where, acting as an artificial electron acceptor, MB is reduced to leukomethylen blue, which in turn reduces metHb to Hb (Coleman and Coleman, 1996). The later metHb reduction pathway requires NADPH as a cofactor, which is normally furnished by the intracellular hexose monophosphate shunt (also called pentose phosphate shunt), with glucose-6-phosphate dehydrogenase (G6PD) being a key enzyme in this multistep pathway. A deficiency in G6PD, due to a sex-linked inherited disorder among nearly 100 million people of African, Asian, or Mediterranean origin, limits the supply of intracellular NADPH, which renders affected individuals more susceptible to metHb-forming chemicals or drugs. Chemically induced methemoglobinemia in these individuals may not respond to MB administration, which, if commenced, may even exacerbate possible ongoing hemolytic anemia due to scarcity of NADPH (Bradberry, 2003; Bloom and Brandt, 2001).

Chemicals that cause methemoglobinemia vary, with some being able to oxidize hemoglobin both in vitro and in vivo (e.g., sodium nitrite and phenylhydroxylamine). Other chemicals are only active in vivo (e.g., aniline and nitrobenzene) due to requirement for enzymatic activation. A third group, typified by potassium ferricyanide, is active in Hb solutions or blood lysates but not in intact cells in vitro (Smith, 1996).

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 (Enzyme Commission [EC] 1.6.99.1, common name reduced nicotine adenine dinucleotide phosphate [NADPH] dehydrogenase).[4] This enzyme catalyzes the following general reaction: NADPH + H^+ + acceptor = $NADP^+$ + reduced acceptor (International Union of 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).

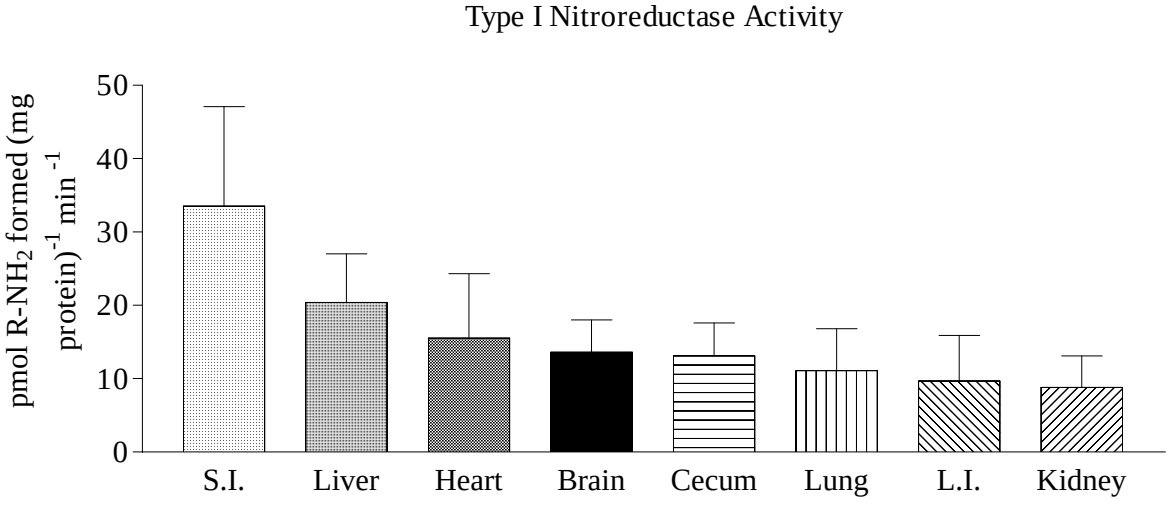


Figure 3-4. Type I nitroreductase activity in male Sprague-Dawley rats.

Note: Results are expressed as pmol of reduced nilutamide (R-NH_2) formed per milligram protein per minute (mean ± standard error of the mean; 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 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-

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

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 ± standard error of the means of determinations in three animals/group, with all determinations in triplicate.

Source: Reddy et al. (1976).

Facchini and Griffiths (1981) demonstrated that little or no metHb was formed when blood was incubated with nitrobenzene in vitro.[5] 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.

[5] While nitrobenzene is generally regarded as unable to directly oxidize Hb in whole blood cells, due to the absence of nitroreductases normally present in bacteria, mitochondria, or microsomes, one study reported metHb formation (10% above background oxidation) when human or rabbit hemolysates were incubated for 5 hours with 5 mM nitrobenzene (Kusumoto and Nakajima, 1970 [based on Vasquez et al., 1995]). Though these findings may indicate direct oxidation (i.e., no prior metabolic activation was required), it is noteworthy that several factors may distinguish this in vitro finding from whole animal studies in that the process of metHb formation here seems to be slow and it requires high concentrations of substrate, which, in itself, may have caused dissociation of the tetrameric structure of Hb and/or possibly lowered its redox potential, resulting in a more direct access and permissible electron transfer between nitrobenzene and the heme moiety.

A similar in vitro study utilizing bovine Hb (0.01–0.02 mM) and dinitrobenzene (DNB) isomers (o-, m-, or p-) (120 μM) demonstrated direct formation of metHb from deoxyhemoglobin (dxHb) but not from oxy- or carboxy-hemoglobin (Vasquez et al., 1995). The study concluded that DNB can oxidize dxHb to metHb directly without the prior metabolic activation inferred from in vivo studies; however, the exact mechanism was not known, even though lack of covalent binding of DNB to the heme or globin was considered suggestive of a direct redox reaction (electron transfer) occurring between the heme-bound iron and DNB. It should also be noted that, very likely, DNB has a higher redox potential and, therefore, a greater tendency to be reduced by Hb (resulting in direct metHb formation) than nitrobenzene.

Table 3-2. MetHb 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 ± standard error of the means, three animals/group.

Source: Facchini and Griffiths (1981).

Goldstein et al. (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 had elevated 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 ± standard error of the means of four determinations.
[b]Significantly different from AIN-76A.

Source: Goldstein et al. (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/kg nitrobenzene 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.

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 deviations for three animals/group.
[b]Significantly different from controls.

Source: Levin and Dent (1982).

Collectively, the findings of the studies by Levine and Dent (1982) and by Goldstein et al. (1984) indicate that lack of metHb formation in germ-free animals is not caused by lack of absorption or bioavailability of nitrobenzene, due for instance to intestinal physiological changes in the germ-free animals, but is rather due to absent gut bacteria that have the nitroreductases needed to activate nitrobenzene to metHb forming metabolites.

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.

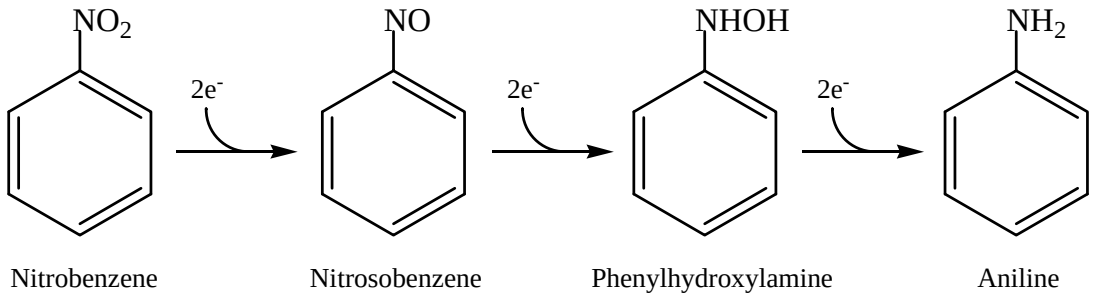


Figure 3-5. Mechanism of bacterial nitrobenzene reduction.

Source: Adapted from Holder (1999).

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).

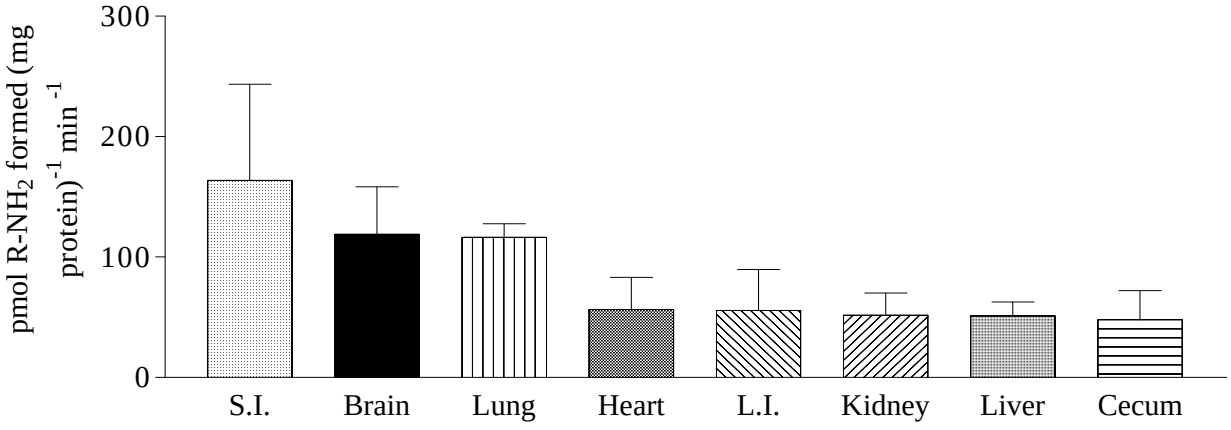


Figure 3-6. Type II nitroreductase activity of male Sprague-Dawley rats.

Note: Results are expressed as pmol of reduced nilutamide ($R\text{-}NH_2$) formed per milligram protein per minute (mean ± standard error of the mean; $n \geq 4$). S.I. = small intestine; L.I. = large intestine.

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 9,000 g 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/minute-mg protein) compared to anaerobic conditions (0.33 nmol/minute-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/minute-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 165,000 g 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 nitro anion 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 concomitant production of superoxide radical and possibly hydrogen peroxide. A metabolic chart in Holder (1999) summarizes the six one-electron reduction step process for nitrobenzene reduction (Figure 3-7).

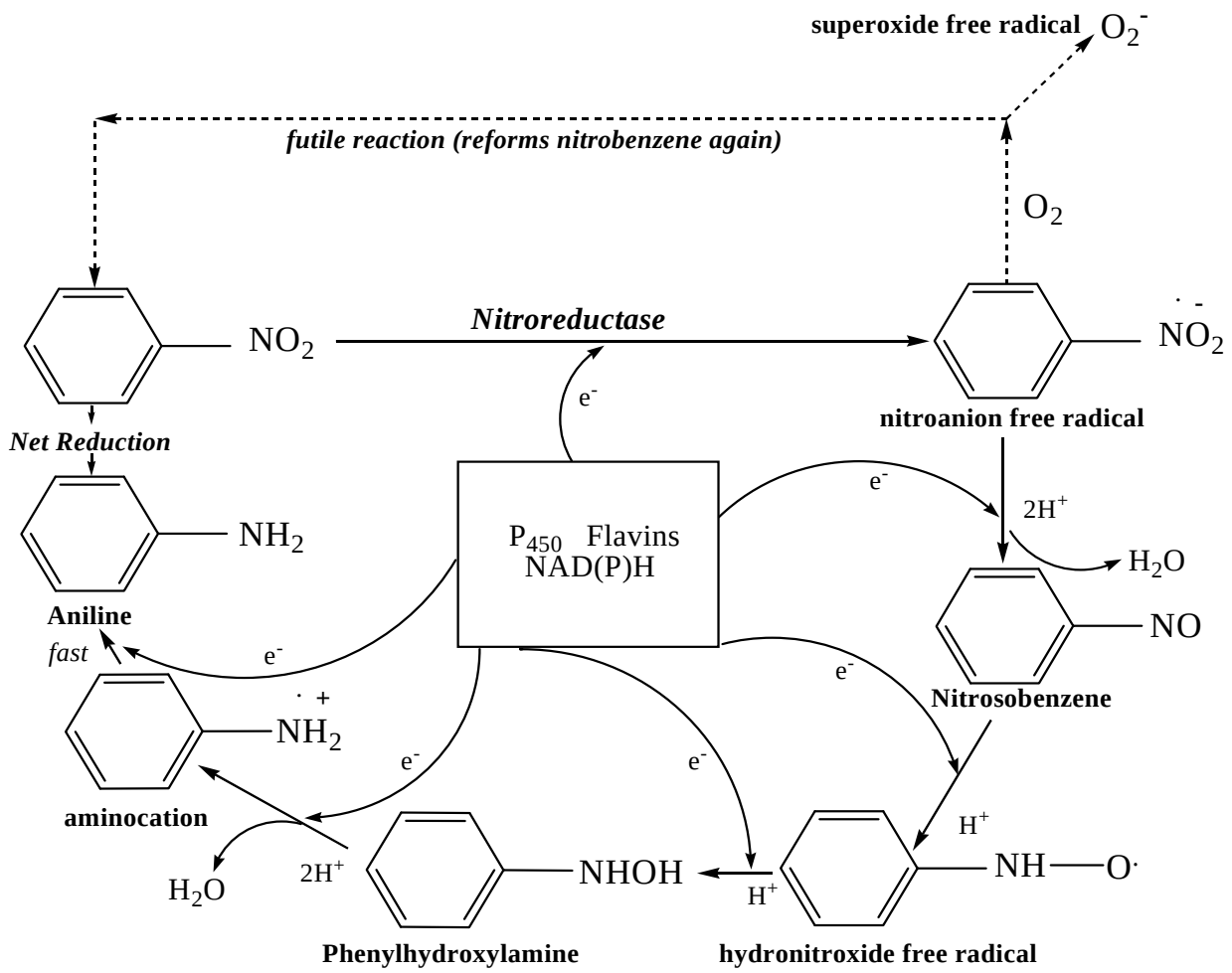


Figure 3-7. Mechanism of microsomal nitrobenzene reduction.

Source: Adapted from Holder (1999).

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 nitro anion 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).

Table 3-5. Enzyme systems in erythrocytes

Enzyme (reference)	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).

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 (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).

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, being an oxygen carrier, they are exposed uninterruptedly to high oxygen tension, RBCs have no capacity to repair damaged components, and Hb is susceptible to autoxidation while 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 and 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 cause the conversion of oxyhemoglobin (oxyHb) to metHb (Figure 3-8).

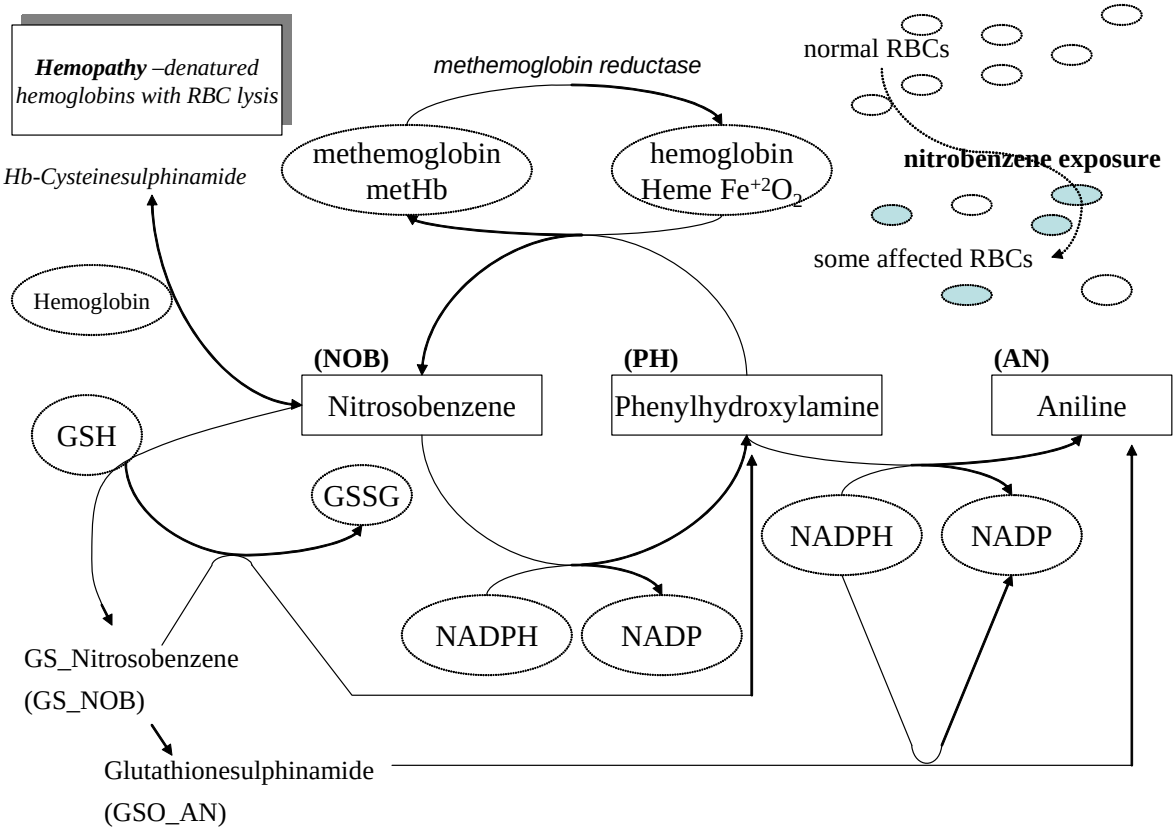


Figure 3-8. Cycling of nitrosobenzene and phenylhydroxylamine in RBCs, resulting in the formation of metHb.

Note: GSH = reduced glutathione, GSSG = oxidized glutathione; GS = glutathionyl conjugate.

Source: Adapted from Holder (1999).

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 the formation of sulfhemoglobin, the formation of an oxidized dimer of glutathione with reformation of phenylhydroxylamine, or 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 having received 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 (IPCS, 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-aminophenol 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, primarily by inhalation, in an occupational setting. 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 by Levin and Dent (1982) on nitrobenzene metabolism in rats 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%, respectively, 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 Levin 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 rat			CD rat		B6C3F1 mouse
	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)		B6C3F1 mouse (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 ± standard error of the means 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 (PBPK) 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 (see section 3).

4.1.1. Oral Exposure

Schimelman et al. (1978) reported on a 48-year-old man who was taken to an emergency room 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 respiration was shallow and irregular. Blood was obtained and was dark brown in color, and methylene blue was administered.[6] 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 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 while pipetting that apparently contained nitrobenzene. The time between ingestion and hospital admission was approximately 1.5–2 hours. On examination, the patient was unconscious, his

[6] Therapeutic interventions for methemoglobinemia include the administration of redox scavengers, with ascorbic acid and/or methylene blue (MB). 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. MB (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, MB reduces the half-life of metHb from 15–20 hours to 40–90 minutes. MB acts as a cofactor to increase erythrocyte reduction of metHb to oxyHb in the presence of NADPH, utilizing the hexose monophosphate shunt pathway. The MB 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 mothballs 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 it was 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 2 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 and 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. 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.

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.

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, intravenous 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 breath, 82% methemoglobinemia	Gastric lavage, 2% thionine in glucose intravenous, oxygen, blood transfusions	Myslak et al. (1971)

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, or 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 *O. 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 intravenous, 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 intravenous, 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 (NTP, 1983a) 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. 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[7] 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 hematologic 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 levels. 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. There was no apparent autolysis among animals that were found dead (all in the 150 mg/kg-day dose group); tissues from these animals were also examined microscopically.

Nine male and three female rats at the 150 mg/kg-day dose level died prior to study completion. The earliest deaths in the 150 mg/kg-day dose male and female rats were at day 67 (week 10) and day 38 (week 6), respectively. In the same group, six males also died on day 73 (week 11) and two more died on day 88 (week 13), while another female rat died on each of days 45 (week 7) and 60 (week 9). 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

[7] Reticulocytes are immature RBCs that are unable to carry and deliver oxygen. Reticulocyte counts are expressed as a percentage of circulating RBCs, which, if increased, may be considered adverse because it may signify loss of RBCs due, for instance, to hemolytic anemia.

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 ± standard deviation)[a]					
	Liver		Kidney		Testis	
	Absolute (mg)	Relative ($\times 10^{-2}$)	Absolute (mg)	Relative ($\times 10^{-3}$)	Absolute (mg)	Relative ($\times 10^{-3}$)
0	11,668 ± 1,309	3.52 ± 0.22	1,025 ± 108	3.10 ± 0.2	1,435 ± 96	4.34 ± 0.26
9.38	13,269 ± 1,555[b]	4.04 ± 0.2[b]	1,085 ± 142	3.30 ± 0.2[b]	1,435 ± 79	4.39 ± 0.35
18.75	14,567 ± 1,168[b]	4.37 ± 0.14[b]	1,115 ± 83	3.36 ± 0.1[b]	1,425 ± 104	4.30 ± 0.23
37.5	15,451 ± 1,327[b]	4.77 ± 0.22[b]	1,070 ± 153	3.30 ± 0.38	1,406 ± 71	4.33 ± 0.15
75	15,679 ± 2,117[b]	5.15 ± 0.15[b]	1,083 ± 104	3.44 ± 0.23[b]	873 ± 476[b]	2.78 ± 1.42[b]
150	11,264	4.79	1,023	4.35	835	3.55

[a]n = 10 in all groups except the 150 mg/kg-day group with one surviving male.
[b]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 ± standard deviation)[a]			
	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[b]	3.76 ± 0.08[b]	615 ± 47	3.13 ± 0.24
18.75	7481 ± 702[b]	3.95 ± 0.18[b]	627 ± 41	3.32 ± 0.14[b]
37.5	8436 ± 587[b]	4.23 ± 0.18[b]	644 ± 52[b]	3.24 ± 0.30
75	9198 ± 713[b]	4.88 ± 0.22[b]	641 ± 68[b]	3.39 ± 0.25[b]
150	9925 ± 436[b]	5.21 ± 0.28[b]	666 ± 40[b]	3.49 ± 0.12[b]

[a]n = 10 in all groups except the 150 mg/kg-day group with 7 surviving females.
[b]Significantly different from control values, as calculated by the authors.

Source: NTP (1983a).

There were statistically significant changes in some hematologic 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. Hematologic 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 ± standard deviations, where n = 10 in each group except for the 150 mg/kg-day group with one male.
[b]Significantly different from controls, as calculated by the authors.

Source: NTP (1983a).

Table 4-6. Hematologic 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,c]	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 ± standard deviations, where n = 10 in each group except for the 150 mg/kg-day group with 7 females.
[b]Significantly different from controls, as calculated by the authors.
[c]Statistics based on nine rats.

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.[8] Splenic corpuscles were small, and the red pulp contained hemosiderin.[9] The severity of splenic congestion was graded by the study authors. Control rats had no splenic congestion or minimal splenic congestion (grade 1). Congestion increased in severity up to moderate in the highest dose group. The incidence of these and other histopathologic lesions in relation to dose is shown in Tables 4-7 and 4-8. Tables 4-7 and 4-8 also report the incidence of splenic congestion of grade 2 or higher.

Table 4-7. Selected histopathology findings 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[a]
Spleen						
Congestion	1/10	4/10	7/10	6/10	10/10	10/10
Congestion ≥ grade 2	0/10	0/10	0/10	0/10	5/10	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

[a]Includes tissue findings in nine rats that died between days 67 and 88.

Source: NTP (1983a).

[8] Splenic congestion is an abnormality that leads to elevated splenic vein pressure, which in turn results in higher sinusoidal pressure, and is commonly observed in laboratory animals in response to a variety of circumstances, including agonal death, method of euthanasia, or exposure to chemicals. Administration to rodents of aromatic amine-type chemicals (e.g., aniline) may cause splenic congestion and hemorrhage, which are accompanied by hemosiderin deposition (brown intracellular pigmentation due to insoluble iron), fatty change, and extramedullary hematopoiesis and fibrosis. These changes have been suggested to result from methemoglobinemia or accumulation in erythrocytes of toxic metabolites that are released in the spleen when RBCs are broken down in the red pulp. Sustained congestion causes the spleen to become more firm, enlarged, and fibrotic and renders the organ susceptible to trauma. Spleen enlargement in humans may be caused by a variety of diseases and, in some instances, is associated with increased workload (such as in hemolytic anemia) or hyperfunction in response to destruction of abnormal RBCs, with symptoms of abdominal pain and early satiety (Greaves, 2007; Cotran et al., 1994).

[9] The red pulp (also called splenic pulp), which may act as a reservoir for storing blood, is a soft mass of dark reddish-brown color resembling coagulated blood, and it is made of a fine reticulum of fibers divided into splenic sinuses and splenic cords. The splenic red pulp may undergo changes due to a variety of factors, including immune stimulation, changes in circulation, accumulation of macrophages, and connective tissue or pigment, and in response to increased demand for filtration of abnormal RBCs (Greaves, 2007; Guyton and Hall, 2000).

Table 4-8. Selected histopathology findings 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[a]
Spleen						
Congestion	2/10	5/10	10/10	10/10	10/10	10/10
Congestion ≥ grade 2	0/10	1/10	3/10	5/10	8/10	9/10
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

[a]Includes tissue findings in three rats that died between days 38 and 60.

Source: NTP (1983a).

It should be noted that the recorded histopathology lesions in the high-dose male and female rats included the findings from animals that died prior to the full 90-day study duration (days 67–88 in males and days 38–60 in females). 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.

Based on the changes in absolute and relative organ weights and the dose-dependent increases in reticulocyte count and metHb concentration, all of which were evident at the lowest administered dose, a lowest-observed-adverse-effect level (LOAEL) of 9.38 mg/kg-day is identified for the subchronic oral effects of nitrobenzene in F344 rats in this study.

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 evident 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)	n	Organ weights in mg (mean ± standard deviation)					
		Liver		Kidney		Testis	
		Absolute (mg)	Relative ($\times 10^{-2}$)	Absolute (mg)	Relative ($\times 10^{-3}$)	Absolute (mg)	Relative ($\times 10^{-3}$)
0	10	1527 ± 286	4.71 ± 0.44	272 ± 35	8.44 ± 0.39	116 ± 7.9	3.66 ± 0.60
18.75	10	1597 ± 137	4.78 ± 0.27	276 ± 23	8.27 ± 0.45	111 ± 12	3.32 ± 0.34
37.5	10	1591 ± 129	4.74 ± 0.33	288 ± 22	8.59 ± 0.52	120 ± 8.3[b]	3.60 ± 0.30[b]
75	9	1709 ± 245	5.02 ± 0.51	300 ± 19[a]	8.84 ± 0.30[a]	113 ± 9.7	3.35 ± 0.31
150	10	1871 ± 172[a]	5.49 ± 0.33[a]	294 ± 20	8.61 ± 0.31	113 ± 16	3.33 ± 0.52
300	7	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.
[b]Summary statistics reflect 9 samples, not 10.

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)	n	Organ weights (mean ± standard deviation)					
		Liver		Kidney		Thymus	
		Absolute (mg)	Relative ($\times 10^{-2}$)	Absolute (mg)	Relative ($\times 10^{-3}$)	Absolute (mg)	Relative ($\times 10^{-3}$)
0	9	1179 ± 58	4.41 ± 0.22	175 ± 14	6.53 ± 0.27	44.14 ± 7.82	1.65 ± 0.26
18.75	9	1278 ± 113[a]	4.64 ± 0.32	179 ± 22	6.52 ± 0.71	51.22 ± 9.94	1.87 ± 0.39
37.5	10	1276 ± 74[a]	4.79 ± 0.32[a]	180 ± 11	6.74 ± 0.46	47.06 ± 9.47	1.76 ± 0.35
75	10	1256 ± 75[a]	4.69 ± 0.19[a]	166 ± 15	6.19 ± 0.44	50.41 ± 8.97	1.89 ± 0.38
150	10	1374 ± 51[a]	5.05 ± 0.14[a]	181 ± 17	6.65 ± 0.56	47.21 ± 13.2	1.73 ± 0.46
300	10	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).

Hematologic 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 RBCs. These changes are documented in Tables 4-11 and 4-12.

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

Dose (mg/kg-day)	n	Hb (g/dL)[a]	Hct (%)[a]	RBCs ($\times 10^6$)[a]	Reticulocytes (%)[a]	MetHb (%)[a]
0	10	15.20 ± 0.66	41.77 ± 2.29	9.27 ± 0.75	5.02 ± 1.0	1.07 ± 0.32
18.75	10	14.59 ± 0.66	39.76 ± 2.89	8.87 ± 0.50	5.81 ± 0.88[c]	2.16 ± 0.32[b,c]
37.5	10	15.02 ± 0.92	41.13 ± 3.48	9.17 ± 0.76	6.95 ± 0.82[b,c]	3.42 ± 0.61[b,c]
75	9	14.63 ± 0.35[b]	39.56 ± 2.66	8.68 ± 0.52	7.85 ± 0.74[b]	4.75 ± 1.03[b]
150	10	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	7	15.45 ± 0.52[d]	36.26 ± 3.30[b,d]	7.79 ± 0.29[b,e]	10.45 ± 1.58[b]	6.72 ± 1.28[b]

[a]Values are means ± standard deviations.
[b]Significantly different from controls, as calculated by the authors.
[c]Summary statistics represent nine samples.
[d]Summary statistics represent six samples.
[e]Summary statistics represent five samples.

Source: NTP (1983a).

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

Dose (mg/kg-day)	n	Hb (g/dL)[a]	Hct (%)[a]	RBCs ($\times 10^6$)[a]	Reticulocytes (%)[a]	MetHb (%)[a]
0	9	15.66 ± 0.61	44.33 ± 3.41	9.54 ± 0.67	4.17 ± 0.35	0.87 ± 0.23
18.75	9	15.70 ± 0.60	44.24 ± 2.32	9.52 ± 0.35	5.54 ± 0.51[b]	1.20 ± 0.22[b]
37.5	10	15.24 ± 0.83	43.86 ± 2.30	9.21 ± 0.60	6.29 ± 0.61[b]	1.45 ± 0.34[b]
75	10	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	10	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	10	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 ± standard deviations.
[b]Significantly different from 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. Selected histopathology findings 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 cells	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. Selected histopathology findings 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 significantly different 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 hematologic 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 hematologic parameters were evident in nitrobenzene-treated rats, with dose-dependent reductions in RBC count, Hct, and 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. Hematologic 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[d] (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[d] (IU/L)	111± 14	81 ± 6[b]	86 ± 6[b]	105 ± 17	89 ± 9	94 ± 10
ALT[d] (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 ± standard deviations for six animals/group, except for the 125 mg/kg-day female group with five animals. The limited information available did not clarify the disposition of the additional female that apparently started on study in the 125 mg/kg-day group.
[b]$p < 0.01$ versus controls, as calculated by the authors.
[c]$p < 0.05$ versus controls, as calculated by the authors.
[d]BUN = blood urea nitrogen; AST = aspartate aminotransferase; ALT = alanine aminotransferase.

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 statistically 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 animals (125 mg/kg) 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 since the average value was only 14% higher than in 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 with the testis in males. A strong dose-dependent increase in absolute spleen weight was observed with males and females with a nearly fourfold 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 those in 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 statistically significantly reduced (70% ↓) in high-dose males and remained reduced by 46% at the end of the recovery period.

Histopathologic 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 two and moderate in three 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 five males and two females and exhibited no change in one male and three females. Brown pigmentation in Kupffer cells was moderate in five males and four females and severe in one male and one female. Absolute kidney weights in males were inconsistent with the histopathologic finding. In the highest dose group, brown pigmentation in tubular epithelium was reported as moderate in five animals and severe in one animal, but no change in absolute kidney weight was reported at the highest dose. 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, and a 3% increase in absolute kidney weight was also observed at the highest dose. All other animals were consistent with controls (no change). 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[b,c] (mg/kg-day)	LOAEL[b,c] (mg/kg-day)	Reference
Rat, F344	6/sex	0, 5, 25, 125 mg/kg-day, gavage, 4 wk	RBC ↓, Hb ↓, 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 ↑	NA	9.4 (M, F)	NTP (1983a)
			Kidney weight↑	NA (M) 9.4 (F)	9.4 (M) 18.8 (F)	
			MetHb ↑ & Hb ↓	NA	9.4 (M, F)	
			Reticulocytes ↑	9.4 (M) NA (F)	18.8 (M) 9.4 (F)	
Mouse, B6C3F1	10/sex	0, 18.8, 37.5, 75, 150, 300 mg/kg-day gavage, 90 d	Liver weight ↑	75 (M) NA (F)	150 (M) 18.8 (F)	
			MetHb ↑	NA (M, F)	18.8 (M, F)	
			Hb ↓	37.5 (M, F)	75 (M, F)	
			Reticulocytes ↑	18.8 (M) NA (F)	37.5 (M) 18.8 (F)	
			Liver cytomegaly	150 (M, F)	300 (M, F)	

[a]Only endpoints with evident dose responses were selected. ↓ or ↑ = decrease or increase in the respective endpoint.
[b]No-observed-adverse-effect levels (NOAELs) and LOAELs determined by nitrobenzene assessment authors.
[c]M = male; F = female; NA = not applicable.

4.2.1.2. *Chronic Studies*

No studies were identified that addressed the chronic toxicity of nitrobenzene 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 and then sacrificed following an i.p. injection with pentobarbital. Samples of blood were taken to measure hematologic 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 hematologic 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 hematologic or clinical chemistry parameters were the increased concentrations of serum metHb (Table 4-17) and a 50% increase 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

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

[a]Values are means ± standard deviations, where n = 5 except for the 16 ppm F344 rat female group with 4 animals.
[b]$p < 0.05$, 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 male 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 (9/9) males, compared with 7/9 high-concentration 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[b,c] (ppm)	LOAEL[b,c] (ppm)	Reference
Rat, F344	10/sex	0, 5, 16, 50 ppm, 6 hr/d, 5 d/wk, 90 d	Methemoglobinemia ↑	NA (M) 5 (F)	5 (M) 16 (F)	CIIT (1984)
			Organ weight ↑	5 (M, F)	16 (M, F)	
			Splenic congestion ↑	NA (M, F)	5 (M, F)	
			Testicular pathology ↑	NA	5	
Rat, CD[c]			Methemoglobinemia ↑	5 (M) 16(F)	16 (M) 50 (F)	
			Spleen weight ↑	16 (M) 5 (F)	50 (M) 16 (F)	
			Splenic congestion ↑	NA	5 (M, F)	
			Liver weight ↑	5 (M, F)	16 (M, F)	
			Testicular pathology ↑	5 (M)	16 (M)	
Mouse, B6C3F1			Methemoglobinemia ↑	16 (M, F)	50 (M, F)	
			Splenic congestion ↑	5 (M) NA (F)	16 (M) 5 (F)	

[a]Only endpoints with evident dose responses were selected. ↑ = an increase in the respective endpoint.
[b]No-observed-adverse-effect levels (NOAELs) and LOAELs determined by nitrobenzene assessment authors.
[c]M = male; F = female; CD = Sprague-Dawley; NA = not applicable.

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 hematologic parameters. For the scheduled interim and final sacrifices, animals were fasted overnight, weighed, and then anesthetized using an i.p. injection of pentobarbital prior to exsanguination. Mice were evaluated at study termination but not at 15 months. Among the hematologic parameters evaluated were WBC counts, RBC counts, Hb, Hct, MCV, mean corpuscular Hb (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 probability of surviving to term in the control groups was 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). The probability of survival was not statistically significantly affected by exposure to nitrobenzene since the actual percentage of mice available during final euthanasia was 55.7, 62.9, 65.7, and 68.6% among males and 44.3, 54.3, 64.3, and 47.1% among females in the control, 5, 25, and 50 ppm nitrobenzene exposure groups, respectively. After rejecting autolyzed specimens from animals that were found dead, all available tissues, including those from animals that were sacrificed in a moribund state, were fixed and evaluated microscopically. The numbers of examined lungs in mice were 68/70, 67/70, 65/70, and 66/70 in males and 53/70, 60/70, 64/70, and 62/70 in females of the 0, 5, 25, and 50 ppm nitrobenzene exposure groups, respectively. Other target organs in mice or rats also had similar or identical numbers of tissues examined per group as specified here for the mouse lung (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 hematologic 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 may indicate a compensatory response to metHb formation.[10] It should also be pointed out that background metHb levels in both strains of control rats were consistently higher at 24 months than at 15 months, resulting in apparently less pronounced relative changes at 24 months than at 15 months among exposed animals (Table 4-20). Furthermore, metHb levels were much lower among control rats in the 90-day inhalation or gavage studies (Tables 4-5, 4-6, and 4-17) than among control rats at 15 or 24 months (Table 4-20). Collectively, these findings may

[10] There is no known information in the literature on a specific possible compensatory response to methemoglobinemia following extended exposure to metHb-forming chemicals. However, two enzyme systems, one in the liver and the other in erythrocytes, may help attenuate metHb formation. Recently, Kurian et al. (2006, 2004) described a liver microsomal reductive pathway in human liver, known as NADH cytochrome b5 reductase, that metabolizes and eliminates arylhydroxylamines, which are known to be rodent carcinogens and may be linked to some human tumors. According to these studies, interindividual variability in expression of this enzyme system is thought to account for cancer susceptibility to arylhydroxylamines. Though not explored in these studies, it is possible that the same liver enzyme system may also partake indirectly in attenuating methemoglobinemia by reducing phenylhydroxylamine (PHA) to aniline, thereby disallowing PHA from undergoing oxidation to nitrosobenzene along with the concomitant conversion of Hb to metHb (Figure 3-8). In another study on a common drug (dapsone), a similar enzyme seems to play a key role in mitigating methemoglobinemia by catalyzing the reduction of the hydroxylamine back to dapsone (Tingle et al., 1997). According to the report by Kurian et al. (2006), a similar or identical soluble enzyme system to the one reported in their studies on arylhydroxylamines is expressed in erythrocytes to maintain Hb in its reduced state (Hultquist and Passon, 1971). This erythrocyte enzyme system is likely NADH-dependent cytochrome b5 methemoglobin reductase that regenerates hemoglobin from metHb as described earlier (footnote 2).

It can be hypothesized that both the liver and erythrocyte NADH-cytochrome b5 reductases are likely critical in attenuating methemoglobinemia. On the one hand, the hepatic enzyme may help keep down the levels of PHA, a key metHb-forming metabolite of nitrobenzene, while on the other hand, metHb is reduced to hemoglobin by the erythrocytic enzyme. While this may apply to healthy individuals, others with congenital deficiency in these enzymes may suffer from worsening methemoglobinemia over extended exposure to nitrobenzene (refer to footnote 2 and section 4.7.3).

indicate a correlation between metHb and age of rats. However, in a 24-months dietary feeding chronic toxicity study of aniline (CIIT, 1982), metHb levels were variable among control F344 rats during the course of the study with no discernible correlation with age. In this study, control levels (% metHb) at 26, 52, 78, and 104 weeks were 1.08, 1.87, 0,96, and 1.39 in males and 2.06, 1.12, 1.95, and 2.72 in females, respectively (CIIT, 1982). Among the high-dose animals (100 mg/kg-day), the levels of metHb were 236, 129, 245, and 261% higher than among male controls and 143, 146, 122, and 111% higher than among female controls at the respective intervals. Some of the variations may be age-dependent changes, but it is also likely, at least partly, that they are a consequence of study-to-study variation or are due to other artifacts. Nonetheless, though it is hard to pinpoint a compensatory response, it can be concluded that methemoglobinemia did not worsen as a function of exposure time to nitrobenzene.

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] Nonneoplastic ♂ A significantly positive nitrobenzene exposure-related trend in incidence for A/B hyperplasia and bronchiolization was observed.[b] Nonneoplastic ♀ A significantly positive nitrobenzene exposure-related trend in incidence for bronchiolization was observed.[b]
Thyroid: follicular cell adenoma	M	Neoplastic ♂ Significantly positive nitrobenzene exposure-related trend in incidence.[b] Nonneoplastic ♂ A significantly positive nitrobenzene exposure-related trend in incidence for follicular cell hyperplasia was observed.[b] Nonneoplastic ♀ A significantly positive nitrobenzene exposure-related trend in incidence for follicular cell hyperplasia was observed.[b]
Mammary gland	F	Neoplastic ♀ Statistically significant difference in incidence for 50 ppm group versus controls when interim sacrifice animals included[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] Nonneoplastic ♂ A significantly positive trend in incidence of centrilobular hepatocytomegaly and multinucleated hepatocytes was observed.[b]
F344/N rat		
Liver: hepatocellular adenoma or carcinoma	M	Neoplastic ♂ Significantly positive nitrobenzene exposure-related trend in incidence.[b] Nonneoplastic ♂ A significantly positive trend in incidence of eosinophilic foci and centrilobular hepatocytomegaly was observed.[b]

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]
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] Nonneoplastic ♂ A significantly positive trend in incidence of tubular hyperplasia was observed.[b]
Endometrial stromal polyp	F	Nonneoplastic ♀ Significantly positive nitrobenzene exposure-related trend in incidence.[b]
Sprague-Dawley rat		
Liver: hepatocellular adenoma or carcinoma	M	Neoplastic ♂ Significantly positive nitrobenzene exposure-related trend in incidence[b] Nonneoplastic ♂ A significantly positive trend in incidence of eosinophilic foci and centrilobular hepatocytomegaly was observed.[b]

[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's exact test, $p < 0.05$, as calculated by the study authors.

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

In mice, RBCs and Hct were statistically significantly lower in 50 ppm males than in controls (8.70 ± 0.12 versus 9.61 ± 0.29 × 10^6 cells/μL and 41.64 ± 0.52 versus 45.06 ± 1.15%, respectively). In common with the rats, 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. An increased incidence of focal inflammation and hypertrophy of the submucosal glands in areas lined by respiratory epithelium was observed in the nasal region of

high-exposure male and female F344 rats. In CD rats, exposure-related lesions in nasal sections consisted of a slight increase in the incidence and severity of inflammatory changes in the anterior section of the nose. 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 with 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 with 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, 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 nonneoplastic lesions included lung, olfactory epithelium, and, in the males, thyroid follicular cells and hepatocytes (Table 4-22). Histopathologic endpoints for the lung included hyperplasia and alveolar bronchiolization.[11] 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 increased at all exposure levels (male 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 statistically significant increases in the number of animals

[11] According to Nettesheim and Szakal (1972), bronchiolization of alveoli are lesions that are thought to arise from the ‘‘colonization’’ of alveolar walls with bronchiolar epithelium either via cell migration through alveolar pores or from the transformation (metaplasia) of alveolar type II cells into bronchiolar-type epithelium. The pathology summary in the nitrobenzene study characterized bronchiolization of the alveolar walls as “a pronounced change in the alveolar epithelium in the region of the terminal bronchioles from a simple squamous to tall columnar epithelium resembling that of the terminal bronchioles” (CIIT, 1993). Section 4.5.2 has additional details on bronchiolization of alveoli in the nitrobenzene study and in other studies of animals exposed to other agents as well as a discussion of the relevance of this finding to humans.

presenting with pigmentation and degeneration of the olfactory epithelium in the nasal region. 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 (male mice: control, 1%; 5 ppm, 2%; 25 ppm, 49%; and 50 ppm, 62%; female mice: control, 0%; 5 ppm, 32%; 25 ppm, 75%; and 50 ppm, 69%). Lesions noted in nasal sections increased in severity with increasing dose (CIIT, 1993); however, severity scores were not reported. A differential response was observed between male and female mice with histopathologic 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. Selected noncancer histopathologic changes in rats as a result of exposure to nitrobenzene via inhalation for 2 years

Target tissue	Exposure concentration (ppm)							
	Males				Females			
	0	1	5	25	0	1	5	25
F344 rats								
Liver								
Eosinophilic foci	26/69	25/69	44/70[a]	57/70[a]	6/70	9/66	13/66	16/70[a]
Centrilobular hepatocytomegaly	0/69	0/69	8/70[a]	57/70[a]	0/70	0/66	0/66	0/70
Spongiosis hepatic	25/69	24/69	33/70	58/70[a]	0/70	0/66	0/66	6/70[a]
Kidney								
Tubular hyperplasia	2/69	2/68	2/70	13/70[a]	0/70	0/66	2/66	2/70
Nose								
Pigmented olfactory epithelium	40/67	53/67	67/70	68/69[a]	37/67	54/65	60/65	66/66[a]
Spleen								
Pigmentation	55/69	63/69	64/70	70/70[a]	62/69	61/66	60/66	68/69[a]
CD rats								
Liver								
Centrilobular hepatocytomegaly	3/63	1/67	14/70[a]	39/65[a]				
Spongiosis hepatic	25/63	25/67	25/70	37/65[a]				
Nose								
Pigmented olfactory epithelium	42/63	49/64	60/66	58/61[a]				
Testis								
Bilateral atrophy	11/62	17/66	22/70	35/61[a]				
Epididymis								
Bilateral hypospermia	8/60	13/65	15/67	32/59[a]				

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

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

Table 4-22. Selected noncancer histopathologic changes in B6C3F1 mice as a result of exposure to nitrobenzene via inhalation for 2 years

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

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

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

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 at terminal sacrifice

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

[a]Only endpoints with evident dose responses were selected. ↑ = increase in the respective endpoint.
[b]No-observed-adverse-effect levels (NOAELs) and LOAELs determined by nitrobenzene assessment authors.
[c]M = male; F = female; CD = Sprague-Dawley.

4.2.3. Dermal Exposure

4.2.3.1. *Subchronic Studies*

NTP sponsored a 90-day skin painting toxicological study (NTP, 1983b) with nitrobenzene in F344 rats and B6C3F1 mice. 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. At 800 mg/kg-day, all rats and 9/10 male and 8/10 female mice 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 in 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

Target tissue	Dose (mg/kg-day)					
	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[b,c] (mg/kg-day)	LOAEL[b,c] (mg/kg-day)	Reference
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			Splenic hematopoiesis ↑ Testicular pathology ↑ Mortality ↑	100 (M, F) 200 (M) NA	200 (M, F) 400 (M) 800 (M, F)	

[a]Only endpoints with evident dose responses were selected. ↑ = an increase in the respective endpoint.
[b]No-observed-adverse-effect levels (NOAELs) and LOAELs determined by nitrobenzene assessment authors.
[c]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. Large 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 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 (Ban et al., 2001), nitrobenzene was used as one of several recognized testicular toxicants to evaluate the utility of different parameters in sperm motion analysis. 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–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 hematologic 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 onward. 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 hematologic parameters, including decreases in RBCs, Hb, and Hct and increases in metHb, MCHb, WBCs, reticulocytes, and erythroblasts. For a number of these parameters, statistically significant differences from 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. Hematologic 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]
MCHb (pg)	17.1 ± 0.4	17.5 ± 0.5	20.8 ± 0.8[b]	24.5 ± 1.0[b]
Reticulocytes (per 1,000 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 ± standard deviations.
[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).

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

Organ[a]	Dose (mg/kg-day)			
	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 grams per 100 grams body weight, means ± standard deviations.
[b]$p < 0.01$ 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.

Among the reproductive/developmental parameters that were evaluated, there were no statistically significant differences from controls in the copulation and fertility indices at any dose level. However, among the dams, only two of nine pregnant females in the high-dose group survived to term, with the subsequent deaths of the two survivors (and their reduced litters) occurring on days 1 and 3 of lactation. In the remaining offspring, pup body weights were statistically significantly decreased at day 0 for both males and females by approximately 10% in the 60 mg/kg-day group. At day 4, body weights in male pups were statistically significantly decreased by about 5% in the 20 mg/kg-day group and by about 25% in both male and female pups in the 60 mg/kg-day group.

A synopsis of no-observed-adverse-effect levels (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 reproductive study with nitrobenzene

Species, strain	Number	Dosing	Effect[a]	NOAEL[b] (mg/kg-day)	LOAEL[b] (mg/kg-day)
Rat, Sprague-Dawley	10/sex	0, 20, 60, 100 mg/kg-day, gavage, up to 54 d	Organ weights ↑. Testicular pathology ↑. Copulation, fertility. Developmental toxicity: day 4 male pup body weights ↓. Mortality.	NA 20 100 NA 60 (M, F_0)	20 (M, F_0) 60 (M, F_0) NA 20 100 (M, F_0)

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

Source: Mitsumori et al. (1994).

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 toxicant. 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 0, 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 have 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 follicle-stimulating hormone 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 deoxyribonucleic acid (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[12] 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 (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 epididymis 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,

[12] Paracrine signaling involves communication between cell "A" releasing a signal and nearby cell "B" receiving the signal. Autocrine signaling involves the release of a signal by cell "A" that is received within cell "A."

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, although 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. The incidence of skeletal variations also did not indicate fetal toxicity. The single exception was a statistically 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 via inhalation 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 hours/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 from the 40 ppm nitrobenzene group 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 sexes, 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 F0, F1, 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 the authors' 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 7–19 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 the 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[b,c] (ppm)	LOAEL[b,c] (ppm)	Reference
Rat, S-D[c]	26 pregnant	0, 1, 10, 40 ppm, 6 hr/d, GDs 6–15, sacrifice on GD 21	Fertility ↓	40	NA	Tyl et al. (1987)
			Skull non-ossification ↑	10	40	
Rat, S-D	30/sex two-generation	0, 1, 10, 40 ppm, 10 wk before mating & through mating, gestation	Testicular pathology ↑	10 (M, F_1)	40 (M, F_1)	BRRC (1985); Dodd et al. (1987)
			Fertility ↓	10 (M, F_1)	40 (M, F_1)	
			Developmental toxicity	40	NA	
Rabbit, New Zealand	12 (22) pregnant	0, 10, 40, 80 (100) ppm, 6 hr/d, GDs 7–19, sacrifice on GD 20 (30)	Fertility ↓	80 (100)	NA	Biodynamics Inc. (1984, 1983)
			Developmental toxicity	80(100)	NA	

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

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 hematologic parameters was monitored, along with such clinical chemistry parameters as the activities of alkaline phosphatase, glutamate pyruvate transaminase, and 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 onward, 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 hematologic responses, the mid- and high-concentration animals displayed statistically significant reductions in Hb concentration and RBC count, while the platelet count, MCV, and 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 osmolarity, 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 toward 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 large 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 in the high-exposure groups either died or were moribund prior to the end of the exposure period. A total of 24 organs and tissues were examined for signs of gross lesions, and the spleen, left kidney, liver, testes, and brain were weighed. Hematologic 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-concentration (10 ppm) groups. Decreased testis weights were observed in the high-concentration (125 ppm) 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 Medinsky and Irons (1985), 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 on 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 blood of rats exposed to nitrobenzene vapors

Group	F344 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: 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 short-term inhalation studies with nitrobenzene

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

[a]Only endpoints with evident dose responses were selected. ↓ or ↑ = decrease or increase in the respective endpoint.
[b]NOAELs and LOAELs determined by nitrobenzene assessment authors.
[c]NA = not applicable.

Few data are available for the oral median lethal dose for nitrobenzene, although Lewis (1992) reported a value of 590 mg/kg in mice. NLM (2003) gives values of 600–640 mg/kg nitrobenzene in rats. DuPont (1981) reported a 4-hour median lethal concentration 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. 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 study (NTP, 1983b) with nitrobenzene in F344 rats and B6C3F1 mice. 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, statistically 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.

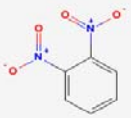
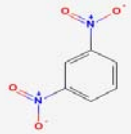
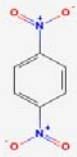
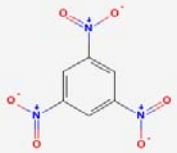
Because of the method of application, the applied dose was unknown. Treatment-related clinical signs, morbidity and mortality, along with associated evidence of incipient methemoglobinemia and other hematologic perturbations, were observed. One hour after application, 15/18 female C3H mice were in partial 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 hematologic 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 (5,000 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 susceptible 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 morphologic changes.

4.4.2. Structure-Activity Relationships

Nitroaromatic compounds related to nitrobenzene include 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 to 1,3-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
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 Cody et al. (1981).
[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, Reddy et al. (1996) 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 weeks	0.54	1.1	Reduced spermatid head count
			NA	0.54	Large reduction in reproductive performance (pups/litter)
Philbert et al. (1987)	Rat (F344)	5 days	NA	20	Ataxia in all male rats
Reddy et al. (1994a)	Rat (F344)	90 days	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 days	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 weeks	NA	4.72	Splenomegaly, fluctuation in Hb levels, atrophy and histopathologic lesions of the testes
Cody et al. (1981)	Rat (Carworth Farms)	16 weeks	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. (2001, 1996)	Rat (F344)	2 years	2.68	13.31	Methemoglobinemia, spleen erythroid cell hyperplasia, decreased body weight
Reddy et al. (1998, 1994a)	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- 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. (1995, 1994a)	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
Narayanan 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	Hematologic 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 hematologic 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 animals receiving 300 mg/kg). 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 mice receiving 300 mg/kg versus 1.03 ± 0.9% in controls). MetHb was not evaluated.

Burns et al. (1994) also observed some treatment-related changes in clinical chemistry parameters, including a dose-dependent increase in the activity of AST (80 ± 9 IU/mL in controls versus 128 ± 16 IU/mL in high-dose mice) and ALT (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 hematologic 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 those 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 by 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°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, nitrobenzene-receiving mice (39.4 ± 1.8 in controls versus 55.3 ± 1.7 in high-dose animals) showed a dose-dependent increase in particle uptake into the livers. 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 were control animals. However, a challenge with 6×10^3 *L. monocytogenes* per 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 resistance 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 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 hematologic 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 statistically 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%, weight/volume, 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 pinprick 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 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 nitrite 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 (up to 4.5% above control) that became statistically significant in the 25 mg/kg group, and absolute brain weights in female rats followed a similar trend that resulted in statistically 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-day for 14 consecutive days. Neurotoxicity was manifest in the 300 mg/kg-day 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 multicenter 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 9000 × g liver 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). Furthermore, several studies from different laboratories (Assmann et al., 1997; Dellarco and Prival, 1989; 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).

In 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, 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 no 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).

The six-step, one-electron-per-step transfer 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. Further work may be needed to see if these in vitro findings are relevant in whole animal or tissue systems and if a mechanism like this could play a role in organ-specific carcinogenesis by nitrobenzene.

Bonacker et al. (2004) recently demonstrated the induction of micronuclei in V79 hamster lung fibroblast cells following exposure to nitrobenzene possibly by affecting tubulin assembly and the spindle apparatus. To further delineate the mechanism by which the micronuclei were formed, the authors used primary syndrome of calcinosis, Raynaud's phenomenon, esophageal motility disorders, sclerodactyly, and telangiectasia (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°C) and disassembly (at 4°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 effect on the gliding velocity of microtubules with a minimal degree of inhibition above 7.5 μM to complete inhibition at 30 μM (Bonacker et al., 2004).

Li et al. (2003a, b), using the ultrasensitive 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 hours 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 (0.125–0.5 mM) and human kidney cells (0.062–0.25 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 mode of action (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 eightfold 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 a single dose of nitrobenzene (310 mg/kg, by mouth) 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, 1998b).

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	TA 98, TA 100, TA 1535, TA 1537	–/–	Haworth et al. (1983)	Reverse mutations
	TA 98, TA 98NR, TA 100, TA 100NR, TA 97a, TA 1535, TA 1537, TA 1537NR, TA 1538	–/ND	Vance and Levin (1984)	
	TA 98, TA 100, TA 1535, TA 1538	ND/–	Anderson and Styles (1978)	
	TA 98, TA 100	–/–	Assmann et al. (1997)	
	TA 98, TA 100	–/ND	Chiu et al. (1978)	
	TA 98, TA 100, TA 1535, TA 1537, TA 1538	–/–	Shimizu et al. (1983)	
	TA 98	ND/–	Ho et al. (1981)	
	TA 1538	–/–	Garner and Nutman (1977)	
	TA 98, TA 100	ND/–	Dellarco and Prival (1989)	
	TA 98, TA 100 TA 98[c] TA 100[c] TA 98NR[c]	–/– +/– –/– –/–	Suzuki et al. (1987, 1983)	Positive in the presence of norharman as comutagen
Mammalian cells in vitro				
Human lymphocytes		+	Huang et al. (1996, 1995)	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		+	Mattioli 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
Sprague-Dawley rats	Primary rat kidney cells	+	Robbiano et al. (2004)	DNA damage and MN
Male Sprague-Dawley rats		+	Mattioli et al. (2006)	DNA damage and UDS

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

Test system	Cell/strain	Result[a] (+/– S9)	Reference	Comments[b]
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]CA = chromosomal aberration; MN = micronuclei; UDS = unscheduled DNA synthesis; SCE = sister chromatid exchange.
[c]In the 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 or 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 partial protection from nitrobenzene-induced cell death. The authors concluded that nitrobenzene causes cellular damage by reactive oxygen species and that nitrobenzene was a non-genotoxic 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} or median effective concentration) was calculated to be 42 and 30 µg/mL in KB and AGMK cells, respectively.

4.5. SYNTHESIS OF MAJOR NONCANCER EFFECTS

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 hematologic 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 histopathologic 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 (Morgan et al., 1985; Bond et al., 1981). 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 susceptible to developing histopathologic lesions in the nasal passages and lungs compared with male and female F344 rats (CIIT, 1993). A summary of the MOA 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 (1999) 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).

Small amounts of methemoglobin are continually produced due to autoxidation of hemoglobin during the normal respiratory function of loading and unloading of oxygen by erythrocytes. Under normal conditions, the level of metHb in RBCs is kept below 1% of total Hb (Harrison, 1977); however, a normal range in healthy humans is generally considered to be 0–3% (Lee and Ferguson, 2007). (See footnotes 1 and 3 under section 3 for additional

information on metHb and methemoglobinemia.) Tissue hypoxia may develop due to the presence of excessive amounts of metHb, which is incapable of transporting oxygen in the body. 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).

There appears to be a progression of incrementally more severe symptoms in humans with increasing metHb concentration. Most patients tolerate low levels (<10%) of metHb fairly well despite appearance of cyanosis (bluish color) of lips and ears at levels as low as 6% in those with light complexions; however, some otherwise normal patients may experience difficulty tolerating metHb levels between 10 and 15% (Coleman and Coleman, 1996). In general, a metHb level of 20% may be considered too high for a patient to be considered asymptomatic, although patient discomfort may largely depend on the rapidity with which metHb accumulates. Fatigue, dyspnea, headache, nausea, and tachycardia may occur at metHb levels of 30 to 50%, while lethargy, stupor, and deteriorating consciousness occur as levels approach 55%. Higher levels may cause cardiac arrhythmia, circulatory failure, and neurological depression, while levels at or above 70% are usually fatal (Coleman and Coleman, 1996).

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. Hematologic 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 statistically significantly different in

the 110 mg/kg and higher dose groups. An increase in metHb in response to orally administered nitrobenzene in Sprague-Dawley rats 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-day). These findings contrast with those of Burns et al. (1994), who, while reporting a number of hematologic 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 (IPCS, 2003).

Of particular note in the NTP (1983a) study of nitrobenzene, the F344 rats experienced significant mortality at a dose that also produced a 12–13% increase in metHb levels. It is not clear what process led to these deaths. Further support for mortality being a nitrobenzene-related effect at comparatively low metHb levels was seen in the Mitsumori et al. (1994) study, with 20% of the male Sprague-Dawley rats administered 100 mg/kg-day nitrobenzene dying (within 35 days of exposure) at an average metHb level of 6.8% in the surviving rats and 100% of the pregnant female rats in the 100 mg/kg-day group dying by 41 days of exposure (metHb not measured).

Closely related to the formation of metHb in nitrobenzene-treated rodents (especially rats) is the range of changes induced in other hematologic 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 hematologic 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 4-29). Burns et al. (1994) documented a similar suite of hematologic 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 morphologic 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 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 two-electron 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 one-electron additions, with the resultant formation of the nitro anion free radical. As depicted by Holder (1999), the nitro anion free radical can be further reduced in RBCs to nitrosobenzene and phenylhydroxylamine, both of which participate in the formation of metHb. However, the nitro anion 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, measured at the interim sacrifice (15 months). This suggests that male Sprague-Dawley rats may form metHb more readily than F344 rats of either sex, male B6C3F1 mice, or female B6C3F1 mice, for which 1, 25, and 5 ppm, respectively, are NOAELs for metHb measured at study termination (24 months) and for metHb in F344 rats at interim sacrifice. MetHb levels in mice were not measured at 15 months. This study also reported bronchiolization of the alveoli in both male and female B6C3F1 mice. Unlike the systemic effects, this portal-of-entry effect was detectable in ≥87% of mice at the lowest dose tested (5 ppm) and nearly 100% of animals at 50 ppm. Bronchiolization of the alveoli was not detectable in controls. Pulmonary effects have also been observed in subchronic inhalation studies in both F344 rats and B6C3F1 mice (CIIT, 1984). In male F344 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.

According to Nettesheim and Szakal (1972), bronchiolization of alveoli are lesions that may arise from the "colonization" of alveolar walls with bronchiolar epithelium either via cell migration through alveolar pores or from the transformation (metaplasia) of alveolar type II cells into bronchiolar-type epithelium. The pathology summary in CIIT (1993) characterized bronchiolization of the alveolar walls as "a pronounced change in the alveolar epithelium in the region of the terminal bronchioles from a simple squamous to tall columnar epithelium

resembling that of the terminal bronchioles" (CIIT, 1993). Bronchiolization was dose related in severity such that this epithelial change tended to be located almost entirely in the region of the terminal bronchioles in animals of the low-dose exposure group, while the lesions were more florid and involved a large proportion of the lung parenchyma in mice that were exposed to the mid or high concentration of nitrobenzene. In contrast, alveolar/bronchiolar hyperplasia was characterized by a more discrete lesion than that described for bronchiolization of the alveolar walls. In hyperplasia "the epithelium involved was similar in appearance but tended to proliferate into the alveolar spaces in multiple fronds, forming a space-occupying lesion which did not exhibit compression of adjacent tissues" (CIIT, 1993).

Alveolar bronchiolization does not appear to be a pre-neoplastic event or a prerequisite to lung neoplasia (Cattley et al., 1994; CIIT, 1993). While incidences of alveolar bronchiolization were high among all nitrobenzene-exposed male and female mice, only male mice had increased incidences of combined adenomas and carcinomas of the lungs. Furthermore, there were no incidences of alveolar bronchiolization among nonexposed control mice, but incidences of combined adenomas and carcinomas of the lungs were 19 and 13% in the control males and females, respectively. Therefore, there is no apparent association in the nitrobenzene carcinogenicity study between findings of lung neoplasia and bronchiolization in mice. Similar findings and conclusions were derived from another study following a lifetime exposure to p-nitrotoluene (NTP, 2002).

In an NTP dietary feeding study of p-nitrotoluene (at 0, 1,250, 2,500, and 5,000 ppm), the incidence and severity of alveolar bronchiolization was increased dose dependently in mice (males: 0/50, 20/50, 30/50, and 48/50; females: 0/50, 33/50, 41/50, and 49/50) but not in rats. Also, the combined incidence of alveolar/bronchiolar adenoma or carcinoma was significantly increased in the 5,000 ppm male mice and exceeded the historical control range while female mice had no increased lung neoplasia. Bronchiolization was considered a metaplastic change and not a pre-neoplastic lesion based on lack of relationship between incidences of lung neoplasia and bronchiolization (NTP, 2002).

Alveolar bronchiolization does not seem to be mouse specific nor does it seem to be tightly linked to lung neoplasia (as for instance being a pre-neoplastic event) since the same pathology has been described in other species, including humans, in the absence or presence of lung neoplasia. In rats or mice, continued inhalation of low levels of toxic oxidant gases, such as ozone or synthetic smog, or exposure to particulate irritants, such as silica or calcium chromate, may result in bronchiolization of alveoli (Friemann et al., 1999; Muhle et al., 1995; Pinkerton et al., 1993; Nettesheim and Szakal, 1972). In several instances, bronchiolization was evidenced in the absence of lung neoplastic or pre-neoplastic changes (Friemann et al., 1999; Pinkerton et al., 1993; Nettesheim and Szakal, 1972). In addition to bronchiolization, increased incidences of bronchoalveolar hyperplasia and lung tumors were also found in rats (Muhle et al., 1995).

Nonetheless, being a cancer precursor, per se, does not necessarily disqualify an endpoint from being used to develop an RfC.

The findings of another study indicate that alveolar bronchiolization may not be dependent on hyperplasia or DNA proliferation following a single intratracheal instillation of quartz in rats (Friemann et al., 1999). Bronchiolization, described as an outgrowth of bronchiolar epithelium to alveolar walls, was increased (more than 30-fold) at 12 and 18 months (but not at 3 or 6 months) after a single quartz instillation. In contrast, bronchoalveolar hyperplasia was increased between 6 and 12 months but not at 18 months, while DNA proliferation was increased (without achieving statistical significance) throughout the 18 months and seemed to peak at 12 months, followed by a drop at 18 months. One of the conclusions in this study was that "alveolar bronchiolization was not regularly associated topographically with the development of fibroproliferative lesions, which is usually observed after exposure to chrysotile asbestos" (Friemann et al., 1999).

Bronchiolization of alveoli was also found in bleomycin-induced pulmonary fibrosis in baboons (Collins et al., 1982) and in human lung cancer biopsies (Jensen-Taubman et al., 1998) as well as in cancer-free patients with idiopathic pulmonary fibrosis/usual interstitial pneumonia (Chilosi et al., 2003). While most biopsied tissues came from lung cancer patients (specifically, non-small-cell lung cancer), some specimens were from non-lung cancer patients with findings of alveolar bronchiolization in 12 and 8% of the specimens, respectively (Jensen-Taubman et al., 1998).

In addition to bronchiolization of the alveoli in mice in the 2-year inhalation bioassay by CIIT (1993), nitrobenzene also resulted in dose-related changes in the nasal passages in mice and rats. In male and female mice, nitrobenzene caused an increased incidence of olfactory epithelial degeneration at all exposure concentrations in female mice and at mid and high concentrations in male mice. CIIT (1993) noted that these changes were consistent with degenerative changes in the olfactory bulbs of rats following inhalation of nitrobenzene as described by Beauchamp et al. (1982). In the CIIT (1993) inhalation study in rats, olfactory changes in the nasal passages in both F344 and CD rat strains were less marked than in mice, consisting of an increased incidence of focal inflammation and hypertrophy of the submucosal glands in areas lined by respiratory epithelium in high-exposure F344 rats, a slight treatment-related increase in the incidence and severity of inflammatory changes in the anterior section of the nose in CD rats, and pigment accumulation within the olfactory region in both rat strains.

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 hematologic 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. Short-term inhalation studies of nitrobenzene toxicity in experimental animals also have resulted in metHb formation (Medinsky and Irons, 1985; CIIT, 1984).

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, 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 Inc., 1984, 1983).

In the 2-year and 90-day inhalation studies of nitrobenzene (Cattley et al., 1994; CIIT 1993, 1984), nonneoplastic lesions of the liver included both morphologic 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 the 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 nitro anion 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 nitro anion 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 (nitro anion, 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 variations 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 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 (IPCS, 2003; Holder, 1999). 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-per-step transfer 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; however, the bulk of experimental evidence from genotoxicity assays has provided negative results. 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).

4.6. EVALUATION OF CARCINOGENICITY

4.6.1. Summary of Overall Weight of Evidence

According to the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), nitrobenzene is characterized as "likely to be carcinogenic to humans" for any route of exposure. 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 kidney tumors in male F344 rats, 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 incidences at the highest dose were elevated statistically significantly compared to controls. 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, 2005a) 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; however, there is no experimental evidence supporting oxidative stress as a carcinogenic MOA (refer to section 4.6.3).

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 statistically significant 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, statistically significant increasing trends 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, 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 gland, adenocarcinoma	0/30 (0%)	Not evaluated	Not evaluated	2/34[b] (6%)
	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 tumor incidences in this table displayed statistically significant ($p < 0.05$), dose-related trends in the Cochran-Armitage test.

[b]The incidence among all high-dose female mice (including interim sacrifice at 15 months) was statistically significantly higher than in the control group (5/60 [10%] versus 0/48, respectively), while the incidences between these two groups at the terminal sacrifice only (in table) were not statistically significantly different.

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. The observations of hepatocellular neoplasia in rats strengthen the relevance of the animal studies to humans because the spontaneous incidence of hepatocellular neoplasia was lower in F344 or CD rats than in B6C3F1 mice and the exposure concentration of nitrobenzene was lower for rats than for mice. Neoplastic effects were also observed 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 EPA's technical reports on *Assessment of Thyroid Follicular Cell Tumors* (U.S. EPA, 1998b) and *Alpha$_{2u}$-Globulin: Association with Chemically Induced Renal Toxicity and Neoplasia in the Male Rat* (U.S. EPA, 1991b) to make this determination. Other evidence that supports the classification of nitrobenzene as a likely human carcinogen is the known carcinogenicity of aniline, a metabolite of nitrobenzene (U.S. EPA, 1994c). A recent study 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; however, further research will be needed to characterize the DNA adducts and their toxicological relevance.

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. 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 data gap.

4.6.3. Mode-of-Action Information

Based on the studies discussed in section 4.4.5, 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.

Using a weight-of-evidence approach of the mutagenicity study findings, a mutagenic MOA is not considered a significant contributor to the carcinogenic potential of nitrobenzene. As discussed in section 3.3, nitrobenzene undergoes reductive and oxidative metabolism, including generation of free radicals (e.g., nitro anion and superoxide) and propagation of redox cycling. It is possible that tumors may arise from oxidative stress resulting from nitrobenzene metabolism if the cellular defenses are overwhelmed as proposed recently by Hsu et al. (2007). Under oxidative stress conditions, there may be several possible scenarios by which reactive chemical species (including oxygen radicals) could facilitate tumor development, including direct DNA oxidative damage, lipid peroxidation, protein damage (including DNA repair enzymes), or modulation of DNA methylation (Halliwell, 2007). However, there is no experimental evidence linking any of these processes to nitrobenzene exposure and tumor formation. Also lacking are actual studies and information on the status of the in situ antioxidant defenses, especially under similar nitrobenzene exposure conditions that gave rise to tumors. Demonstrating a correlation among exposure to nitrobenzene, status of antioxidant defenses, and changes in specific toxicity endpoints characteristic of oxidative stress would be critical to establishing a link between nitrobenzene-induced carcinogenicity and oxidative stress.

Other possible MOAs by which nitrobenzene may cause tumors, including cytotoxicity followed by increased cell proliferation resulting in promotion of initiated cells, formation of DNA adducts, or disruption of intercellular communication, also remain unexplored. Due to the paucity of information on the MOA of carcinogenicity, the MOA framework described in the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a) has not been applied, the observed tumors are deemed relevant to humans and a linear low-dose extrapolation as a default option is applied.

The thyroid and kidney tumors observed in experimental animals may be suggestive of rodent-specific MOAs; however, as discussed below, experimental data required by EPA's guidance for excluding these tumors are lacking (U.S. EPA, 1998b; U.S. EPA, 1991b). Tumors of the rodent thyroid may develop by the following hypothesized MOA. A sustained increase in conjugating enzymes alters the hypothalamic-pituitary-thyroid axis by increasing the clearance of T4 (via glucuronidation) and T3 (via sulfation) in rodents, which causes a compensatory increase in circulating TSH and ultimately follicular cell activation (U.S. EPA, 1998b). Since the levels of T3 and T4 are tightly regulated in humans, chemicals that cause tumors of the thyroid via this MOA are not relevant to humans. However, specific data are not available to support this MOA, such as studies determining the effects of nitrobenzene on circulating blood levels of TSH, T4, and T3. Since these data are not available for nitrobenzene, the thyroid tumors are considered relevant for assessing carcinogenic risk to humans (U.S. EPA, 1998b). Similarly, tubule tumors of the male rat kidney are hypothesized to occur by the following MOA. After chronic exposure to some chemicals, α_{2u}-globulin-induced 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 toxicity observed in rodents via this MOA is not relevant for assessing human risk. However, relevant data are not available for nitrobenzene.

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 ("Hb 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 (G6PD) 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 generally negative, metHb levels were not examined in the offspring (BRRC, 1985, 1984; Biodynamics Inc., 1984, 1983). Hence, uncertainty exists as to the susceptibility of the test species' Hb 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 metHb. There are at least two inherited diseases that affect an organism's susceptibility to metHb formation (Goldstein et al., 1969). First, genetic deficiency of NADH cytochrome b_5 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 G6PD deficiency (see section 4.7.1), 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., 1962). Within the U.S., 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 (RfD)

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 (BMD), with uncertainty factors (UFs) generally applied to reflect limitations of the data used.

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

There are no lifetime nitrobenzene exposure studies by the oral route, and no human studies other than case reports of accidental nitrobenzene poisonings are known. Of the animal studies of oral exposure to nitrobenzene, the 90-day gavage study (10 animals/dose/sex) conducted by NTP (1983a) is the most relevant study for deriving an RfD for nitrobenzene, because it is the longest duration study available and used several dose levels. Several other studies are available but are less suitable for developing a reference value (e.g., reproductive toxicity studies using a one-time administration or a single dose level (Kawashima et al., 1995a, b; Levin et al., 1988; Bond et al., 1981) or relatively short duration (Koida et al., 1995; Matsuura et al., 1995). A 28-day toxicity study (Shimo et al., 1994), a reproductive study (Mitsumori et al., 1994), and an immunotoxicology study (Burns et al., 1994) were also considered, as summarized below.

When a well-characterized PBPK model is available, route-to-route extrapolation from a suitable chronic inhalation study can inform the oral database. As described in sections 4.2.2.2 and 5.2, there is a 2-year inhalation bioassay (Cattley et al., 1994) (see section 5.3). However, the absence of well-characterized PBPK modeling for nitrobenzene precludes a route-to-route extrapolation to derive an RfD.

Figure 5-1 is an exposure-response array that presents NOAELs, LOAELs, and the dose range tested corresponding to selected health effects observed in relevant subchronic and reproductive oral toxicity studies.

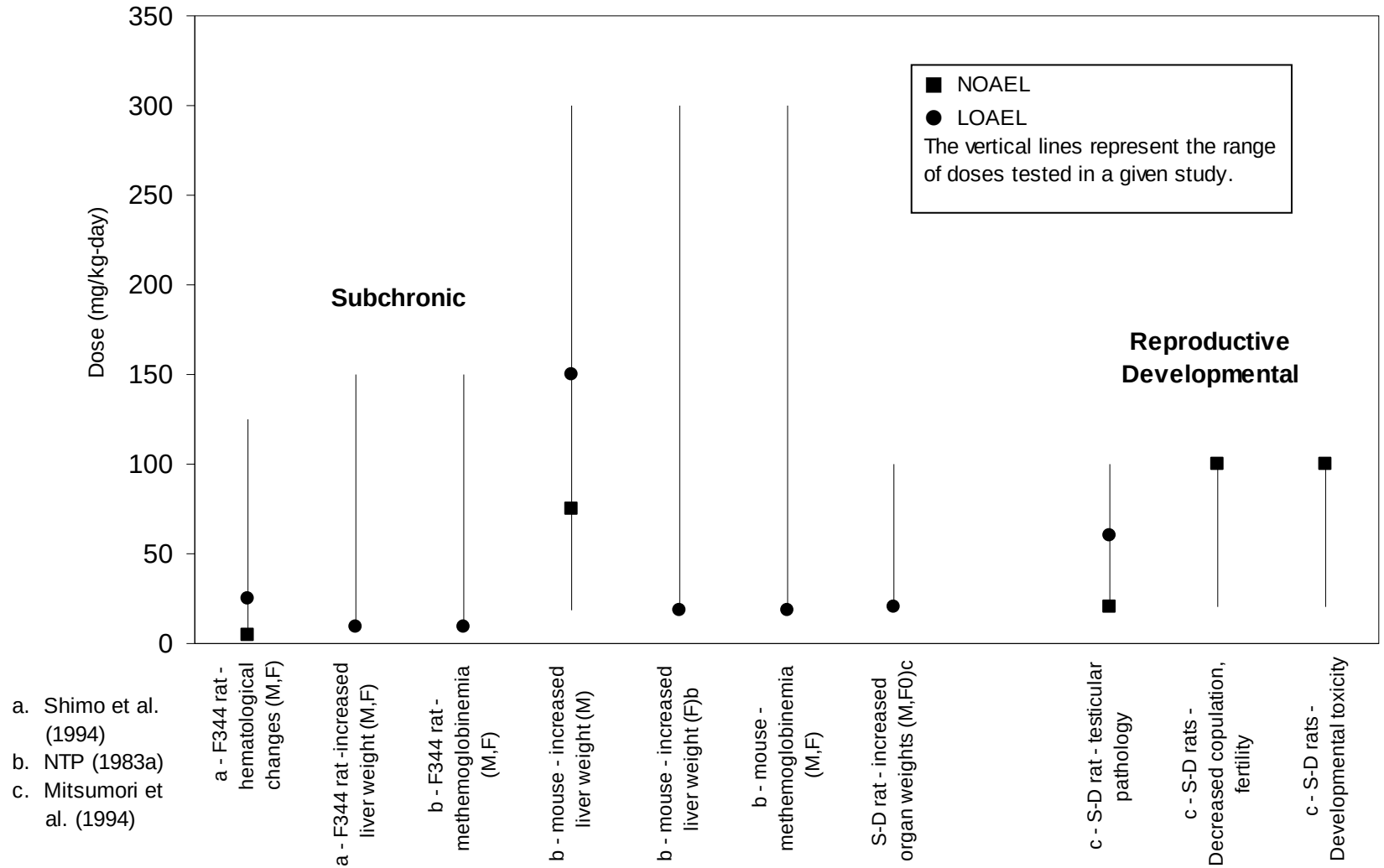


Figure 5-1. Exposure-response array of selected subchronic and reproductive-developmental toxicity effects by the oral route.

Shimo et al. (1994) conducted a 28-day gavage toxicity study of nitrobenzene in F344 rats (six/sex/group) at doses of 0, 5, 25, and 125 mg/kg-day. Animals were evaluated for generalized signs of toxicity, body and organ weight changes, food consumption, histopathology, and hematologic and clinical chemistry parameters. There were clinical signs as well as a marked reduction in body weight increase in the high-dose rats. Organ weight changes were also observed (spleen and liver were increased and testes were decreased) and were corroborated by histopathology findings (e.g., spleens had increased congestion, brown pigmentation in red pulp, and extramedullary hematopoiesis, while testis had epithelium degeneration and atrophy of seminiferous tubules). However, the most sensitive changes were dose-dependent hematologic changes, including reductions in RBC count, Hct, and Hb concentration, in addition to increases in MCV and the WBC count; metHb concentrations were not reported. The NOAEL and LOAEL for these reported changes were 5 and 25 mg/kg-day, respectively.

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 4-29, 4-30, and 4-31). While this dose was more than twice the lowest dose used in the NTP (1983a) study, the dose-response relationships of the effects common to both studies were consistent with those in the NTP study.

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 effect of nitrobenzene on the spleen and hematology parameters. However, 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 biologically significant endpoints, including changes in absolute and relative organ weights, changes in hematologic 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.

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) 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, decreases in testis weight (~40%) were generally seen only at the two highest doses in rats (75 and 150 mg/kg-day), accompanied by an up to 90% lethality (NTP, 1983a). Similarly, a decrease in testis weight (~30%) was only observed with the highest dose in male mice (300 mg/kg-day) 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, since more relevant endpoints were identified at lower levels of exposure.

A number of dose-dependent hematologic 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 animal studies and methemoglobinemia has been observed with most human poisonings, these outcomes were considered candidate critical effects. Of the other hematology endpoints that were affected with increasing exposure, reticulocyte levels showed the greatest change, increasing by about 42% in female rats at the lowest exposure level. The remaining hematology parameters were not considered further for deriving the RfD because they were considered manifestations of the same toxicity response and seem to be less sensitive than the endpoints already highlighted.

In summary, the following considerations were used for selecting the 90-day gavage administration study (NTP, 1983a) from among other toxicity studies based on the approach outlined in U.S. EPA (2002):

- Route-to-route extrapolation from the inhalation study is not possible in the absence of well-characterized PBPK modeling for nitrobenzene.
- The NTP (1983a) study has the longest continuous exposure duration via the oral route.
- It included both sexes of rats and mice with five dose groups, plus control, spaced from 9.4–150 mg/kg-day in rats and from 18–300 mg/kg-day in mice.

5.1.2. Method of Analysis—Including Models

BMD software (BMDS), version 1.4.1c (U.S. EPA, 2007), was used to estimate a point of departure (POD) for deriving an RfD for nitrobenzene. Although splenic congestion and changes in reticulocyte counts are considered secondary to the formation of metHb, data on

metHb, splenic congestion, and reticulocyte counts were modeled for purposes of comparison (Table 5-1).

Table 5-1. Summary of effects in F344 rats associated with exposure to nitrobenzene by gavage for 90 days

Dose (mg/kg-day)	n[a]	Reticulocytes (%)[b]	MetHb (%)[b]	Splenic congestion, severity greater than minimal[c]
Males				
0	10	2.23 ± 0.44	1.13 ± 0.58	0/10
9.38	10	2.62 ± 0.45	2.75 ± 0.58[d]	0/10
18.75	10	3.72 ± 0.65[d]	4.22 ± 1.15[d]	0/10
37.5	10	4.75 ± 0.62[d]	5.62 ± 0.85[d]	0/10
75	10	6.84 ± 0.72[d]	7.31 ± 1.44[d]	5/10
150	1	15	12.22	10/10
Females				
0	10	2.60 ± 0.37	0.94 ± 0.03	0/10
9.38	10	3.69 ± 0.32[d]	2.06 ± 0.45[d]	1/10
18.75	10	4.75 ± 0.68[d]	3.62 ± 1.09[d]	3/10
37.5	10	6.28 ± 0.90[d]	5.27 ± 0.76[d]	5/10
75	10	8.72 ± 1.49[d]	6.85 ± 2.25[d]	8/10
150	7	32.07 ± 3.56[d]	12.77 ± 1.83[d]	9/10

[a]Number of animals surviving at terminal sacrifice.
[b]Values are means ± standard deviations.
[c]All animals, including early deaths, underwent histopathologic examination.
[d]Significantly different from controls, as calculated by the authors.

Source: NTP (1983a).

Consistent with the U.S. EPA (2000b) BMD technical guidance, biologically relevant response levels were considered for these endpoints. There is considerable information in the literature concerning management and treatment of methemoglobinemia in humans, while characterization of levels at which cyanosis and other clinical symptoms become apparent vary across the available literature (see section 4.5.1), commonly falling in the range of 6–10%. Unfortunately, little information exists concerning the biological significance of particular metHb levels in rodents and what would correspond to humans, at least regarding relative biological significance. Information that is available suggests that the normal range in rats may not necessarily parallel that in humans. Compared to human erythrocytes, rat erythrocytes are known to have a higher activity of NADH-metHb reductase, the enzyme that spontaneously regenerates Hb from metHb (Smith, 1996). This difference may indicate that rat erythrocytes are more efficient at regenerating Hb from metHb, especially following exposure to metHb-forming agents, although the extent to which this translates into differences in biologically significant metHb levels in rats and humans is unknown. Of particular note in the NTP (1983a) study of nitrobenzene, the F344 rats experienced significant mortality when metHb levels increased above

10% (see both 150 mg/kg-day groups in Table 5-1). Therefore, projecting metHb levels associated with clinical symptoms from humans to rats was considered not to be appropriate in this assessment.

For metHb, a 1 standard deviation (SD) benchmark response (BMR) was considered first. As detailed in the BMD technical guidance (U.S. EPA, 2000b), a 1 SD BMR provides the exposure level at which 10% of those exposed would be expected to exceed the 98^{th} (or 2^{nd}) percentile of the control group's responses. Second, a BMR corresponding to the normal range of 0–3% in humans was also considered (see section 4.5.1), under the unverified assumption that this could describe a comparable range in rats. This second approach was implemented by estimating the exposure level at which 10% of those exposed would be expected to exceed 3% metHb and would be the preferred approach to selecting a BMR if a normal upper limit for metHb in rats had been established at 3% (U.S. EPA, 2000b). In addition, a BMR of 2 SDs was included for comparison.

No information concerning minimally significant increases of splenic congestion or reticulocytes in rats was found. Because increased reticulocyte counts and splenic congestion are understood to be sequelae of methemoglobinemia, BMR selection focused primarily on metHb levels and characterization of normal ranges. BMD modeling carried out for increased reticulocyte counts and splenic congestion used BMRs of 1 SD and 10% extra risk, respectively, for comparison with the results from modeling metHb levels.

The endpoints were modeled in terms of administered dose. In the NTP (1983a) study, the animals were gavaged 7 days/week, thus no adjustment from intermittent to continuous exposure was required.

The BMD modeling results for metHb levels, reticulocyte count, and splenic congestion in male and female F344 rats are provided in Appendix B-1 and summarized in Table 5-2. For male rats, the 95% lower bound on the BMD (BMDL) corresponding to 10% of an exposed population exceeding a 3% metHb level was nearly twice as high as the BMDL corresponding to a 1 SD increase in the control mean. The $BMDL_{2SD}$ was twofold higher than the $BMDL_{1SD}$. Results for the female rats were in a similar range. As noted in Appendix B-1, however, the female rat metHb data were somewhat unusual in that there was very little variability in the control values, and an estimate of the SD was assumed to be the same as that associated with the low-dose females. Therefore, the female rat metHb results are shown for consistency but were not considered suitable for deriving an RfD.

Table 5-2. Summary of noncancer BMD modeling of selected endpoints from F344 rats exposed by gavage to nitrobenzene for 90 days, using NTP (1983a) bioassay data

Endpoint	Sex[a]	Model used	*p* value	BMR	BMD (mg/kg-day)	BMDL (mg/kg-day)
MetHb	M[b]	Hill	0.42	10% extra risk of exceeding 3% metHb	4.9	3.2
				1 SD	3.0	1.8
				2 SD	5.7	3.9
	F[b]	Hill	0.41	10% extra risk of exceeding 3% metHb	8.2	6.3
				1 SD	4.7	3.1
				2 SD	7.2	5.2
Reticulocyte count	M[b]	Linear	0.16	1 SD	9.4	7.9
	F[b]	Hill	0.58		2.7	1.8
Splenic congestion	M	Multistage	1.0	10% extra risk of mild or moderate congestion	54.6	37.8
	F	Multistage	1.0		7.8	5.6

[a]M = male, F = female.
[b]Highest dose not included in BMD modeling.

The results of modeling reticulocyte counts and splenic congestion fall in a range similar to that of the metHb results, reinforcing the latter results, with the exception of the male rat splenic congestion. The BMDL was roughly 10-fold higher than that for splenic congestion in the female rats and for the other endpoints ($BMDL_{1SD}$s) but is consistent with splenic congestion being secondary to methemoglobinemia.

Based on the considerations above, the POD for developing an RfD was the $BMDL_{1SD}$ derived from the male rat metHb data. The BMDL corresponding to 10% of an exposed population exceeding 3% metHb was roughly twofold higher but not clearly linked to biological significance for rats. The use of a 1SD BMR in developing an RfD for humans engenders an assumption of analogous variability between rats and humans, i.e., that humans would be expected to demonstrate a 10% extra risk of exceeding the normal range of an unexposed population. This does not necessarily imply that the mean metHb level would be shifted by an amount equal to the rat SD (0.5%) in a human population exposed at the BMDL.

5.1.3. RfD Derivation—Including Application of Uncertainty Factors (UFs)

The RfD for nitrobenzene was calculated as follows:

$$\text{BMDL} \div \text{UF} = \text{RfD}$$
$$1.8 \text{ mg/kg-day} \div 1{,}000 = 2 \times 10^{-3} \text{ mg/kg-day}$$

The composite UF of 1,000 follows from considering these areas of uncertainty and variability:

- An intraspecies UF of 10 was applied to account for human variability and to protect potentially sensitive humans (e.g., G6PD deficiency or chronic congenital methemoglobinemia) and life stages (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 UF 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.
- A UF to account for the extrapolation from a LOAEL to a NOAEL was not applied because the BMR of 1 SD was determined to represent a minimal biologically significant level of change.
- A subchronic-to-chronic UF of 3 was applied to account for less-than-lifetime exposure in the principal study. A chronic oral study is not available. In studies by the inhalation route, the severity of hematologic effects (e.g., metHb, reticulocyte count, and splenic congestion) did not increase between subchronic (CIIT, 1984) and chronic (CIIT, 1993) exposure durations (see section 4.5.2). Nonetheless, other toxicity endpoints may result from chronic oral exposure due to route-specific differences in metabolism, pharmacokinetics, and/or pharmacodynamics that were not observed in the subchronic oral study or the inhalation studies. In particular, several studies of gut bacterial metabolic activation (nitro reduction) support the possibility of higher relative concentrations of active metHb-forming metabolites than would be expected following exposure by the inhalation route (see section 3.3.1).
- An UF of 3 for database deficiencies was applied. The database of oral studies includes the principal study, a 90-day gavage study in two species and both sexes (NTP, 1983b); a reproductive/developmental study (Mitsumori et al., 1994) and two male reproductive toxicity studies (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 possible route-specific differences in metabolism, pharmacokinetics, and/or pharmacodynamics suggest uncertainty in the potential for transgenerational effects from longer-term oral exposures.

5.1.4. Previous RfD Assessment

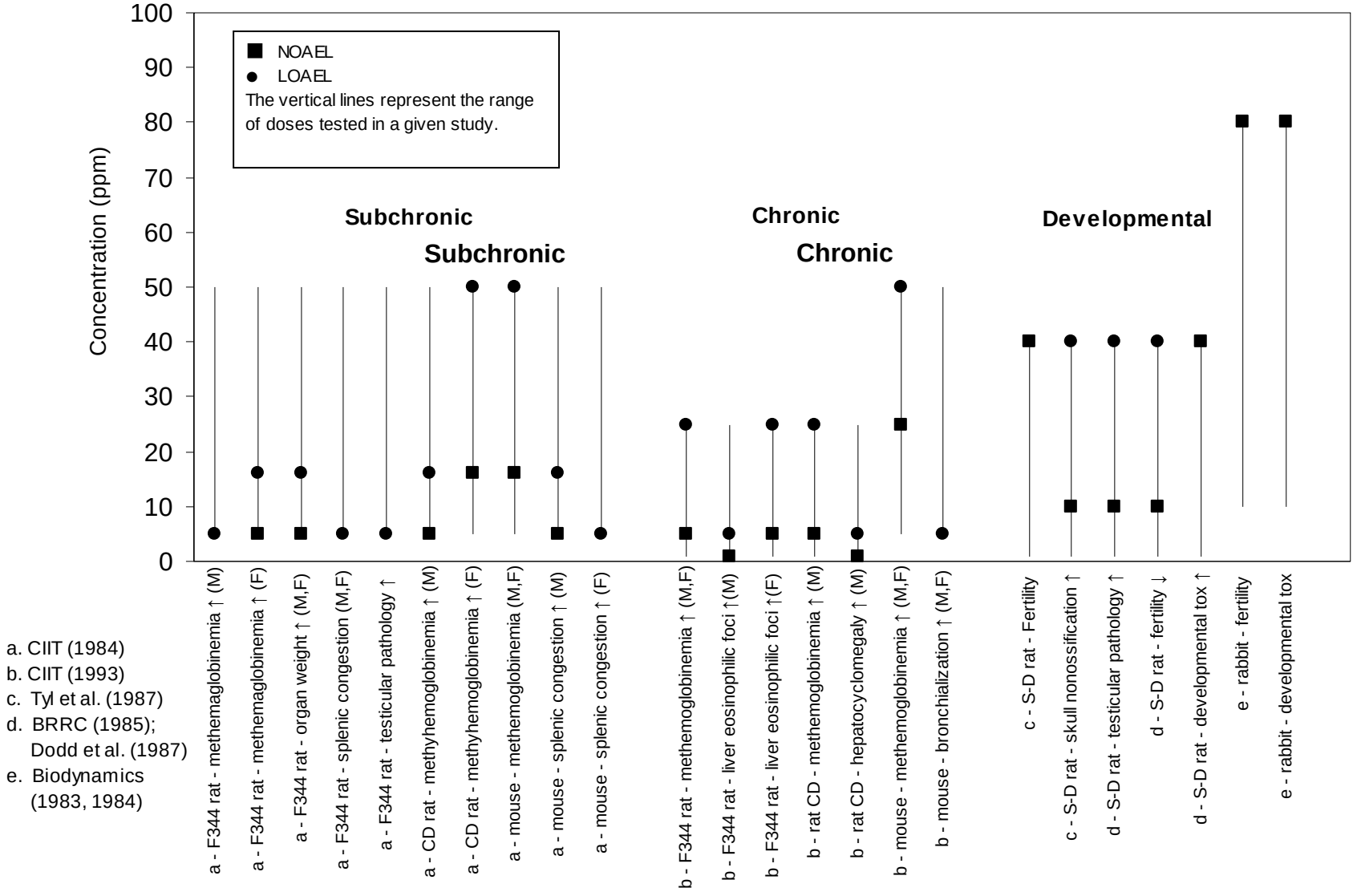
The previous IRIS assessment based the RfD for nitrobenzene of 5×10^{-4} mg/kg-day on a 90-day inhalation study in F344 rats and B6C3F1 mice (CIIT, 1984). Critical endpoints included methemoglobinemia and histopathologic lesions to the adrenal gland, kidney, and liver. A route-to-route extrapolation 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 by using default assumptions for 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.

5.2. INHALATION REFERENCE CONCENTRATION (RfC)

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 addition, portal-of-entry effects following chronic inhalation exposure to nitrobenzene included bronchiolization of the alveoli and olfactory degeneration in both male and female B6C3F1 mice (CIIT, 1993). Pulmonary effects were also observed in subchronic inhalation studies in both F344 rats and B6C3F1 mice (CIIT, 1984). The DuPont (1981) and Medinsky and Irons (1985) studies were not considered as principal studies for RfC derivation because both studies had short exposure times (14 days) and comparatively high levels of exposure (10–125 ppm nitrobenzene). Figure 5-2 is an exposure-response array that presents NOAELs, LOAELs, and the dose range tested corresponding to selected health effects observed in chronic, subchronic, and reproductive inhalation toxicity studies of nitrobenzene.

A 90-day subchronic study was conducted by 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. Signs of hemolytic anemia were evident in rats while methemoglobinemia was consistently observed in both species (Table 4-18). Pulmonary effects were also observed in F344 rats and B6C3F1 mice. In male F344 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.



a. CIIT (1984)
b. CIIT (1993)
c. Tyl et al. (1987)
d. BRRC (1985); Dodd et al. (1987)
e. Biodynamics (1983, 1984)

Figure 5-2. Exposure-response array of selected subchronic, chronic, and reproductive toxicity effects by the inhalation route.

The 2-year study, also conducted by CIIT (Cattley et al., 1994; CIIT, 1993), is the most suitable study for derivation of an RfC 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, and 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 hematologic parameters. According to the study authors, and as noted under section 4.2.2.2, the proportion of study animals surviving to study termination was not statistically significantly affected by exposure to nitrobenzene.

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 in female rats. The incidence of renal tubular hyperplasia in male F344 rats showed a statistically significant positive trend. 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 metHb was observed at all exposure levels in male CD rats and only at the highest concentration with male and female F344 rats. At terminal sacrifice, a statistically significant increase in metHb was observed with both sexes of mice at the highest concentrations tested. An approximate twofold increase in metHb 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). Hct and Hb were reduced only in female mice, being statistically significantly different at the 5 ppm concentration and lower concentrations, albeit still statistically 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 to not be treatment related because of the lack of a dose response.

Exposure-related degeneration and loss of olfactory epithelium were observed in mice of both sexes, with the females being more sensitive than the males. At the highest concentration tested (50 ppm), the incidence was 62% in males and 69% in females. Bronchiolization of the alveoli was also 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 sensitive effects observed following nitrobenzene exposure were degeneration and loss of the olfactory epithelium and bronchiolization of the alveoli in mice. Degeneration and loss of the olfactory epithelium occurred in a concentration-dependent manner, with high incidences (≥62%) at the highest exposure in both males and females, while females were more sensitive than males at the lowest exposure, with 19/60 females responding versus 1/66 males (Table 5-3). The study report (CIIT, 1993) indicated that the severity increased with increasing exposure but provided no further details.

Table 5-3. Incidence of histopathologic lesions in mice following chronic nitrobenzene inhalation

Histopathologic lesion	Sex[a]	Exposure level (ppm)			
		0	5	25	50
Olfactory epithelium degeneration, loss[b]	M	1/67	1/66	32/65	41/66
	F	0/52	19/60	47/63	42/61
Bronchiolization of the alveoli[b]	M	0/68	58/67	58/65	62/66
	F	0/53	55/60	63/64	62/62

[a]M = male, F = female.
[b]Significant positive trend by Armitage-Cochran test in both sexes, $p < 0.05$.

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

As discussed in section 4.5.2, exposure-related olfactory changes in the nasal passages were also observed in both F344 and CD rat strains in the CIIT (1993) study, although the changes were less marked than in mice, and in the olfactory bulbs of rats exposed to nitrobenzene by inhalation in Beauchamp et al. (1982). Olfactory degeneration was considered as a candidate critical effect for the derivation of the RfC.

Bronchiolization of the alveoli occurred with high incidence (≥87%) in both males and females in the exposed 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. According to the report, the change was concentration related in severity; however, no additional information on severity was reported (CIIT, 1993). In the low-concentration exposed 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, with animals in the mid-concentration group being slightly less affected than the high-concentration animals.

As discussed in section 4.5.2, bronchiolization of the alveoli is a histologically distinct lesion that has been seen in various species, including mice and humans, and that may indicate a variety of pathological conditions, including inflammation, chemical irritation, or exposure to carcinogens. Bronchiolization was also considered as a candidate critical effect for the derivation of the RfC.

Methemoglobinemia was not chosen as a candidate critical endpoint for the inhalation RfC. As explained in section 4.2.2.2, the biological significance of the hematologic findings, including methemoglobinemia, in the chronic inhalation study (Cattley et al., 1994) is unclear. In several instances, the differences between the dosed groups and the controls were minimal or decreased with increasing length of exposure. In most instances, methemoglobinemia was notably increased only at the highest nitrobenzene exposure, while time-related trends were not clear-cut due to a possible compensatory response among all exposed rat groups (Table 4-20).

5.2.2. Methods of Analysis—Including Models

BMD modeling (U.S. EPA, 2000b) was used to analyze the incidence data for bronchiolization of the alveoli and olfactory degeneration from CIIT (1993) as shown in Table 5-3. All of the available dichotomous models in U.S. EPA's BMDS (version 1.4.1c) were fit to the incidence data for bronchiolization of the alveoli and olfactory degeneration. Consistent with U.S. EPA (2000b) BMD technical guidance, consideration was given to identifying biologically relevant response levels for developing RfDs. Insufficient information was available to identify minimally adverse levels of response for either bronchiolization of the alveoli or olfactory degeneration. A BMR of 10% is generally used to facilitate a consistent basis of comparison across assessments and was used for these endpoints in the absence of information regarding the level of change considered to be biologically significant.

The BMD modeling results for bronchiolization of the alveoli and olfactory epithelium degeneration are summarized in Table 5-4. Detailed model output is in Appendix B-2.

Table 5-4. Modeling results for bronchiolization of the alveoli and olfactory degeneration in mice

Endpoint	Sex	Model	*p* Value	BMC_{10} (ppm)[b]	$BMCL_{10}$ (ppm)[b]
Olfactory epithelium degeneration, loss	Males	Probit	0.38	12.3	10.0
	Females[a]	Gamma, multistage (1^o), Weibull	0.50	1.75	1.42
Bronchiolization of the alveoli	Males	Log-logistic[a]	0.028	0.13	0.083
	Females	Log-probit	0.12	0.40	0.28
		Log-logistic	0.80	0.18	0.022

[a]High-dose group excluded.
[b]BMC = benchmark concentration; BMCL = 95% lower bound on the BMC.

Data sources: Cattley et al. (1994); CIIT (1993).

Dose-response modeling provided satisfactory descriptions of the olfactory epithelium degeneration data (with adequate goodness-of-fit *p* values > 0.1 and low chi-squared residuals). The greater sensitivity in female mice noted above was estimated to correspond to an approximate 10-fold difference between the 95% lower bound on the benchmark concentrations ($BMCL_{10}$s) for male and female mice in this study, at 10 ppm for males versus 1.4 ppm for females.

Modeling was not quite as successful for the bronchiolization data. The male mice data only supported a model with a goodness-of-fit *p* value of 0.02 for the best fit. The female mice data supported two adequate but somewhat equivocal fits. That is, the slightly better fitting model (log-logistic; better fit owing to a lower chi-squared residual at the lowest dose) led to a $BMCL_{10}$ (0.022 ppm) about 10-fold lower than its benchmark concentration (BMC_{10}) (0.18 ppm), while the only other model providing an adequate fit to these data (the log-probit model) yielded a more precise $BMCL_{10}/BMC_{10}$ range of 0.28/0.40 ppm, both measures slightly higher than the first model's BMC_{10}.

The male mice bronchiolization data were problematic to fit with available models because the response at the low end of the plateau of responses was underestimated. Further, there is no way to tell how far into the lower exposures the plateau really extends, since most of the dose-response relationship has not been captured. It is therefore possible that a $BMCL_{90}$, for example, more closely fitting the observed data could be overestimated, aside from the accuracy of any extrapolations to lower doses. In addition, despite the relatively better fitting models for the female mice, these data are as uncertain as for the males regarding the extent of the response plateau.

On the other hand, the study report noted that the severity of bronchiolization was dose related, increasing with increasing exposure. Because no data on the quantification of severity was provided in the study report, it is unknown what impact the consideration of such data might have on the dose-response relationship, although it is plausible that the reported low-exposure

response was overstated relative to that at the higher exposures. Given these two divergent possibilities, use of the modeled response appears to be a reasonable compromise.

Because of the uncertainty evident in modeling the bronchiolization data, the NOAEL/LOAEL approach was also considered for estimating the POD. The highest $BMCL_{10}$ estimated, 0.28 ppm, is about twofold lower than the LOAEL (5 ppm) divided by the LOAEL-to-NOAEL UF of 10, or 0.5 ppm. A LOAEL at an 87% response level, however, is arguably less informative than a $BMCL_{10}$ for characterizing exposure response in the low-exposure region, since it is not clear that a 10-fold UF applied to such a high response can provide an adequate estimate of a minimally biologically significant response level.

Therefore, the BMD approach was used for characterizing a POD for the bronchiolization data. An average of the three $BMCL_{10}$s— 0.1 ppm—was considered as one POD for developing the RfC, while acknowledging the uncertainty associated with the somewhat extreme degree of extrapolation to estimate a $BMCL_{10}$ from either the male or female mice bronchiolization data.

5.2.3. Evaluation of Human Equivalent Concentrations

While the dose-response modeling shows bronchiolization to be a more sensitive response in mice than olfactory degeneration, the $BMCL_{10}$s for both endpoints were considered for extrapolation to human equivalent concentrations (HECs), since this process can have different impacts depending on the respiratory sites affected. This process involves two main steps, adjustment to equivalent continuous lifetime exposures, followed by adjustment to human equivalents.

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, in the absence of information to the contrary (U.S. EPA, 2002). In the chronic studies (Cattley et al., 1994; CIIT, 1993), animals were exposed for 6 hours/day, 5 days/week. Therefore, the POD adjusted for continuous exposure (POD_{ADJ}) for inhalation of nitrobenzene is as follows:

$$POD_{ADJ} = POD\ (\text{in ppm}) \times 6\ \text{hours}/24\ \text{hours} \times 5\ \text{days}/7\ \text{days} \qquad (5\text{-}1)$$

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

EPA guidance for RfC derivation provides procedures for determining an HEC from the POD_{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 versus systemic and, in the former case, extrathoracic, thoracic, tracheobronchial, or pulmonary). The effects considered, bronchiolization and olfactory degeneration, were pulmonary and extrathoracic effects, respectively. 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 by using methods for category 1 gases for portal-of-entry effects and category 3 methods for systemic effects (U.S. EPA, 1994b). The olfactory degeneration is more clearly a portal-of-entry effect; hence, the method for category 1 gases was used to derive its HEC. Because the bronchiolization occurred on the boundary with systemic circulation, HECs consistent with this endpoint being either a portal-of-entry or a systemic effect were estimated.

EPA's *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b) suggests that HECs be estimated by applying to the duration-adjusted exposure level, here the POD_{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 and extrathoracic regions as follows:[13]

$$RGDR_{PU\ or\ ET} = (MV_a/S_{a,PU\ or\ ET}) \div (MV_h/S_{h,PU\ or\ ET}) \quad (5\text{-}2)$$

where

MV_a = minute volume for mice = 0.06 m^3/day (see Appendix B-2)
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
$S_{a,ET}$ = default extrathoracic surface area for mice = 3 cm^2
$S_{h,ET}$ = default extrathoracic surface area for humans = 200 cm^2

Finally, the POD_{HEC} is derived as follows:

$$POD_{HEC} = POD_{ADJ} \times RGDR \quad (5\text{-}3)$$

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

Olfactory Degeneration

The POD_{HEC} for olfactory degeneration using the methods described above is estimated as follows:

$$POD_{ADJ} = 1.42 \text{ ppm} \times 6 \text{ hours}/24 \text{ hours} \times 5 \text{ days}/7 \text{ days} \times 5.04 \text{ (mg/m}^3\text{)/ppm}$$
$$= 1.275 \text{ mg/m}^3$$

Substituting the POD_{ADJ} and appropriate values for surface area for the extrathoracic region into equation 5-2, the $RGDR_{PU}$ is calculated as follows:

$$RGDR_{PU} = (0.06 \text{ m}^3\text{/day})/(3 \text{ cm}^2) \div (20 \text{ m}^3\text{/day})/(200 \text{ cm}^2) = 0.2$$

Finally, the POD_{HEC} is calculated as follows:

$$POD_{HEC} = 1.275 \text{ mg/m}^3 \times 0.2 = 0.26 \text{ mg/m}^3$$

Bronchiolization of the Alveoli

The POD_{HEC} for bronchiolization of the alveoli using the methods described above is estimated as follows:

$$POD_{ADJ} = 0.1 \text{ ppm} \times 6 \text{ hours}/24 \text{ hours} \times 5 \text{ days}/7 \text{ days} \times 5.04 \text{ (mg/m}^3\text{)/ppm}$$
$$= 0.09 \text{ mg/m}^3$$

Substituting the POD_{ADJ} and appropriate values for surface area for the pulmonary region into equation 5-2, the $RGDR_{PU}$ is calculated as follows:

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

Finally, the POD_{HEC} is derived as follows:

$$POD_{HEC} = 0.09 \text{ mg/m}^3 \times 3.24 = 0.29 \text{ mg/m}^3$$

Alternatively, under the assumption that bronchiolization of the alveoli might be a systemic effect, interspecies extrapolation to an HEC involves consideration of the nitrobenzene air:blood partition coefficients for humans and rats (U.S. EPA, 1994b). In the absence of such data, as in this case, the ratio of animal to human air:blood partition coefficients is assumed to be unity. The $POD_{HEC} = 0.09 \text{ mg/m}^3$.

5.2.4. RfC Derivation—Including Application of Uncertainty Factors (UFs)

Because the POD_{HEC}s for the two respiratory effects—bronchiolization of the alveoli and olfactory degeneration—were similar in value, at 0.29 and 0.26 mg/m^3, respectively, the effects were considered co-critical effects for purposes of deriving the RfC and the lower of the two values, 0.26 mg/m^3, was chosen as the POD.

The RfC was calculated based on the POD_{HEC} values for these co-critical effects by application of UFs as follows:

$$RfC = POD_{HEC} \div UF \quad (5\text{-}4)$$

$$RfC = 0.26 \text{ mg/m}^3 \div 30 = 0.0085 \text{ mg/m}^3 = 9 \times 10^{-3} \text{ mg/m}^3$$

The composite UF of 30 follows from considering these areas of uncertainty and variability:

- An intraspecies UF of 10 was applied to account for human variability and to protect potentially sensitive humans and life stages (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.
- A UF of 3 was applied to account for uncertainty in extrapolating from laboratory animals to humans. This value is adopted by convention, where a dosimetric adjustment from an animal-specific POD_{ADJ} to a POD_{HEC} already has been incorporated. Application of a full UF 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 an HEC as described in the RfC methodology (U.S. EPA, 1994b). The toxicodynamic uncertainty is also accounted for to a certain degree by the use of the applied dosimetry method.
- A UF to account for extrapolation from a LOAEL to a NOAEL was not used because the current approach is to address this extrapolation as one of the considerations in selecting a BMR for BMD modeling. In this case, a BMR of a 10% change in either of the respiratory effects was selected under an assumption that it represents a minimal biologically significant change.
- A subchronic-to-chronic UF for extrapolation to lifetime exposure was not applied since the data used originated from a 2-year (lifetime) chronic study.
- A UF of 1 was applied to account for database deficiencies. The inhalation database is considered complete because it includes developmental toxicity studies in rats (Tyl et al., 1987) and rabbits (Biodynamics Inc., 1984), a two-generation reproduction study in rats (Dodd et al., 1987), and a 2-year toxicity study in mice and two strains of rats (Cattley et

al., 1994; CIIT, 1993) in addition to short-term toxicity studies in mice and two strains of rats (Medinsky and Irons, 1985; DuPont, 1981).

5.2.5. Previous RfC Assessment

An inhalation 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 that supported quantitative cancer assessment.

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

A 2-year inhalation cancer bioassay (CIIT, 1993, published as Cattley et al. [1994]) was used for development of an inhalation unit risk (IUR) for nitrobenzene. 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]). Ranges of exposure concentrations were selected based on a subchronic study by the same route. 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 (see section 4.2.2.2 for additional details).

Increased incidences of neoplasms with increasing nitrobenzene exposure were observed in both mice and rats. 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 originating from the same cell type represent stages along a continuum of carcinogenic effects resulting from the same mechanism, as recommended by the EPA cancer guidelines (U.S. EPA, 2005a).

For example, hepatocellular adenomas or carcinomas were consistently seen in males of both rat strains (i.e., F344 and CD) and also in female F344 rats. The incidence of these neoplasms in male CD rats was lower than in male F344 rats. Table 5-5 summarizes the incidences of hepatocellular neoplasms, by type and combined, among terminally sacrificed F344 rats. The hepatocellular adenomas were described as being spherical, sharply demarcated, and compressed to surrounding normal parenchyma, and they consisted of well-differentiated hepatocytes arranged in sheets or irregular cords. Hepatocellular carcinomas were generally larger and more irregular than the hepatocellular adenomas, caused a marked compression of surrounding parenchyma, and had a distinctive feature of a trabecular (rod-shaped) pattern. The carcinomas were composed of pleomorphic cells arranged in sheets or thickened disorganized cords (Cattley et al., 1994). Based on the overlapping characteristics of the two forms of hepatocellular tumors, it was judged reasonable to analyze both forms together.

Table 5-6 presents an overview of the tumor incidence data from Cattley et al. (1994). 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 neoplasia in female B6C3F1 mice. A slightly elevated incidence of thyroid neoplasia, 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.

Table 5-5. Hepatocellular neoplastic findings in F344 rats exposed to nitrobenzene via inhalation for 2 years

F344 rat	Nitrobenzene concentration (ppm)							
	Male				Female			
	0	1	5	25	0	1	5	25
Adenoma[a]	1/43	3/50	3/47	15/46	0/49	2/50	0/50	3/49
Carcinoma[a]	0/43	1/50	2/47	4/46	0/49	0/50	0/50	2/49
Adenoma[a] or carcinoma	1/43	4/50	5/47	16/46	0/49	2/50	0/50	4/49

[a]Liver tumor incidences are based on terminal sacrifice data.

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

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%)
		Nitrobenzene concentration (ppm)			
		0	**1**	**5**	**25**
Rat, male, F344	Liver, hepatocellular adenoma or carcinoma	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).

Statistically significant increasing 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. Moreover, no corresponding renal neoplasia 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 there was no evidence or concern that nitrobenzene exposure would be associated with more severe neoplasia at this site.

The strongest dose response for hepatocellular adenomas or carcinomas occurred in male F344 rats; therefore, this data set and the data sets for kidney and thyroid adenomas or carcinomas in male F344 rats were chosen for cancer dose-response assessment. In addition, thyroid and lung adenomas or carcinomas in male B6C3F1 mice were considered for cancer dose-response assessment. As indicated in section 4.6.2, the relevance of hepatocellular neoplasia in rats to the overall assessment of nitrobenzene carcinogenicity in humans is strengthened by the facts that the spontaneous incidence of hepatocellular neoplasia is higher in

B6C3F1 mice than in F344 or CD rats (Cattley et al., 1994) and that B6C3F1 mice have been known to more likely develop hepatocarcinogenicity in response to chemical exposures than do rats (Goodman et al., 1985). While the liver tumors in the male F344 rat appear to be the most sensitive cancer endpoint, IURs were also calculated for the thyroid and kidney tumors in order to characterize potential total risk (see sections 4.6.2 and 4.6.3 concerning the human relevance of these tumors).

5.3.2. Benchmark Concentration Modeling

Because there are no biologically based dose-response models suitable for the tumor data identified above, these data were modeled by using the multistage model, as implemented by BMDS 1.4.1c (U.S. EPA, 2007). This model has the following 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.

A 10% BMR was used with each tumor type (U.S. EPA, 2005a). BMC modeling results are shown in Appendix B-3.

Male rats were slightly more sensitive to nitrobenzene carcinogenicity, displaying lower values for the 95% lower bound at 10% extra risk ($BMCL_{10}$) than male mice. Consequently, the modeling results for male rats form the basis for IUR derivation, with support from the male mouse data. Table 5-7 shows the estimated BMC_{10}s, $BMCL_{10}$s, and chi-square *p* values derived for the three tumor types modeled for male F344 rats.

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[a]	BMC_{10} (ppm)	$BMCL_{10}$ (ppm)	χ^2 *p* value for lack of fit
Kidney	Tubular adenoma or carcinoma	22.8	16.8	1.0
Thyroid	Follicular cell adenoma or carcinoma	13.6	7.8	0.37
Liver	Hepatocellular adenoma or carcinoma	6.8	4.4	0.63

[a]See Appendix B-3.3 for modeling results.

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

The current *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a) stipulate that the method used to characterize and quantify cancer risk from a chemical is determined by what is known about the MOA 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 mutagenic MOA because of DNA reactivity or if another MOA that is anticipated to be linear is applicable. An assumption of nonlinearity is appropriate when the MOA theoretically has a threshold (e.g., when the carcinogenic action is secondary to another toxic effect that itself has a threshold). Low-dose extrapolation could also include other nonlinear modeling approaches if indicated by the MOA or other data. If the MOA of carcinogenicity is not adequately understood, a linear dose-response relationship at low doses is assumed and the linear extrapolation is used (U.S. EPA, 2005a).

The available evidence suggests that nitrobenzene is not, or is at most weakly, mutagenic (see section 4.4.5). In addition, nitrobenzene has been shown to undergo redox cycling (see section 3.3) with the possibility that it may cause oxidative stress (see section 4.6.3). 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 carcinogenic MOA. Accordingly, the low-dose linear approach is used for the derivation of carcinogenic potency.

In order to derive an inhalation risk unit (IUR), the BMCLs from inhalation exposure to nitrobenzene reported in Table 5-8 were converted to mg/m^3 (1 ppm = 5.04 mg/m^3 under 0.15 mm Hg at 25°C) and adjusted for continuous exposure as follows:

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

The tumor types associated with nitrobenzene exposure in rats are systemic effects. As was discussed for deriving an RfC from the bronchiolization data, interspecies extrapolation to a HEC involves consideration of the nitrobenzene air:blood partition coefficients for humans and rats (U.S. EPA, 1994b). Since these coefficients were not available, the ratio of animal to human air:blood partition coefficients is assumed to be unity.

The slope of the dose response from the $BMC_{10/HEC}$ for each site (derived by dividing the BMR [e.g., BMR = 10% or 0.10] by the $BMC_{10/HEC}$) is provided to illustrate statistical uncertainty. Estimates of these slopes based on kidney, thyroid, or liver tumors in male F344 rats are shown in Table 5-8.

Table 5-8. Cancer risk estimates from nitrobenzene tumor incidence in male F344 rats, based on the slope to background from the BMC

Target organ/tumor type	BMC_{10} (ppm)	$BMC_{10/HEC}$[a] (mg/m^3)	Slope from the $BMC_{10/HEC}$[b] $(\mu g/m^3)^{-1}$
Kidney tubular adenoma or carcinoma	22.8	20.5	5×10^{-6}
Thyroid follicular cell adenoma or carcinoma	13.6	12.2	8×10^{-6}
Hepatocellular adenoma or carcinoma	6.8	6.1	2×10^{-5}

[a]HEC = BMC × 5.04 mg/m^3 × 5/7 × 6/24, assuming ratio of animal to human air:blood partition coefficients is 1.
[b]Slope from the BMC_{HEC} = BMR (0.1)/BMC_{HEC}.

Estimated IURs are calculated by dividing the BMR (e.g., BMR = 10% or 0.1) by the $BMCL_{HEC}$. Estimates of the IURs based on kidney, thyroid, or liver tumors in male F344 rats are shown in Table 5-9.

Table 5-9. IURs for nitrobenzene, based on tumor incidence in male F344 rats, based on the slope to background from the BMCL

Target organ/tumor type	BMCL (ppm)	$BMCL_{HEC}$[a] (mg/m^3)	Estimated IUR[b,c] $(\mu g/m^3)^{-1}$
Kidney tubular adenoma or carcinoma	16.8	15.1	7×10^{-6}
Thyroid follicular cell adenoma or carcinoma	7.8	7.0	1×10^{-5}
Hepatocellular adenoma or carcinoma (combined)	4.5	4.1	2×10^{-5}

[a]$BMCL_{HEC}$ (adjusted) = BMCL × 5.04 mg/m^3 × 5/7 × 6/24 assumes ratio of animal to human air:blood partition coefficients is 1.
[b]IUR = BMR (0.1)/$BMCL_{HEC}$.
[c]These IURs should be used with caution at exposure concentrations above the $BMCL_{HEC}$s, because above these levels the responses are not expected to continue linearly (i.e., responses cannot exceed 100%).

The highest IUR estimated for nitrobenzene was from liver adenomas or carcinomas, at 3×10^{-5} $(\mu g/m^3)^{-1}$. The IUR associated with thyroid tumors was about threefold lower than that for liver tumors, and the IUR associated with kidney tumors was about fourfold lower than that for liver tumors.

With a multiplicity of tumors, as is the case for nitrobenzene, there is a concern 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. Two approaches were considered in which a composite IUR for tumors of the liver, kidney, and thyroid was developed (see Appendix B-3.3). The two approaches supported each other. The composite IUR, rounded to one significant figure, is 4×10^{-5} $(\mu g/m^3)^{-1}$, two fold higher than the IUR based on liver adenomas or carcinomas, 2×10^{-5} $(\mu g/m^3)^{-1}$. The recommended upper bound estimate on human extra cancer risk from continuous lifetime inhalation exposure to

nitrobenzene is 4×10^{-5} $(\mu g/m^3)^{-1}$, reflecting the exposure-response relationships for liver, thyroid, and kidney cancer.

5.3.4. Uncertainties in Cancer Risk Values

Extrapolation of study data to estimate potential risks to human populations from exposure to nitrobenzene has engendered some uncertainty in the results. The uncertainty falls into two major categories: model uncertainty and parameter uncertainty. Model uncertainty "refers to a lack of knowledge needed to determine which is the correct scientific theory on which to base a model," whereas parameter uncertainty "refers to a lack of knowledge about the values of a model's parameters" (U.S. EPA, 2005a). In the absence of a biologically based model, a multistage model was the preferred model because it has some concordance with the multistage theory of carcinogenesis and serves as a benchmark for comparison with other cancer dose-response analyses. It is unknown how well this model or the linear low-dose extrapolation predicts low-dose risks for nitrobenzene. Also, while the male mice did not appear to have as strong a carcinogenic response as the male rats, it is not known which species is more relevant for extrapolation of risk to humans.

Parameter uncertainty can be assessed through confidence intervals and probabilistic analysis. Each description of parameter uncertainty assumes that the underlying model and associated assumptions are valid. Some uncertainty in the animal dose-response data can be assessed through the ratio of BMDs to their BMDLs. For the tumors evaluated here, the ratio was below a factor of 2, which is a typical degree of statistical uncertainty.

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 that highest levels after an oral dose to female Wistar rats were 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 reductase enzymes as catalysts and NAD(P)H as the cofactor: an aerobic three-step,

two-electrons-per-step process in intestinal microflora that operates at a high metabolic rate and an anaerobic six-step, one-electron-per-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, 1999). 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 metHb from iron (III) to its normal, oxygen-carrying iron (II) state. Severe cases have been known to have a fatal outcome, particularly in children. 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 had methemoglobinemia (>12%) and considerable blood pathology (decreased Hb, Hct, and RBC count and 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. Splenic congestion and effects on some hematologic values were observed at low doses. 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 (1983a) study 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 good laboratory practice (GLP) and contemporary requirements for chronic studies. Both the 90-day and the 2-year studies were carried out by 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 pathological 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 ≥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 are 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 G6PD 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 potentially 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). Mortality was high among the high-dose females (7/9 and 2/2 died during gestation and lactation, respectively). 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 reproductive/developmental inhalation 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, 1999; 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 RBCs, with resulting hemolysis, anemia, and splenic congestion.

There is, as yet, no hypothesis concerning the development of olfactory degeneration, bronchiolization of the alveoli, or 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 MOA 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, 2005a), nitrobenzene is "likely to be carcinogenic to humans." This descriptor is based on the induction of 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 in a 2-year inhalation bioassay. There are no studies that document the carcinogenicity of nitrobenzene in humans.

There are no nitrobenzene exposure data or studies in humans from which to assess a potential mechanism of action for cancer. Nitrobenzene caused neoplasia in a 2-year chronic inhalation study (Cattley et al., 1994; CIIT, 1993) in a dose-related manner 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 a causative agent (U.S. EPA, 1994c).

Based on the results of genotoxicity tests, nitrobenzene does not seem to induce tumor formation via a mutagenic MOA. This determination is based on the almost exclusively negative results in salmonella assays (Ames tests; the only exception is TA 98 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. Other MOAs, including oxidative stress, formation of DNA adducts, disruption of intercellular communication, or cytolethality with subsequent regenerative hyperplasia, a promotion-based MOA, have not been experimentally validated in connection with nitrobenzene. Also, experimental data for excluding thyroid and kidney tumors observed in experimental animals based on rodent-specific MOAs are lacking (U.S. EPA, 1998b, 1991b). Additionally, 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 MOA exists in animals that might not be relevant to humans. Therefore, a final conclusion on a cancer MOA cannot be determined at this time. This is reflected in the use of a linear approach as a default option 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 hematologic 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 1.4.1c), with 10% extra risk as the BMR for splenic congestion of grade 2 (mild) or higher, and with 1 SD as the BMR for reticulocyte count and metHb levels. Splenic congestion and increased reticulocyte count were considered to be sequelae of methemoglobinemia, and the BMD modeling of these endpoints generally supported that assumption. The resulting POD, based on the exposure at which 10% of an exposed population would be expected to exceed the 98% upper limit of an unexposed population's metHb levels, was 1.8 mg/kg-day. After application of a composite UF of 1,000, the oral RfD was identified as 2×10^{-3} mg/kg-day.

The composite UF consists of an interspecies UF of 10 for extrapolation from animals to humans, an intraspecies UF of 10 to adjust for sensitive subpopulations (most importantly small children), a subchronic-to-chronic UF of 3 to correct for the less-than-lifetime exposure duration in the principal study, and a database deficiency UF of 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 such deficits in the database.

6.2.2. Inhalation RfC

A few studies with nitrobenzene in human research subjects have been conducted 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 by using F344 and CD rats as well as B6C3F1 mice of both sexes (CIIT, 1984). Exposure concentrations were 0, 5, 16, and 50 ppm for 6 hours/day, 5 days/week. This study identified a variety of hematologic 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. Olfactory degeneration was a sensitive endpoint in female mice and to a lesser extent in male mice. Bronchiolization of the alveoli and olfactory degeneration were chosen as co-critical effects for deriving the RfC, over methemoglobinemia, because of the greater extent and increasing severity of both endpoints with increasing concentration compared to the lack of a clear concentration-dependent response for methemoglobinemia at final sacrifice.

The effects selected for RfC derivation, bronchiolization of the alveoli and olfactory degeneration in male and female B6C3F1 mice in the chronic study, were considered portal-of-entry effects. The POD_{ADJ} for bronchiolization, derived by BMD modeling, was 0.9 mg/m^3, which was converted to a POD_{HEC} of 0.29 mg/m^3. The POD_{ADJ} for olfactory degeneration, also derived by BMD modeling, was 1.27 mg/m^3, which was converted to a POD_{HEC} of 0.26 mg/m^3, essentially the same as the POD_{HEC} derived for bronchiolization. The lower POD, 0.26 mg/m^3, was chosen as the POD. A composite UF of 30 was applied to the POD_{HEC}, resulting in an RfC of 9×10^{-3} mg/m^3. The composite UF included an interspecies UF of 3 for animal-to-human pharmacodynamics extrapolation, because an HEC pharmacokinetic adjustment had already been incorporated. An intraspecies UF of 10 was applied to allow for sensitive human populations, and a database deficiency UF of 1 was used.

Confidence in the principal study is high because it was a 2-year bioassay with a sufficient number of animals, and it is reasonable to assume that the endpoint is relevant to humans. Confidence in the database is rated high due to the existence of a 2-year inhalation study, a two-generation reproductive and developmental toxicity study, and a subchronic inhalation study. The overall confidence in the RfC evaluation is medium due to a concern that a NOAEL was not identified for the incidence of bronchiolization of the alveoli in all exposure groups.

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 (U.S. EPA, 1994c).

6.2.4. Inhalation Cancer Risk

The mode of carcinogenic action of nitrobenzene remains poorly understood but, based on available studies, is not likely due to mutagenicity. 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. According to the *Guidelines for Carcinogen Risk Assessment*

(U.S. EPA, 2005a), the default approach in such a case is to use a linear dose extrapolation approach. Nitrobenzene caused tumors in multiple organs in both sexes in two species (rat and mouse) and in two different strains of rats in a 2-year inhalation study (Cattley et al., 1994; CIIT, 1993). Nitrobenzene increased the incidence of 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. IURs were developed for each of these tumor types, and liver tumors appear to be the most sensitive tumor type. The IUR for liver tumors is 2×10^{-5} $(\mu g/m^3)^{-1}$. Composite IURs that account for all tumor types were also developed using two approaches—a Bayesian approach and a tumor-bearing animal approach. Both approaches provided an IUR of 4×10^{-5} $(\mu g/m^3)^{-1}$, two fold higher than the IUR based on liver tumors alone. The recommended upper bound estimate on human extra cancer risk from continuous lifetime inhalation exposure to nitrobenzene is 4×10^{-5} $(\mu g/m^3)^{-1}$, an estimate that reflects the exposure-response relationships for liver, thyroid, and kidney cancer.

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APPENDIX A: SUMMARY OF EXTERNAL PEER REVIEW AND PUBLIC COMMENTS AND DISPOSITION

The *Toxicological Review of Nitrobenzene* has undergone formal external peer review performed by scientists in accordance with EPA guidance on peer review (U.S. EPA, 2006a). The external peer reviewers were tasked with providing written answers to general questions on the overall assessment and on chemical-specific questions in areas of scientific controversy or uncertainty. A summary of significant comments made by the external reviewers and EPA's responses to these comments arranged by charge question follow. In many cases the comments of the individual reviewers have been synthesized and paraphrased in development of Appendix A.

EXTERNAL PEER REVIEWER COMMENTS

A. General Comments

1. Is the *Toxicological Review* logical, clear and concise? Has EPA objectively and transparently represented and synthesized the scientific evidence for noncancer and cancer hazard?

Comment: Most reviewers agreed that the presentation, for the most part, is logical, clear, and transparent. Some reviewers commented that editorial corrections of typographical errors will be needed, including explanation of certain qualitative descriptors, such as "significantly" or "substantial amounts."

Response: Typographical errors were corrected and the use of qualitative descriptors, such as "significant(ly)" or "substantial amounts," was explained according to the context in the respective cited literature.

Comment: One reviewer commented that "readability of the document" would be improved by providing, at the end of each section, summaries of key points and comparisons across studies in addition to graphical presentation of tabular data to help follow dose-response patterns.

Response: In addition to the study-by-study tabular presentation of findings, summaries by type of study, duration, and/or route of exposure, including dosing regimens, key findings, and NOAEL/LOAEL, were already provided throughout section 4 (see for instances Tables 4-16, 4-18, 4-19, 4-23, 4-28, 4-34, and 4-36). Sections 4.5 and 4.6 are summaries of the noncancer

and cancer studies presented in the earlier parts of section 4. Graphical exposure-response arrays were added to section 5 as Figures 5-1 and 5-2.

Comment: One reviewer commented on the need to have additional discussion or a checklist of criteria for selecting principal studies.

Response: A list of reasons for selecting the 90-day gavage study for deriving the RfD was added at the end of section 5.1.1. As specified under sections 5.2.1 and 5.3, the 2-year inhalation study was chosen for the RfC and cancer assessments because it was the only lifetime study by this route and it included both sexes of two species with large group sizes and properly spaced exposure levels.

Comment: Another reviewer commented that the document had no information on exposures in the workplace or in the environment and recommended reviewing some of the "workplace biomonitoring and somewhat older foreign studies," including epidemiologic data on other structurally related nitro aromatics, and describing concentration levels that were found at different sites and in different matrices. No specific information on the recommended studies was provided.

Response: Information on environmental releases of nitrobenzene and potential exposure through food was added to section 2 of the *Toxicological Review*. Information on exposures through air and water in the workplace or the environment and epidemiologic data on related nitroaromatic compounds were either not found or lacked data on health effects associated with the exposures, making it unsuitable for drawing conclusions about health hazards. A number of published case reports of nitrobenzene poisoning are summarized in section 4 of the *Toxicological Review*; however, there are no reports of epidemiologic studies of the human health impacts of nitrobenzene exposure in the workplace or environment.

2. Are you aware of additional studies that should be considered in the assessment of the noncancer and cancer health effects of nitrobenzene?

Comment: Three reviewers said they were not aware of additional studies but one of the three urged the authors to check on and provide a discussion about additional workplace/epidemiologic literature studies from foreign countries as was recommended by another reviewer. Another commented that some information on use patterns and potential exposures might be useful.

Response: The scope of IRIS assessments generally includes only the hazard identification and dose-response portions of the risk assessment, which users combine with exposure information to conduct a risk assessment. Biomonitoring data and ambient concentrations are usually included in IRIS assessments only insofar as they are part of an epidemiology study that also provides response data, or they illustrate specific aspects of the hazard identification section such as toxicokinetic features of the chemical (e.g., measurements of metabolites in urine) or susceptible subpopulations (e.g., measurements in breast milk indicating high infant exposure). Consequently, much of the recommended literature is outside the scope of this assessment. EPA obtained a bibliography from the reviewer but did not find any relevant studies on the list that were not already cited in the *Toxicological Review* except for some early studies from the 1950s and 1960s that are not available in English.

Comment: One reviewer suggested having additional coverage of background information on metHb production (not specifically for nitrobenzene) and the health effects associated with varying levels of metHb in the blood.

Response: Additional information on metHb formation and methemoglobinemia was added to the *Toxicological Review* as footnotes 1 and 3 in sections 3.2 and 3.3. Information on clinical effects in humans associated with metHb formation is summarized in sections 4.1 and 4.5.1.

Comment: Another reviewer cited the ability of nitroaromatics to directly produce metHb without activation by microsomes or reducing/anaerobic environment as described in a reference on dinitrobenzene by Vasquez et al. (1995).

Response: A discussion of the possibility of direct metHb generation by nitroaromatics was added to the *Toxicological Review* as footnote 5 in section 3.3.1.

Comment: One reviewer raised additional issues, including possible alterations in absorption due to changes in gut morphology in the germ-free antibiotic-treated animal (see Heneghan [1984]), and questioned the validity of the assumption that reactive intermediates pass through intestinal membranes rather than preferentially react with the gut contents.

Response: The issue of absorption (bioavailability) of nitrobenzene in germ-free animals is discussed in text added to section 3.3.1 of the *Toxicological Review*. Information on gut morphology and function of the alimentary tract in relation to germ colonization was evaluated in the cited reference (Heneghan, 1984) but was considered not to be informative with regards to possible alteration in nitrobenzene absorption in the germ-free animal.

As discussed in section 3.2, nitrobenzene or its metabolites were widely distributed among the major organs and tissues following [^{14}C]-nitrobenzene administration in rats where the highest levels were in blood, apparently due to covalent binding to hemoglobin and plasma proteins (Albrecht and Neumann, 1985; Goldstein and Rickert, 1984). While reactive intermediates of nitrobenzene may also likely react with gut contents, these findings as well as others on systemic toxicity and metabolism in germ-free and conventional animals clearly show that nitrobenzene and its metabolites are appreciably absorbed following administration by the oral route.

B. Oral Reference Dose (RfD)

1. Is the selection of the NTP (1983) study as the principal study scientifically justified? Is the rationale transparently and objectively described?

Comment: Despite some reservations, all reviewers agreed that the NTP (1983a) study is the most relevant and is well justified. Two reviewers indicated that the transparency and rationale for the selection could be improved by comparing/discussing (in section 5.1) this study with other candidate studies, including the lower doses used in a shorter-term (28-day) study. Other reviewers wanted a more focused discussion of how the study was selected.

Response: The considerations listed for evaluating studies outlined in U.S. EPA (2002a) were used to select the principal study for deriving an RfD for nitrobenzene. The rationale for selecting the 90-day gavage administration study (NTP, 1983a) was added at the end of section 5.1.1. A discussion of Shimo et al. (1994), the 28-day study, was added to section 5.1.1. The LOAELs and NOAELs reported for selected endpoints in Shimo et al. (1994) and NTP (1983a) are provided in Table 4-16 and Figure 5-1.

Comment: One reviewer expressed concern about using "a one-time bolus dose" in a gavage study as being unrepresentative of what may be observed following actual continuous exposures as in the 2-year inhalation bioassay. However, the reviewer conceded that route-to-route extrapolation from the inhalation study would be very difficult in the absence of well-characterized PBPK modeling for nitrobenzene. Therefore, using the NTP (1983a) 90-day oral study is scientifically justified, especially since there was good agreement between routes of exposure and toxicity outcomes.

Response: EPA agrees with the comment on the difficulty of route-to-route extrapolation from the inhalation study. These comments are captured under the material that was added to section 5.1.1 on selection of the principal study.

Comment: One reviewer thought that there may be room for additional coverage of the influence of route of exposure or organ on nitrobenzene metabolism and distribution including organ-specific metabolizing enzymes and reactions.

Response: As summarized in section 3.3 of the *Toxicological Review*, information on nitrobenzene metabolism is derived from nitrobenzene metabolite identification in the urine from humans and animals and from in vitro metabolism by liver microsomes or erythrocytes. The metabolite findings from these studies indicate that oxidative (possibly cytochrome P450 dependent) and reductive pathways, including conjugation, are involved but do not yield information on tissue-specific roles of different metabolizing enzymes. The *Toxicological Review* also covers in vitro studies on types I and II (oxygen-insensitive and oxygen-sensitive) nitroreductases in various tissue homogenates by following nilutamide (a nitroaromatic drug) reduction ($R\text{-}NO_2 \rightarrow R\text{-}NH_2$) under aerobic and anaerobic conditions. Although nitrobenzene was not studied, the findings on nilutamide demonstrated that the highest levels of nitroreductases are in intestinal microflora, but various organs also have the same activities. However, there are no specific studies on the influence of route of administration (e.g., oral versus inhalation) on formation of nitrobenzene metabolites and on route-dependent pathways or involvement of other metabolizing enzymes.

2. Splenic congestion (increased by 10%), methemoglobin levels (increased by 1 SD), and reticulocyte count (increased by 1 SD) relative to control values serves as the basis for the RfD. Is the selection of the splenic congestion, metHb levels, and reticulocyte count as the co-critical effects for deriving the RfD scientifically justified? Has the rationale for selection of these critical effects been transparently and objectively described? Is it appropriate to derive point of departure by averaging BMDLs across sexes and co-critical effects?

Comment: Three reviewers agreed with the three selected endpoints, including rationale, justification, and averaging of BMDLs. One reviewer agreed with the choice of endpoints but commented that biological justification for the selection of BMR should be provided and that more explanation is needed for averaging the BMDLs. Another reviewer provided extensive comments on data presentation and BMD modeling used to derive the POD.

Response: The rationale for the selection of the three endpoints was expanded to clarify the likely interdependence of the observed effects. The data considered for RfD derivation were also summarized in section 5.1 and in the relevant appendices.

The individual animal data were obtained to determine whether splenic congestion (and other histopathologic lesions) had been only noted as present (as analyzed for the *Toxicological Review*) or graded, since this could affect the BMR. Splenic congestion had been graded; control

rats of both sexes had none or only minimal congestion (grade 1), while congestion increased in severity up to moderate in the most highly exposed rats, including those that died early in the study. Footnotes were added to clarify how many animals with this finding died early. The BMR for splenic congestion was reconsidered in terms of having at least mild congestion (grade 2). The *Toxicological Review* was revised to take into consideration the severity of splenic congestion.

Individual hematology data were examined to assist in addressing the comments regarding heterogeneous variances of the continuous variables. There were no apparent reasons to consider excluding any of the individual values for the highlighted endpoints as outliers. The modeling was revised to consider only monotonic dose-response shapes and the reviewer suggestions for modeling variances. Model results were generated for all cases previously missing results.

EPA disagrees with the comment on the necessary degree of consistency of the fitted models with limited data in the high-dose group(s). Even with increased consistency accomplished by using the monotonic dose-response shapes, it is more important to fit the data at the lower end of the dose response, where mortality did not have as obvious an impact on the responses. Consequently, a number of model fits dropped the high dose. These instances were identified more clearly.

Regarding biological justifications for BMR selection, it is not clear what level of effect constitutes a minimal biologically significant degree of change for the metHb and reticulocyte effects in rodents and how changes in these effects relate quantitatively to humans. The NTP has regarded similar changes in rat metHb to be biologically significant (e.g., p-chloroaniline [NTP, 1989]). However, additional analyses and text were added in section 5.1 to address the biological significance of metHb formation and issues related to endogenous levels in humans. Methemoglobin changes in male rats were identified as the most sensitive effect. Accordingly, the critical effect was changed to reflect a reliance on metHb data in male rats with a POD based on a 1SD increase from the control mean.

Comment: One reviewer stated that it would be useful to include "a clear and transparent definition and description of the adverse consequences of splenic congestion," since the pathophysiological consequences of "brown pigmentation" and "red pulp" mentioned in section 4.2 are not clear. This reviewer also requested further explanation of how increased metHb and reticulocyte count are considered adverse.

Response: These comments were addressed by providing appropriate discussion or footnotes in section 4.2.1.1.

Comment: One reviewer suggested consideration of the reversibility of the selected endpoints upon reduction or discontinuation of exposure and the compensatory response following extended exposure.

Response: In the NTP (1983a) study, there is no information on reversibility of the endpoints that were selected for the RfD. However, changes in hematologic parameters, including methemoglobinemia, as well as increased spleen weight were reversed after discontinuation of exposure in other studies, including the 28-day gavage administration study (Shimo et al., 1994) and the 2-week inhalation studies by DuPont (1981) and Medinsky and Irons (1985). The following is a further discussion of the issues of reversibility and possible compensatory response to methemoglobinemia while keeping in mind that other related findings, including hemolytic anemia, reticulocytosis, and spleen congestion (or splenomegaly), are interrelated in the sense that they are all indicative of erythrocyte toxicity by nitrobenzene or other nitroaromatic and aromatic aniline chemicals.

As discussed in the *Toxicological Review*, metHb, which is an abnormal form of Hb with diminished affinity for oxygen, is formed and metabolized back to Hb at roughly equal rates under physiological conditions. Methemoglobinemia may develop when generation of metHb, following for instance exposure to a chemical, exceeds the normal compensatory physiological reductive capacity within erythrocytes mainly carried out by the NADH-dependent cytochrome b5 metHb reductase. After discontinuation of exposure, methemoglobinemia may be reversed as evidenced, for instance in the study by Medinsky and Irons (1985), after 14 days of discontinuation of exposure to nitrobenzene (section 4.4.1, including Table 4-35). Reversibility of methemoglobinemia also seems to take place in humans following discontinuation of exposure and treatment with methylene blue and ascorbic acid as summarized in some of the case reports (section 4.1). However, reversibility may be hampered in some individuals due to congenital deficiencies in the reductase enzyme or in G6PD (see sections 3.3 and 4.7.3).

The issue of a possible compensatory response to methemoglobinemia was addressed in section 4.2.2.2 and under footnote 10 of the *Toxicological Review*, where it is concluded that it is difficult to convincingly identify a compensatory response in the chronic inhalation study, and there is no known specific information in the published literature that addresses this topic. A similar comment on the possibility of a compensatory response to methemoglobinemia in relation to selecting a UF of 3 for less-than-lifetime exposure in the principal oral study was also raised and responded to in the first comment under RfD question 4 (Q4).

Finally, an RfD is an estimate of a daily oral exposure that is likely to be without appreciable risks to health during a lifetime, which implies that the RfD includes continuous lifetime exposures.

3. Are the uncertainty factors applied to the point of departure for the derivation of the RfD scientifically justified and transparently and objectively described?

Comment: Two reviewers agreed that the type and value of the selected UFs appeared to be transparently and objectively described but one considered the total value of 1,000 to be relatively large and suggested that considering data on relative animal-to-human toxicity for structurally similar compounds might help mitigate some of the uncertainties. Two reviewers questioned applying the UF for subchronic-to-chronic exposure or using an interspecies 10-fold UF, which implies that humans are more sensitive to metHb than are rodents. Some of the suggestions included (1) providing a rationale with direct dose-response comparison between rodents and humans regarding sensitivity to metHb by estimating the ingested dose from nitrobenzene human ingestion studies or utilizing clinical data on oxidatively damaging drugs and (2) including information on what is known about drug sensitivity due to G6PD deficiency.

Response: In the absence of comparative chemical-specific data, humans are generally assumed to be more sensitive than animals on a mg/kg-day basis, based on their relative size. Application of a full UF of 10 is warranted to account for uncertainty in extrapolating from laboratory animals to humans based on two areas of uncertainty, namely toxicokinetic and toxicodynamic differences. There are no available in vivo data that compare human sensitivity relative to that of rodents towards metHb induction by nitrobenzene or related nitroaromatic chemicals. Also, it is not feasible to compare doses from existing human toxicity reports based on a single ingestion exposure on the order of grams to chronic doses of nitrobenzene in the microgram to milligram range used in animal studies. EPA's practice is that, unless data support the conclusion that the test species is more or equally as susceptible to the pollutant as humans and in the absence of any other specific toxicokinetic or toxicodynamic data, a default factor of 3 (in conjunction with HEC derivation) or 10 is applied (U.S. EPA, 2002a).

In a comparative study using in vivo and in vitro techniques, Tingle et al. (1997) concluded that humans are more sensitive than rats or mice to hematotoxicity (mainly metHb formation) from dapsone (diaminodiphenyl sulfone), an antibacterial and anti-inflammatory drug used to treat leprosy and certain skin conditions.

Species differences were also described in the activity of red cell spontaneous (NADH) metHb reductase with rat, mouse, guinea pig, and rabbit having higher activity than humans, while horse and pig have lower rates than humans (Smith, 1996). Based on this review, rodents are more apt to repair metHb than are humans, since the relative erythrocyte metHb reductase activity between rats and humans ranged from 1.3- to 5.0-fold and between mice and humans was 9.5-fold. Also, the rat is nearly twice as efficient as humans in the methylene blue stimulated (NADPH) erythrocyte metHb reductase activity (Smith, 1996). In the absence of actual data, other pharmacokinetic differences between rodents and humans may also exist.

Therefore, the 10-fold UF for extrapolating from rats to humans was retained.

The issue of applying a subchronic to chronic UF of 3 in relation to lack of apparent worsening methemoglobinemia was also raised and responded to in the last comment under RfD Q2 and in the first comment under RfD Q4.

Comment: One reviewer suggested factoring in "the species differences among rodents and the dependence on gut flora," and questioned the rationale for applying a factor of 10-fold for animal-to-human extrapolation for the oral RfD and only threefold for the inhalation RfC.

Response: The response to the previous comment addressed the issue of applying a 10-fold factor for animal-to-human extrapolation for deriving an RfD. Regarding possible species differences in gut microflora, there is no information that indicates that metabolism of nitroaromatics by gut flora is specific to rodents.

As stated in section 5.2.4, the interspecies UF of 10 to extrapolate from animals to humans would account for two areas of uncertainty, namely toxicokinetic and toxicodynamic differences, each of which comprises $10^{0.5}$ of the total UF. Based on the applied RfC methodology (U.S. EPA, 1994), the toxicokinetic component was addressed by the HEC dosimetric adjustment from an animal-specific $LOAEL_{ADJ}$ to a human $LOAEL_{HEC}$. Therefore, a UF of 3 was applied to account for the toxicodynamic uncertainty in derivation of the RfC. There is little information to inform nitrobenzene toxicokinetics via the oral route; consequently, the toxicokinetic portion of the UF is retained.

Comment: Another reviewer answered "no" and suggested providing documentation of guidelines or criteria of how UFs were selected in order to improve the transparency.

Response: A review document on the RfD and RfC by the Risk Assessment Forum Technical Panel (U.S. EPA, 2002a) discusses derivation of reference values, which takes into consideration and accounts for five areas of uncertainty or variability in the available data by assigning a factor (UF) for each. The exact value of each UF depends on the quality of the studies, the extent of the database, and scientific judgment. The five areas of uncertainty/variability for deriving an RfD and RfC were identified in section 5 (sections 5.1.3 and 5.2.4, respectively) and justification was provided for each selected UF, including revisions based on the external peer review comments.

4. An uncertainty factor of 3 was selected to account for less-than-lifetime exposure in the principal oral study. Is the choice of this UF scientifically justified and transparently and objectively described?

Comment: One reviewer provided no comments and another indicated insufficient familiarity with the use of UFs to comment while two reviewers agreed that using a UF for less-than-lifetime exposure is reasonable or is consistent with Agency policy. Two of the four reviewers suggested discussing the basis for the selection or describing the guidelines that were followed. Three reviewers questioned the need for a UF for lifetime extrapolation from a subchronic study, indicating that the extent of methemoglobinemia seemed to be independent of exposure duration in the inhalation studies, with one reviewer finding the selection rationale "to be a bit problematic." The same reviewer also cited an apparent selectivity in presenting the rationale for applying the factor based on cited route-specific metabolite formation (under section 5.1.3) compared to formation of nitrosobenzene from inhaled nitrobenzene in Figure 3-8. This reviewer also questioned why a similar compensatory response to methemoglobinemia, as the one seen in the inhalation studies (discussed in Appendix B-2), would not be operative when nitrobenzene is administered orally. The second reviewer stated that "it doesn't seem likely that additional toxicity not identified in the sub-chronic studies would occur in the chronic bioassays." The third reviewer stated, "There is the experience with the inhalation studies that suggests no such factor is needed."

Response: Considerations for deriving reference values and selection of UFs are discussed in the response to a similar comment under the previous question (RfD, Q3).

EPA agrees with the comment that formation of the nitro anion free radical and nitrosobenzene are not route specific; however, inadequate data exist from the oral route to evaluate the formation of these metabolites and greater concentrations may be generated following exposure by the oral route compared to exposure via inhalation due to gut bacterial microflora activation (nitro reduction). A similar comment was also made by a reviewer under RfD Q5. Section 5.1.3 was modified to resolve apparent inconsistencies.

As explained in section 4.2.2.2 and in the response to a comment on a compensatory response under charge question 2 of the section on the oral RfD, the extent of methemoglobinemia as a function of extended exposure in humans remains largely unknown. According to the data in Table 4-20, metHb levels were not increased when exposure to nitrobenzene via inhalation was extended from 15 months (interim sacrifice) to 24 months (terminal sacrifice) in both rat strains. Additionally, other toxicity endpoints may result from chronic oral exposure due to route-specific differences in metabolism, pharmacokinetics, and/or pharmacodynamics that were not observed in the subchronic oral study or in the inhalation studies. Therefore, a subchronic-to-chronic UF of 3 is appropriate to account for the uncertainty regarding the compensatory response and for potential additional effects, other than the above-mentioned hematologic endpoints, that might occur with chronic exposure.

5. An oral database uncertainty factor of 3 was applied. The database of oral studies includes the principal study (NTP, 1983b) (*sic*), 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 comparing nitrobenzene to dinitro- and trinitrobenzene; and a multidose immunological study in mice (Burns et al., 1994). However, due to a lack of an oral multigeneration reproductive toxicity study and in light of evidence of male reproductive toxicity, a factor of 3 was applied. Is the choice of an UF of 3 scientifically defensible given the available oral and inhalation databases? Does the available data suggest that the oral exposures may result in new adverse effects at oral doses equivalent to or lower than the inhalation concentrations used in the multigeneration reproductive and developmental study by Dodd et al. (1987)?

Comment: One reviewer indicated that weighing scientific information and presenting a number of reasons for choosing a UF of 3 strengthened EPA's discussion on deriving an oral RfD. Another reviewer stated that a UF may be justified based on enhanced gut bacterial metabolic activation (nitro reduction), resulting in higher concentrations of different active metabolites than would be possible by inhalation exposure, which makes it hard to predict oral versus inhalation potential long-term effects on reproduction. Both of these reviewers, however, reiterated the need to specify the guidance used by EPA for selecting the UFs.

Response: Refer to the response given under RfD Q3 on a similar comment on EPA guidance for selecting UFs.

Comment: One reviewer stated: "At most, I would suggest a factor of 3 for these two UFs combined (i.e., subchronic to chronic extrapolation and database deficiency), pending more complete investigation of the route-to-route extrapolation that might allow a fuller integration of the oral and inhalation databases." This reviewer also cited a lack of major differences in metabolism or systemic response between oral and inhalation exposures.

Response: Route-to-route extrapolation from the inhalation study would be difficult in the absence of a well-characterized PBPK model for nitrobenzene. It is also difficult to calculate dose equivalency between oral and inhalation routes in the absence of an equivalent metric for the measured changes between the routes. Two reviewers offered a similar opinion (under RfD Q1 and RfD Q5) that comparison of dose equivalency between the oral and inhalation exposure routes was not feasible.

Furthermore, there are knowledge gaps in route-specific comparative metabolism or systemic responses that prevent a determination of whether differences exist following chronic exposure to nitrobenzene by the oral versus inhalation routes. The database and subchronic-to-

chronic UFs are intended to address two different uncertainties. The database UF is intended to account for the potential for deriving an underprotective RfD/RfC as a result of an incomplete characterization of the chemical's toxicity Another UF is applied when a study of subchronic duration is used for a chronic exposure scenario. It accounts for the uncertainty associated with the possibility that other or more severe effects might have been observed if the duration of exposure was longer.

Comment: One reviewer questioned the need for a database UF since reproductive toxicity occurred at a dose greater than that used for setting a POD.

Response: EPA agrees that in the one-generation oral study by Mitsumori et al. (1994) reproductive toxicity (specifically testicular pathology and male fertility) was a less sensitive endpoint than other endpoints (such as spleen or liver weights or hematology). However, when an RfD or RfC is based on animal data, EPA generally applies a database UF of 3 if either a developmental (prenatal) toxicity study or a two-generation reproduction study is missing or a factor of 10 if both are missing (U.S. EPA, 2002a). EPA is not aware of specific data to suggest that reproductive effects may worsen with extended mating in a two-generational study; however, EPA similarly has no data to establish that reproductive effects would not occur at lower doses following multigeneration exposure. In light of this uncertainty, EPA considers a database UF of 3 to be appropriate.

Comment: A reviewer commented that it is unlikely that oral exposures may result in new adverse effects at oral doses equivalent to or lower than the inhalation concentrations used in the multigeneration reproductive and developmental study by Dodd et al. (1987). This reviewer also commented that "it is difficult, if not impossible, to calculate dose equivalence between oral and inhalation routes without a precise basis and understanding for the mechanism of action for the biologic effect and for an equivalent metric for the measured changes between the routes."

Response: The issue of the feasibility of route-to-route extrapolation from the inhalation study in the absence of well-characterized PBPK modeling for nitrobenzene was discussed earlier (see comment under RfD Q1). It is not possible to determine oral equivalent doses to the inhalation concentrations of the multigeneration reproductive study. Also, in the absence of a multigeneration reproductive study by the oral route, it is difficult to predict the type or extent of adverse outcomes in parental animals or their offspring.

C. Inhalation Reference Concentration (RfC)

1. Is bronchiolization of the alveoli the most scientifically justifiable endpoint on which to base the RfC? Have the rationale and justification for this selection been transparently and objectively described? Are there any other studies that you believe would be justified scientifically as the basis for the RfC?

Comment: None of the reviewers suggested other studies for deriving the RfC. Two reviewers were concerned that bronchiolization of alveoli may not be the most justifiable endpoint or that scientific justification was not provided. Another reviewer disapproved (saying "no") of the choice, adding that "this endpoint is highly species-specific." Two other reviewers considered the selection appropriate but recommended further discussion of possible species specificity and relevance to humans. Additional specific concerns raised by one or more of these five reviewers were difficulty in distinguishing bronchiolization as a noncancer endpoint, likelihood that it is a pre-neoplastic event, unknown relevance to humans, lack of information on whether the effect on lung tissue was the result of metabolism and thus requiring information on metabolic competencies of lung cell types, and whether a similar finding may occur in humans.

Of the remaining two reviewers, one stated that the chosen endpoint is valid based on the route-specific respiratory outcome and the last reviewer indicated that the discussion of the reasoning for choosing bronchiolization was good but still found it "hard to completely dismiss the methemoglobinemia that is concordant with effects seen in the oral studies."

Response: There is no available information on metabolism of nitrobenzene in mice and human lung tissue. Alveolar bronchiolization, which may be a metaplastic change or a colonization process of alveolar walls with bronchiolar epithelium, has been identified in response to particulate irritants and oxidant gases in several studies with various species, including humans. Highlights of these findings, including lack of association between alveolar bronchiolization and lung neoplasia, were added to section 4.5.2. This endpoint was retained for deriving an RfC, and additional language was incorporated under section 5.2.1. The revised Sections 4.5.2 and 5.2.1 address the concerns that were raised regarding the relevance and justification of bronchiolization as an endpoint for RfC.

Additional internal review clarified that the olfactory epithelial degeneration and loss in female mice was at least as sensitive an endpoint, in terms of expected human equivalent concentration, as bronchiolization. The RfC derivation has been modified to include olfactory degeneration as a co-critical endpoint.

2. If bronchiolization of the alveoli is the most scientifically justifiable endpoint on which to base the RfC, is the LOAEL-to-NOAEL approach the best method for deriving the RfC?

Comment: Two reviewers disagreed with the approach, citing difficulty or inability to compare bronchiolization to other endpoints in the analysis with one suggesting that BMD modeling can still be done, since not all doses gave a 100% response. Another reviewer commented that, although using a LOAEL seemed odd, it was difficult to see how BMD modeling could be used and recommended some discussion about the flat dose-response pattern and about considering bronchiolization possibly to be a systemic effect.

Response: On further review, neither approach is clearly superior for the bronchiolization data. Consequently, both BMD modeling and the NOAEL/LOAEL approach are considered in the document. The BMDLs for male and female mice were lower than LOAEL/10 (5 ppm/10, or 0.5 ppm) at about 0.1–0.3 ppm. Neither approach validates the other, but for the purpose of developing a reference value they do not suggest radically different starting points. Considering the possibly greater crudeness of deriving a suitable POD by dividing a LOAEL by 10, the assessment has been revised to use BMD modeling to characterize the bronchiolization data.

Second, the bronchiolization data were problematic to fit with available models because the response at the low end of a plateau of responses like this one is often underestimated. This was true for the male mice data, while some of the models did fit the female mice data adequately. Regardless, there is no way to tell how far into the lower exposures the plateau may really extend for either data set. On the other hand, the study report did note that the severity of bronchiolization was dose related, increasing with increasing exposure. Since no data clarifying severity grading were provided, it is not clear how much of an impact considering severity would have on the dose-response relationship, although it is possible that the reported low exposure response was overstated relative to that at the higher exposures. Given these two divergent possibilities, use of the modeled response is a reasonable compromise. This discussion has been summarized in the assessment.

If it can be assumed that bronchiolization is a systemic effect rather than a site-of-contact effect, then EPA's current RfC methodology leads to a lower RfC based on bronchiolization than developed in the external peer review draft. Nitrobenzene does have systemic effects, so this assumption is reasonable to consider. If nitrobenzene's impact on the lung is directly proportional to administered concentration, and due only to systemic distribution of a metabolite, then the RfC would be estimated at about threefold lower than an RfC based on site-of-contact effects. This discussion has been summarized in the assessment.

Comment: Another reviewer cited several issues for further discussion, including the observance of bronchiolization in rats, loss of animals and its impact on the assessment, providing a better definition for bronchiolization (see Appendix R of CIIT [1993]), and including information on anatomical or species differences based on its occurrence in mice.

Response: Bronchiolization or lung tumors have not been observed in two strains of rats. Relevant issues on bronchiolization, including the anatomical and species differences in mice, were addressed in the response to the comments of the previous charge question (RfC Q1). A discussion was added to the *Toxicological Review* (sections 4.2.2.2 and 5.2.1) on animal survival to term. A new definition of bronchiolization was also added (section 4.2.2.2).

Comment: Of the remaining three reviewers, one approved of the LOAEL-to-NOAEL approach "based on the data and Agency policy"; another cited a lack of sufficient expertise in modeling analysis to comment on this question but referred to the earlier comment that "bronchiolization was not the best endpoint for these analyses"; and the last reviewer stated that "the only comment would be that the dose response for this endpoint was pretty quirky (plateau). As such, the extrapolation might involve even more uncertainty than usual."

Response: The comment that bronchiolization was not the best endpoint to use was addressed under an earlier question (RfC Q1) as was the issue regarding the dose-response curve for bronchiolization.

3. A database UF of 1 was applied in deriving the RfC because the database includes 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 two 90-day inhalation studies. Is the application of a database UF of 1 scientifically defensible and transparently and objectively described given the available data for nitrobenzene?

Comment: All reviewers agreed that the selected UF of 1 is reasonable, appropriate, or justified, but one reviewer recommended additional discussion and justification for asserting that the literature is complete when the analyses are based on a few limited studies. Another reviewer commented on "the inter-connectedness of the database UF for inhalation and for oral routes of exposure" and indicated that the UF choices will be improved if, to the extent possible, these can be integrated. One reviewer recommended discussing the confidence in choosing a UF of 1 in addition to the already discussed rationale for having a complete inhalation database.

Response: Section 5.2.4 was modified, and additional discussion was added regarding completeness of the database as well as rationale and confidence in selecting a database UF of 1.

D. Carcinogenicity of Nitrobenzene

1. Under EPA's 2005 *Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgr-d.htm), nitrobenzene is classified as *likely to be carcinogenic to humans*. Have the rationale and justification for this designation been transparently and objectively described? Do the available data support the conclusion that nitrobenzene is a likely human carcinogen? If the weight of the evidence supports the descriptor *likely to be carcinogenic to humans*, is it appropriate to describe nitrobenzene as a case that lies on the low end of the range of this descriptor?

Comment: Most reviewers agreed with the classification of carcinogenicity but two reviewers recommended having additional discussion and justification, including description of criteria and explanation of why "suggestive" was not used instead. One reviewer provided no comment on this question.

One reviewer concurred with the statement that nitrobenzene "lies at the low end of the range" for the "likely to be carcinogenic in humans" descriptor. Four reviewers remarked that the meaning of the statement was not clear, citing the need for additional information on the basis of ranking and how it was arrived at, including examples and comparisons with other chemicals. One reviewer questioned the designation "at the low end of the range" based on uncertainties about the MOA and findings of tumors at multiple sites in two species with some showing clear dose-response relationship.

Response: The considerations used to assign the descriptor are listed in section 2.5 of the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a). The carcinogenicity descriptor "likely to be carcinogenic to humans" was used rather than "suggestive evidence of carcinogenicity" because nitrobenzene induced tumors in two species and sexes of laboratory animals, two strains of rats (F344 and male CD), and at multiple sites. The "suggestive evidence of carcinogenicity" descriptor covers a spectrum of evidence associated with lower levels of concern for carcinogenicity such as a single positive cancer result (in one species) in an extensive database that includes negative studies in other species. Upon further evaluation, the text indicating that the designation lies at the low end of the range of the "likely descriptor" has been removed. The text in sections 4.6.1 and 6.1.6 has been augmented to better describe the designation.

2. The two-year inhalation cancer bioassay (CIIT, 1993; published as Cattley et al., 1994) was used for development of an inhalation unit risk (IUR). Is this study the most appropriate selection for the principal study? Has the rationale for this choice been transparently and objectively described?

Comment: All reviewers that commented on this question agreed that the selection was appropriate, with some reviewers indicating that it was the only viable choice due to lack of alternatives. Three reviewers stated that the rationale and/or justification were well documented or well described. One reviewer requested more information on how the study was selected, while another indicated that including additional information from the original study (e.g., design and animal loss) would improve transparency.

Response: EPA has followed the guidelines for evaluating cancer bioassays presented in section 2 of the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a). The 2-year inhalation cancer bioassay, conducted by CIIT (Cattley et al., 1994; CIIT, 1993) is the only study available. It is a well-conducted lifetime exposure study that evaluated two species of both sexes in laboratory animals. The study design and reporting are stated as meeting acceptable GLP standards. Ranges of exposure concentrations were properly selected based on a subchronic study by the same route. The study design is described in section 4.2.2.2. Additional information on study design including animal survival has been added to the *Toxicological Review* (sections 4.2.2.2 and 5.2.1).

Comment: A reviewer indicated that further discussion is needed for "the lack of consistency with the oral studies, even for systemic endpoints."

Response: There are no oral cancer bioassays available. In general, there seems to be good agreement in the noncancer toxicity outcomes following oral and inhalation exposure of rats and mice to nitrobenzene. For instance, irrespective of the exposure route, among the most common findings in the 90-day inhalation and gavage studies were methemoglobinemia, hemolytic anemia, and target organ toxicity in spleen, liver, kidney, and testis (CIIT, 1984; NTP, 1983a). However, there were portal-of-entry findings specific to exposure via the inhalation route, including bronchial hyperplasia among F344 rats (both sexes) and nasal lymphoid hyperplasia/inflammation in CD rats (CIIT, 1984).

3. Data on hepatocellular tumors in F344 rats were used to estimate the IUR. Are the reasons for basing the quantitative assessment on hepatocellular tumors in male F344 rats scientifically justified and transparently described? For calculating the IUR, adenomas and carcinomas were combined. Has EPA's justification for this approach been objectively and transparently presented? Is combining adenomas and carcinomas the most scientifically justifiable approach for these tumors? Please suggest any other scientifically justifiable approaches for calculating the IUR.

Note: This charge question overlaps somewhat with Q4 below in that both questions concerned interpreting multiple tumor types in rats for extrapolation to humans and asked if the risk estimate should be based on hepatocellular tumors only or if there are other methods for deriving the IUR. For clarity, issues concerning the interpretation of the hepatocellular tumors alone are addressed here, while those concerning total risk, involving additional tumor types, are addressed in the comments and responses for Q4.

Comment: The reviewers generally agreed that hepatocellular tumors in rats were the most biologically significant result and generally agreed with combining hepatocellular adenomas and carcinomas for this assessment. One reviewer expressed no opinion, and, among the rest, three recommended providing additional justification or other information in the document. One of these three reviewers recommended clarifying why male rat hepatocellular tumors were selected by stating that this choice is the most health protective and recommended presenting only the combined modeling results instead of displaying separate modeling for each tumor type. Another of these reviewers recommended providing more of the raw data on the histology of hepatocellular tumors provided in the CIIT (1993) report, to help understand whether it is scientifically feasible to combine adenomas and carcinomas and to achieve greater scientific transparency. The third reviewer provided no specific comments.

Response: The document has been clarified to indicate that, when there are multiple significant tumor types and when there are no data to the contrary (such as, lack of human relevance for particular MOAs), total risk is one of the considerations weighed in recommending a risk value. The incidence data for the separate hepatocellular tumor types have been clarified and the modeling results have been removed.

Key nonneoplastic and neoplastic liver histopathology findings in F344 rats are included in Tables 4-21 and 4-41 of the *Toxicological Review*. Information has been added to section 5.3.1 on the histology of hepatocellular tumors, including an additional summary table (Table 5-5) of incidences of hepatocellular neoplasms, by type and combined, among terminally sacrificed F344 rats.

Comment: One reviewer critiqued the selection of different BMRs for different endpoints and suggested having some consistency across data sets by picking a BMR level that "corresponds to a response level likely to be within the range of the experimental doses."

Response: Although the use of different BMRs was intended to provide estimates of the low-dose slope most relevant for IUR derivation, there was no difference in IURs within each of these data sets whether the choice of BMR was 5 or 10%. The document has been revised to indicate that the highest IUR rather than the lowest BMDL denoted the most sensitive response,

which was hepatocellular tumors. However, all IUR derivations were revised to use a 10% BMR for consistency.

Comment: One reviewer suggested a different approach to quantifying cancer risk, commenting that the decision to use linear low-dose extrapolation appeared to be inconsistent with earlier statements in the document regarding the weak genotoxicity of nitrobenzene and that the predominately negative genotoxicity evidence was ignored. For this reason, he questioned the validity of estimating cancer risk based on calculating an IUR and concluded that, instead of using an IUR for estimating inhalation cancer risk, a POD approach should be used with a UF of 30–100. This reviewer also suggested that evidence on possible DNA damage (in section 4.6.3) from the in vitro study by Ohkuma and Kawanishi (1999) using calf thymus DNA and nitrosobenzene in the presence of Cu^{2+} was misrepresented and stated that, based on the study's findings, "the conditions under which nitrobenzene would induce DNA damage in vivo are unlikely to occur." This reviewer added that discussion of the study should probably be under the genotoxicity study description section (section 4.4.5).

Response: Following the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), EPA uses linear low-dose extrapolation when evidence is insufficient to establish the MOA for a tumor site, as in this assessment. EPA also notes that there is no empirical indication of low-dose nonlinearity in the observed range of rat hepatocellular tumors.

The summary (under section 4.6.3) of the in vitro study by Ohkuma and Kawanishi (1999) has been moved to section 4.4.5 and has been replaced with new language on the MOA.

4. The IUR was calculated from hepatocellular tumors in male F344 rats. The recommended upper bound estimate on human extra cancer risk from continuous lifetime exposure to nitrobenzene was calculated to be 3×10^{-5} $(\mu g/m^3)^{-1}$. Is it scientifically defensible to base the IUR on liver tumors alone? Have the rationale and justification for this analysis been transparently and objectively described? Is it more appropriate to calculate the IUR using combined tumor incidence of liver, thyroid, and kidney tumors in male F344 rats as done in the alternate derivation of the IUR in the Appendix? If summing of tumors is scientifically justified, is the method used to sum the tumors supported by the science and the data? If not, what alternative method should be used?

Comment: Three reviewers indicated a preference for using the most sensitive tumor site (male rat liver tumors) for the derivation of the IUR over combining the risks from three tumor sites. Two other reviewers did not reject considering total risk but criticized the estimation method used, listing pitfalls of combining unit risks from separate tumor types. One of these reviewers recommended an alternative analysis involving likelihood profile methodology. The

remaining two reviewers did not respond to this question, with one offering the same response as for Q3 regarding adenomas and carcinomas.

The rationale given for not combining different tumor types included that it may not make sense if they have different mechanisms and/or relevance to humans and that a lack of consistency in effects among rodent species and sexes would not support relevance to humans. Several reviewers noted that the additional tumor data did not increase the risk estimate when rounded to one significant digit, 3×10^{-5} $(\mu g/m^3)^{-1}$.

Response: EPA appreciates these comments and the opportunity to improve the transparency of the dose-response assessment. The charge question's unfortunate use of the phrases "summing tumors" and "combined incidence" may have caused confusion based on the responses to this question. "Summing tumors," suggesting a crude summing of the incidences before developing the quantitative result, was not a part of this analysis. "Combined incidence," which suggests that the incidence of animals with liver, thyroid, or kidney tumors was the basis of the quantitation, analogous to the consideration of hepatocellular adenomas and carcinomas discussed in Q3 above, also was not used for this analysis. As described in the external review draft assessment, it was the extra risk estimates for each site that were summed.

In cases of multisite carcinogens, it has been EPA's practice to characterize *total* risk, the risk that an individual could develop any of the critical tumors, not just the most sensitive tumor type. For example, see the final IRIS assessments for pentachlorophenol (U.S. EPA, 1993), bromate (U.S. EPA, 2001), 1,3-butadiene (U.S. EPA, 2002b), and 1,2-dibromoethane (U.S. EPA, 2004). However, none of the reviewers considered this aspect of cancer risk assessment in their comments. Aside from nitrobenzene-specific issues, the panel's discussion for this charge question was not sufficient to set aside this general practice.

The reviewers who responded negatively to considering nitrobenzene's multiple tumor types primarily cited lack of relevance of the other two types, kidney and thyroid tumors. EPA agrees that it would be inappropriate to include risks from tumor types not expected to be relevant to humans. As discussed in sections 4.6.2 and 4.6.3, however, although the thyroid and kidney tumors observed in male rats and thyroid tumors in male mice may be suggestive of rodent-specific MOAs, the experimental data do not satisfy the criteria set forth in EPA's technical reports on *Assessment of Thyroid Follicular Cell Tumors* (U.S. EPA, 1998b) and *Alpha $_{2u}$-globulin: Association with Chemically Induced Renal Toxicity and Neoplasia in the Male Rat* (U.S. EPA, 1991b) to make this determination. Furthermore, EPA does not require site concordance between species before raising concern for human carcinogenic potential (U.S. EPA, 2005a), given that some human carcinogens have produced tumors in rodents at different sites than were observed in humans. Also, mice and rats often do not respond with the same set of tumor types to any chemical exposure, so lack of consistency between laboratory species does not inform human relevance. Without data to the contrary, the thyroid and kidney tumors are

considered relevant to humans. EPA agrees that the justification in section 5.3 was brief and has provided cross-references to the relevant discussions in chapter 4.

EPA does not agree that it is inappropriate to combine risks if there are different mechanisms involved. Different mechanisms increase the possibility that the different tumor types occur independently of each other (e.g., that the occurrence of a kidney tumor, for instance, is independent of the occurrence of a liver tumor in an individual). The possibility of different mechanisms for different tumor types is exactly why risks from different tumor types *should* be combined, so that the total potential risk—the risk that in an individual could develop a tumor by any of the possible mechanisms—is not underestimated. This is the basis for using a different quantitative method than that used for adenomas and carcinomas resulting from the same mechanism, as for the hepatocellular tumors.

As discussed in the assessment, the National Research Council (NRC, 1994) concluded that an approach based on counts of animals with one or more tumors (or tumor-bearing animals), as used by EPA previously, would tend to underestimate overall risk when tumor types occur independently and that an approach based on combining the risk estimates from each separate tumor type should be used. The quantitative method used in the total risk analysis in the nitrobenzene external review draft has been routinely used (e.g., final IRIS assessments for bromate, 1,3-butadiene, and 1,2-dibromoethane) and received favorable external peer review as recently as March 2008 (1,2,3-trichloropropane). Meanwhile, EPA has evaluated methods for combining risks across tumor sites in response to the NRC (1994) recommendations. EPA applied a Bayesian approach, consistent with the NRC recommendation and consistent with the likelihood profile method that was suggested by one of the external peer reviewers, to the site-specific risk estimates for nitrobenzene. Note that, while the analysis recommended by the reviewer could not be readily carried out without software development, the comparable Bayesian analysis was straightforward to implement with available software.

The estimate of the IUR for total risk (3.8×10^{-5} $(\mu g/m^3)^{-1}$) is slightly higher than that in the external peer review draft (3.49×10^{-5} $(\mu g/m^3)^{-1}$). EPA also conducted an analysis using the tumor-bearing animal approach, which yielded an IUR of 4.1×10^{-5} $(\mu g/m^3)^{-1}$. These approaches are discussed and results provided in Appendix B-3. *All* approaches considered for estimating overall risk provided a higher IUR than liver tumors alone (2.5×10^{-5} $(\mu g/m^3)^{-1}$), and the *Toxicological Review* has been revised to recommend an IUR of 4×10^{-5} $(\mu g/m^3)^{-1}$, replacing the IUR of 3×10^{-5} $(\mu g/m^3)^{-1}$, which was based on liver tumors alone.

Comment: One reviewer remarked that some consideration may be warranted of possible enhanced susceptibility from early-life exposure to carcinogens, as per U.S. EPA (2005b) guidance and based on specific examples with other chemicals. This reviewer also noted five review publications on early-life exposure and susceptibility to carcinogens for consideration in the *Toxicological Review*.

Response: In general, differences in susceptibility with respect to early life stages may not be fully accounted for because cancer slope factors are usually based on effects observed following exposures to adult humans or sexually mature animals. The *Supplemental Guidance for Assessing Susceptibility from Early-life Exposure to Carcinogens* (U.S. EPA, 2005b) provides an approach for developing lifetime cancer risk estimates that account for possible age-related differences in susceptibility and exposure. The guidance takes into consideration the MOA, if known, information on chemical-specific susceptibility differences between adults and juveniles, if available, and the methodology (linear versus nonlinear) used for estimating cancer risk, in addition to applying age-dependent adjustment factors (ADAFs) for mutagenic carcinogens. According to the guidance, unless chemical-specific data are available, ADAFs are currently only applied to carcinogens that are known to act via a mutagenic MOA.

All five review articles suggested by the peer reviewer are not specific to nitrobenzene. Among the covered issues and examples were genotoxic carcinogens as well as factors that influence susceptibility at different prenatal and/or neonatal stages of development, including some of the known molecular mechanisms, age-related differences in drug metabolizing enzymes, oncogenes, tumor-suppressor genes, and certain growth regulatory genes.

PUBLIC COMMENTS

No public comments were submitted on this assessment.

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APPENDIX B: DOSE-RESPONSE MODELING

APPENDIX B–1: Dose-Response Modeling for Derivation of an RfD for Nitrobenzene
APPENDIX B–2: Dose-Response Modeling for Derivation of an RfC for Nitrobenzene
APPENDIX B–3: Dose-Response Modeling of Carcinogenicity Data for Nitrobenzene

APPENDIX B–1: Dose-Response Modeling for Derivation of an RfD for Nitrobenzene

B–1.1. METHODS

The models in U.S. EPA's BMDS (version 1.4.1c) were fit to multiple data sets presented in a 90-day study of gavage exposure to nitrobenzene in F344 rats and B6C3F1 mice (NTP, 1983a). The endpoints considered for modeling were splenic congestion, methemoglobin (metHb), and reticulocyte levels in male and female rats (Table B-1.1).

Table B-1.1. Summary of splenic congestion, reticulocyte count (%) and metHb levels (%) in male and female F344 rats exposed by gavage to nitrobenzene for 90 days

Dose (mg/kg-day)	Splenic congestion, severity greater than minimal[a]	Hematology endpoints		
		n[b]	Reticulocytes (%)[c]	MetHb (%)[c]
Males				
0	0/10	10	2.23 ± 0.44	1.13 ± 0.58
9.38	0/10	10	2.62 ± 0.45	2.75 ± 0.58[d]
18.75	0/10	10	3.72 ± 0.65[d]	4.22 ± 1.15[d]
37.5	0/10	10	4.75 ± 0.62[d]	5.62 ± 0.85[d]
75	5/10	10	6.84 ± 0.72[d]	7.31 ± 1.44[d]
150	10/10	1	15	12.22
Females				
0	0/10	10	2.60 ± 0.37	0.94 ± 0.03
9.38	1/10	10	3.69 ± 0.32[d]	2.06 ± 0.45[d]
18.75	3/10	10	4.75 ± 0.68[d]	3.62 ± 1.09[d]
37.5	5/10	10	6.28 ± 0.90[d]	5.27 ± 0.76[d]
75	8/10	10	8.72 ± 1.49[d]	6.85 ± 2.25[d]
150	9/10	7	32.07 ± 3.56[d]	12.77 ± 1.83[d]

[a]All animals, including early deaths, were subject to histopathologic examination
[b]Number of surviving animals at termination.
[c]Values are means ± SD.
[d]Significantly different from controls, as calculated by the authors.

Source: NTP (1983a).

The dose levels used were the administered doses reported in the study, since there was no information supporting other dose metrics. In accordance with the U.S. EPA (2000b) BMD technical guidance, biologically relevant response levels were considered for developing RfDs where possible. Insufficient information was available to identify minimally adverse levels of response for increased reticulocyte counts and splenic congestion. For methemoglobinemia, BMRs based on 3% metHb being the upper limit of a normal range for humans was considered, as well as a 2 SD increase in the control mean (see section 5.1.2), BMRs of a 10% increase in

extra risk and a change in the mean equal to 1 SD from the control mean were estimated for dichotomous and continuous data, respectively, as standard points of comparison.

Models were run using the default restrictions on parameters provided in BMDS, primarily to avoid biologically implausible dose-response shapes. Specifically, the restrictions included limiting shape parameters to be ≥1 for the gamma, log-logistic, log-probit, and Weibull (dichotomous) models and power and Hill (continuous) models and allowing only monotonic fits of the multistage (dichotomous) and polynomial (continuous) models.

For continuous data, the assumption of constant variance across dose groups was evaluated for each model. If the variances were statistically homogenous ($p > 0.05$), then fit of the various models to the means was evaluated while assuming a constant variance. If variances were heterogeneous, then variance was modeled as a power function of the mean. For data sets where the fit of the variance model was adequate ($p > 0.05$), the fit to the means of the various models was evaluated contingent on the use of the variance model.

B–1.2. RESULTS

The BMD modeling results are summarized in Table B-1.2. This table shows the final BMDs and BMDLs derived for each endpoint modeled for male and female rats. The remainder of this section shows detailed summaries of the modeling results for splenic congestion, metHb concentration, and reticulocyte count.

Table B-1.2. Summary of PODs derived from BMD modeling of NTP (1983a) bioassay data for male and female rats exposed by gavage to nitrobenzene for 90 days

Endpoint (data type)	Sex	Model	BMR	BMD (mg/kg-day)	BMDL (mg/kg-day)	Output
Spleen congestion (dichotomous)	M	Gamma, multistage	10% extra risk of mild or moderate congestion	54.6	37.8	I
	F	Multistage, log-probit		7.8	5.6	II
MetHb (%) (continuous)	M [a]	Hill	1 SD = 0.5%	3.0	1.8	III
			Point BMR = 2% [b]	4.9	3.2	
			2 SD = 1.0%	5.7	3.9	
	F [a]	Hill	1 SD = 0.4%	4.7	3.1	IV
			Point BMR = 1.9% [c]	8.2	6.3	
			0.8%	7.2	5.2	
Reticulocytes (%) [a] (continuous)	M [a]	Linear	1 SD = 0.6%	9.4	7.9	V
	F [a]	Hill	1 SD = 0.3%	2.7	1.8	VI

[a] High-dose group (150 mg/kg-day) dropped.
[b] Point BMR = the mean of a distribution of metHb with 10% of the (exposed) population exceeding 3% metHb.

B-1.2.1. Male F344 rat spleen congestion

Adequate fit ($p > 0.1$) with all of the models.

Gamma and multistage gave best fits (low Akaike Information Criteria [AICs], most biologically plausible dose-response shapes); gamma output provided.

BMD = 51.9–54.6 mg/kg-day
BMDL = 35.0–37.8 mg/kg

Model fit to means	χ^2	df	*p* Value for model fit	AIC	BMD (mg/kg-day)	BMDL (mg/kg-day)
Gamma (power ≥1)	0.05	5	1.00	15.96	54.6	37.8
Logistic	<0.01	4	1.00	17.86	70.6	41.6
Log-logistic (slope ≥1)	<0.01	5	1.00	15.86	66.4	40.6
1-Stage multistage (5th degree)	0.23	5	1.00	16.3	51.9	35.0
Probit	<0.01	4	1.00	17.86	66.5	38.9
Log-probit (slope ≥1)	<0.01	4	1.00	17.86	64.4	39.4
Weibull (power ≥1)	<0.01	4	1.00	17.86	66.6	36.9

OUTPUT I. Male F344 rat spleen congestion

```
 ======================================================================
         Gamma Model. (Version: 2.7;  Date: 01/18/2007)
         Input Data File: G:\_BMDS\NB_RAT_SPLEEN.(d)
         Gnuplot Plotting File:  G:\_BMDS\NB_RAT_SPLEEN.plt
                                                  Tue Feb 12 10:50:13 2008
 ======================================================================

 BMDS MODEL RUN
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   The form of the probability function is:

   P[response]= background+(1-background)*CumGamma[slope*dose,power],
   where CumGamma(.) is the cummulative Gamma distribution function


   Dependent variable = sp_cng_m
   Independent variable = mg_kg_d
   Power parameter is restricted as power ≥1

   Total number of observations = 6
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008


                  Default Initial (and Specified) Parameter Values
                     Background =    0.0454545
                          Slope =    0.0123945
                          Power =          1.3


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Background    -Power
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )

                    Slope
     Slope              1
                                 Parameter Estimates
```

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0	NA		
Slope	0.234878	0.0216068	0.19253	0.277227
Power	18	NA		

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-6.93147	6			
Fitted model	-6.98023	1	0.0975094	5	0.9998
Reduced model	-33.7401	1	53.6173	5	<.0001

AIC: 15.9605

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0.000
9.3750	0.0000	0.000	0	10	-0.000
18.7500	0.0000	0.000	0	10	-0.003
37.5000	0.0043	0.043	0	10	-0.208
75.0000	0.4951	4.951	5	10	0.031
150.0000	0.9995	9.995	10	10	0.072

Chi^2 = 0.05 d.f. = 5 P-value = 1.0000

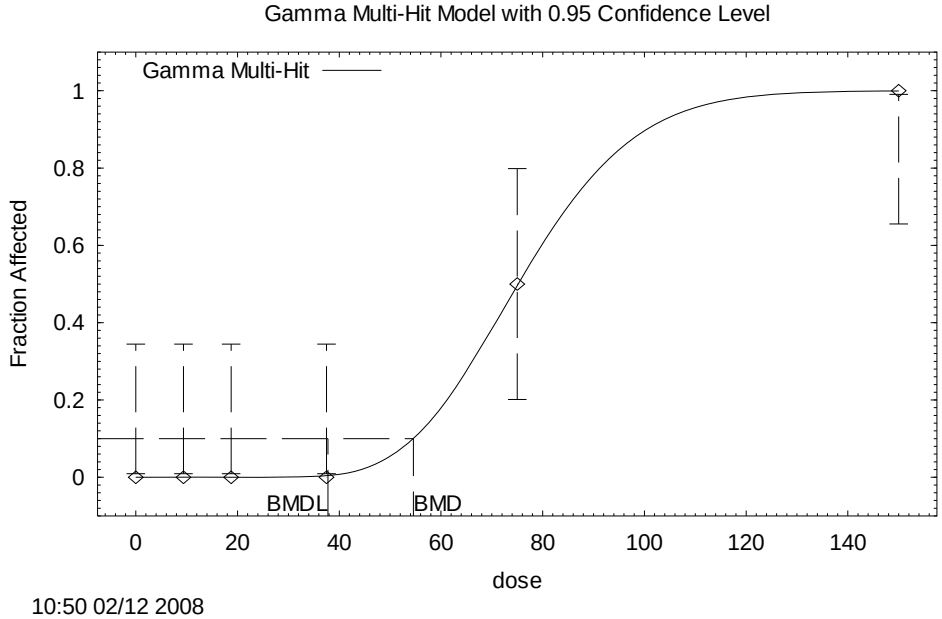
Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 54.5885
BMDL = 37.7754



B-1.2.2. Female F344 rat spleen congestion

Adequate fit ($p > 0.1$) with all of the models, all have plausible shapes and small residuals.

Multistage and log-probit models gave best fits (lowest AICs, essentially the same); BMDs and BMDLs averaged.

BMD = 7.8 mg/kg-day
BMDL = 5.6 mg/kg-day

Model fit to means	χ^2	df	*p* Value for model fit	AIC	BMD (mg/kg-day)	BMDL (mg/kg-day)
Gamma (power ≥1)	0.60	4	0.96	53.69	6.5	4.2
Logistic	0.14	4	1.00	53.24	8.7	3.0
Log-logistic (slope ≥1)	5.80	4	0.21	58.99	16.5	11.5
Multistage, 1-stage	**0.59**	**5**	**0.99**	**51.71**	**5.9**	**4.2**
Probit	5.99	4	0.20	59.65	16.7	12.1
Log-probit (slope ≥1)	**0.23**	**5**	**1.00**	**51.31**	**9.7**	**6.9**
Weibull (power ≥1)	0.60	4	0.96	53.70	6.1	4.2

OUTPUT II. Female F344 rat spleen congestion

```
=================================================================
         Multistage Model. (Version: 2.8;  Date: 02/20/2007)
         Input Data File: C:\BMDS\UNSAVED1.(d)
         Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                                Fri Feb 01 14:03:30 2008
 =================================================================

 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
                -beta1*dose^1-beta2*dose^2-beta3*dose^3-beta4*dose^4-beta5*dose^5)]

   The parameter betas are restricted to be positive


   Dependent variable = sp_cong_f
   Independent variable = mg_kg_d

 Total number of observations = 6
 Total number of records with missing values = 0
 Total number of parameters in model = 6
 Total number of specified parameters = 0
 Degree of polynomial = 5


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     Background =    0.0671025
                        Beta(1) =    0.0160015
                        Beta(2) =            0
                        Beta(3) =            0
                        Beta(4) =            0
                        Beta(5) =            0
          Asymptotic Correlation Matrix of Parameter Estimates
```

(*** The model parameter(s) -Background -Beta(2) -Beta(3) -Beta(4) -Beta(5)
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	Beta(1)
Beta(1)	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0	*	*	*
Beta(1)	0.0178413	*	*	*
Beta(2)	0	*	*	*
Beta(3)	0	*	*	*
Beta(4)	0	*	*	*
Beta(5)	0	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-24.5458	6			
Fitted model	-24.8535	1	0.615336	5	0.9873
Reduced model	-41.0539	1	33.0162	5	<.0001

AIC: 51.7069

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0.000
9.3750	0.1540	1.540	1	10	-0.473
18.7500	0.2843	2.843	3	10	0.110
37.5000	0.4878	4.878	5	10	0.077
75.0000	0.7377	7.377	8	10	0.448
150.0000	0.9312	9.312	9	10	-0.389

Chi^2 = 0.59 d.f. = 5 P-value = 0.9883

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

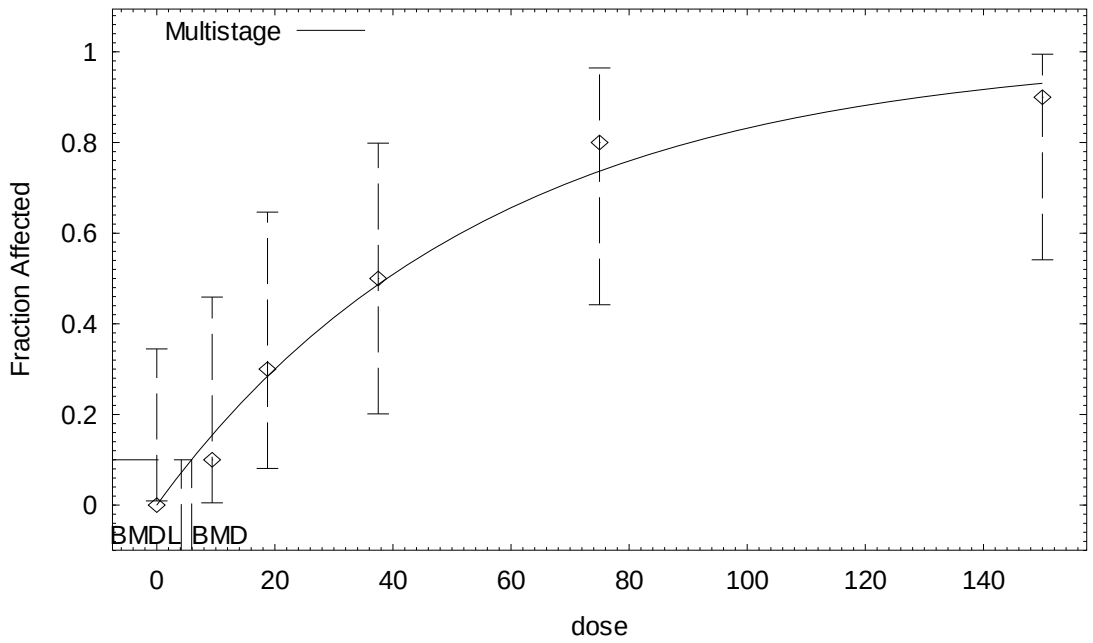
Confidence level = 0.95

BMD = 5.90543

BMDL = 4.17053

BMDU = 11.3289

Taken together, (4.17053, 11.3289) is a 90 % two-sided confidence interval for the BMD




```
==========================================================================
         Probit Model. (Version: 2.9;  Date: 09/23/2007)
         Input Data File: C:\BMDS\UNSAVED1.(d)
         Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                                    Fri Feb 01 14:05:29 2008
 ==========================================================================

 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = Background
               + (1-Background) * CumNorm(Intercept+Slope*Log(Dose)),

   where CumNorm(.) is the cumulative normal distribution function


   Dependent variable = sp_cong_f
   Independent variable = mg_kg_d
   Slope parameter is restricted as slope ≥1

   Total number of observations = 6
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


                 Default Initial (and Specified) Parameter Values
                    background =            0
                     intercept =     -3.60482
                         slope =            1


           Asymptotic Correlation Matrix of Parameter Estimates
           ( *** The model parameter(s)  -background    -slope
```

have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	intercept
intercept	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
background	0	NA		
intercept	-3.55163	0.20686	-3.95707	-3.1462
slope	1	NA		

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-24.5458	6			
Fitted model	-24.6565	1	0.221419	5	0.9989
Reduced model	-41.0539	1	33.0162	5	<.0001

AIC: 51.313

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0.000
9.3750	0.0945	0.945	1	10	0.060
18.7500	0.2675	2.675	3	10	0.232
37.5000	0.5290	5.290	5	10	-0.184
75.0000	0.7781	7.781	8	10	0.167
150.0000	0.9277	9.277	9	10	-0.338

Chi^2 = 0.23 d.f. = 5 P-value = 0.9987

Benchmark Dose Computation

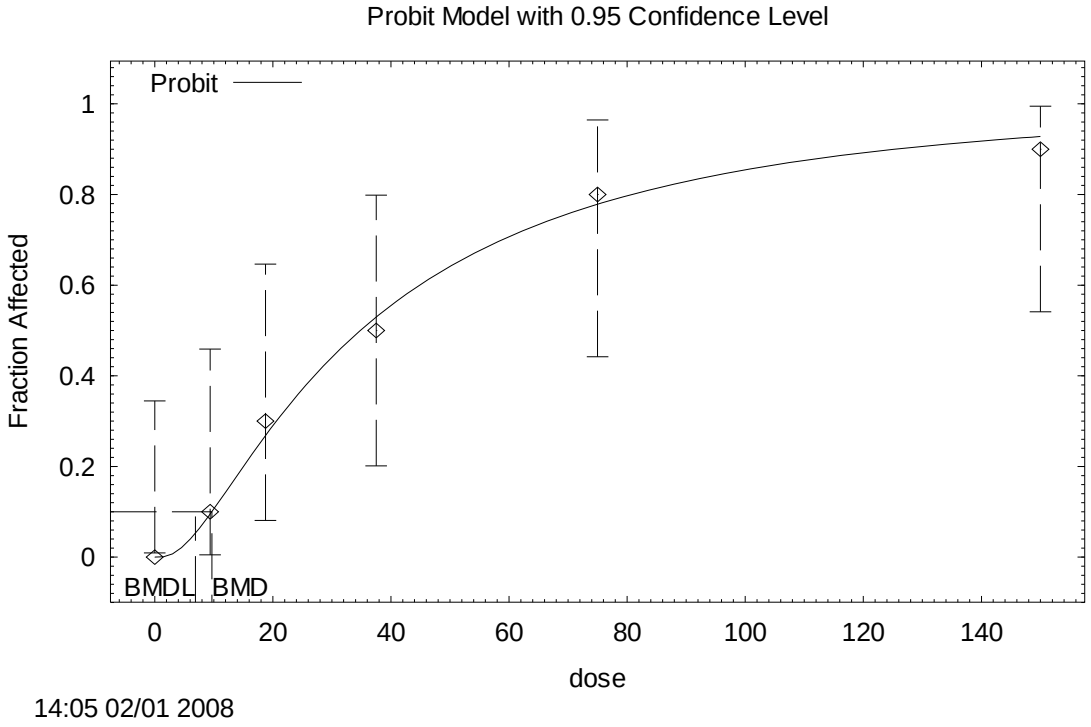
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 9.68021

BMDL = 6.88503


Probit Model with 0.95 Confidence Level
Probit
Fraction Affected
1
0.8
0.6
0.4
0.2
0
BMDL
BMD
0
20
40
60
80
100
120
140
dose
14:05 02/01 2008

B-1.2.3. Male F344 rat metHb

Nonhomogenous variance.

No reasonable fits, including high dose, which only had one observation; dropped high-dose group.

Adequate fit ($p > 0.1$) to means with Hill model.

Model fit to means (without high dose group)	df	*p* Value for variance model	*p* Value for overall model fit	AIC for fitted model	BMR	BMD (mg/kg-day)	BMDL (mg/kg-day)
Linear and higher order polynomial models (coefficients restricted to be positive); Power model (power ≥1)	3	0.27	<0.01	66.09	1 SD	5.5	4.6
Hill (power ≥1)	**1**	**0.27**	**0.42**	**46.38**	1 SD = 0.5%[a]	3.0	1.8
					Point BMR = 2%[b]	4.9	3.2
					2 SD = 1.0%	5.7	3.9

[a]As estimated for the control group by the fitted variance model. Note that the Hill model run used 0.5% as an absolute deviation from the control mean rather than a BMR of 1 SD because of a software issue that prevented estimation of a BMDL; the approaches are equivalent.
[b]The BMR corresponds to the mean metHb level at which 10% (of those exposed) would be expected to exceed 3% metHb, an upper limit on the normal range in humans.

OUTPUT III. Male F344 rat metHb

```
================================================================
         Hill Model. (Version: 2.11;  Date: 01/18/2007)
         Input Data File: G:\_BMDS\NB_RMALE_METHG.(d)
         Gnuplot Plotting File:  G:\_BMDS\NB_RMALE_METHG.plt
                                                Tue Feb 12 17:04:56 2008
 ================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = intercept + v*dose^n/(k^n + dose^n)

   Dependent variable = RESPONSE
   Independent variable = mg_kg_d
   Power parameter restricted to be greater than 1
   The variance is to be modeled as Var(i) = exp(lalpha  + rho * ln(mean(i)))

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008


                  Default Initial Parameter Values
                         lalpha =    -0.044823
                            rho =            0
                      intercept =        1.131
                              v =        6.176
                              n =      7.43183
```

k = 18.7564

Asymptotic Correlation Matrix of Parameter Estimates

	lalpha	rho	intercept	v	n	k
lalpha	1	-0.88	-0.27	0.087	-0.085	0.055
rho	-0.88	1	0.25	-0.099	0.098	-0.063
intercept	-0.27	0.25	1	-0.15	0.18	-0.031
v	0.087	-0.099	-0.15	1	-0.94	0.98
n	-0.085	0.098	0.18	-0.94	1	-0.94
k	0.055	-0.063	-0.031	0.98	-0.94	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
lalpha	-1.47644	0.434447	-2.32794	-0.624943
rho	0.924156	0.300902	0.3344	1.51391
intercept	1.14391	0.159504	0.831293	1.45654
v	8.33828	2.62299	3.1973	13.4793
n	1.17121	0.377134	0.432036	1.91038
k	31.363	19.3683	-6.59809	69.3241

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Est Mean	Obs Std Dev	Est Std Dev	Scaled Res.
0	10	1.13	1.14	0.58	0.509	-0.0803
9.375	10	2.75	2.77	0.583	0.766	-0.0928
18.75	10	4.22	4.09	1.15	0.917	0.436
37.5	10	5.62	5.75	0.85	1.07	-0.365
75	10	7.31	7.27	1.44	1.2	0.0869

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = exp(lalpha + rho*ln(Mu(i)))
Model A3 uses any fixed variance parameters that were specified by the user

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	# Param's	AIC
A1	-21.245411	6	54.490822
A2	-14.899496	10	49.798991
A3	-16.860218	7	47.720436
fitted	-17.188393	6	46.376786
R	-67.618347	2	139.236693

Explanation of Tests

```
 Test 1:  Do responses and/or variances differ among Dose levels?
          (A2 vs. R)
 Test 2:  Are Variances Homogeneous? (A1 vs A2)
 Test 3:  Are variances adequately modeled? (A2 vs. A3)
 Test 4:  Does the Model for the Mean Fit? (A3 vs. fitted)
 (Note:  When rho=0 the results of Test 3 and Test 2 will be the same.)
```

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	105.438	8	<.0001
Test 2	12.6918	4	0.01288
Test 3	3.92144	3	0.2701
Test 4	0.65635	1	0.4179

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels It seems appropriate to model the data

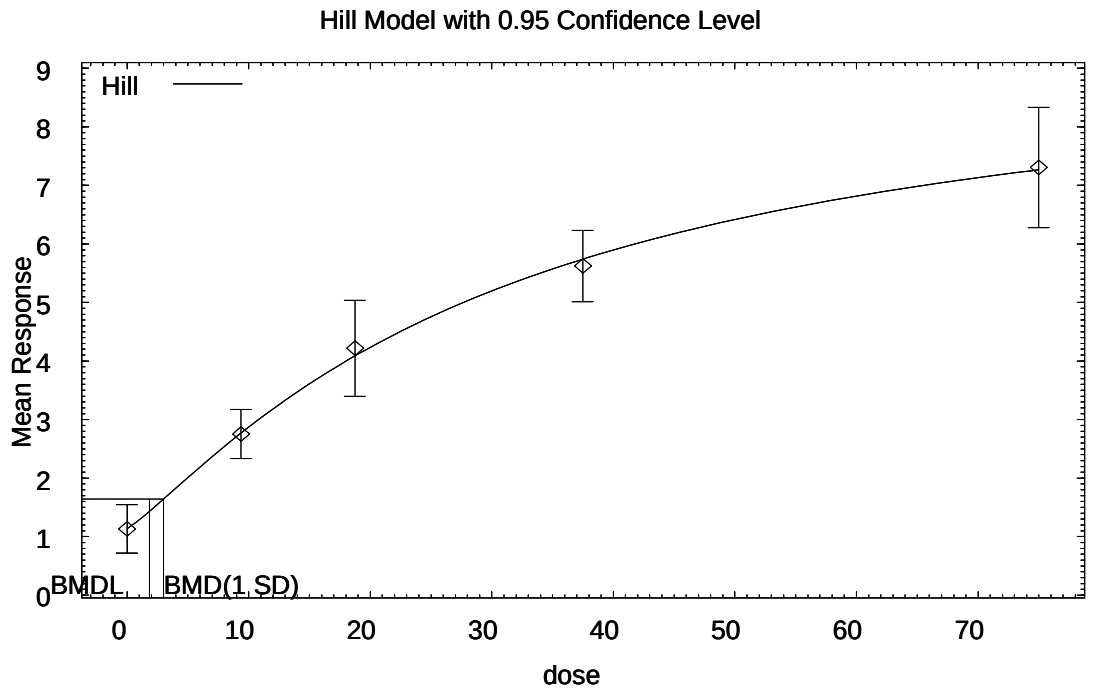
The p-value for Test 2 is less than .1. A non-homogeneous variance model appears to be appropriate

The p-value for Test 3 is greater than .1. The modeled variance appears to be appropriate here

The p-value for Test 4 is greater than .1. The model chosen seems to adequately describe the data

Benchmark Dose Computation

Specified effect = 0.5	Specified effect = 2	Specified effect = 1
Risk Type = Absolute risk	Risk Type = Point risk	Risk Type = Absolute risk
Confidence level = 0.95	Confidence level = 0.95	Confidence level = 0.95
BMD = 2.99149	BMD = 4.92646	BMD = 5.71947
BMDL = 1.84409	BMDL = 3.18476	BMDL = 3.93129



B-1.2.4. Female F344 rat metHb

Nonhomogenous variance.

Adequate fit ($p > 0.1$) to means with Hill model after dropping high dose and transforming data (see below).

Model fit to means	df	*p* Value for variance model fit	*p* Value for overall model fit	AIC for fitted model	BMR	BMD (mg/kg-day)	BMDL (mg/kg-day)
Linear and higher order polynomial models (coefficients restricted to be positive); power (power ≥1)	4	<0.01	<0.01	23.2	0.4%[a]	4.7	3.1
Hill (power ≥1)	2	<0.01	0.02	36.2	0.4%[a]	3.0	2.3
High dose group dropped; constant (0.9) subtracted from each data point[b]							
Linear and higher order polynomial models (coefficients restricted to be positive); power (power ≥1)					0.4%[a]		
Hill (power ≥1)[a]	**1**	**0.1**	**0.41**	**46.23**	0.4%[a]	**4.5**	**2.6**
					Point BMR = 1.9%[c]	**8.2**	**6.3**
					0.8%	**7.2**	**5.2**

[a]The SD of the control group was unusually low, at 0.03%, compared with other data sets. A value closer to the SD of the low-dose group was used for the BMR, as an absolute deviation from the control mean.

[b]Dropping the high dose alone did not sufficiently improve the fit of the variance model or the continuous data models. The variance model depends on the values of the means; transformation of the data by subtracting a constant value of 0.9 from each mean produced an adequate fit of the variance model while preserving the overall dose-response relationship. The *p* value for the variance model fit was 0.097 and reproduced the observed SDs sufficiently well in the lower dose groups.

[c]As for the male metHb BMR, this BMR corresponds to the mean metHb level at which 10% (of those exposed) would be expected to exceed 3% metHb, an upper limit on the normal range in humans. Because 0.9% was subtracted from all metHb values in this analysis, the point estimate for the run was 1.0% (1.9–0.9).

OUTPUT IV. Female F344 rat metHb

```
================================================================
         Hill Model. (Version: 2.11;  Date: 01/18/2007)
         Input Data File: G:\_BMDS\NB_NTP83_FRATS_METHG_WO6.(d)
         Gnuplot Plotting File:  G:\_BMDS\NB_NTP83_FRATS_METHG_WO6.plt
                                                Thu Feb 07 11:10:38 2008
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the response function is:

   Y[dose] = intercept + v*dose^n/(k^n + dose^n)


   Dependent variable = RESPONSE
   Independent variable = mg_kg_d
   Power parameter restricted to be greater than 1
   The variance is to be modeled as Var(i) = exp(lalpha  + rho * ln(mean(i)))
```

Total number of dose groups = 5
Total number of records with missing values = 0
Maximum number of iterations = 250
Relative Function Convergence has been set to: 1e-008
Parameter Convergence has been set to: 1e-008

Default Initial Parameter Values

lalpha =	0.339891
rho =	0
intercept =	0.041
v =	5.91
n =	0.338927
k =	53.1402

Asymptotic Correlation Matrix of Parameter Estimates

	lalpha	rho	intercept	v	n	k
lalpha	1	-0.29	-0.4	-0.047	0.018	0.011
rho	-0.29	1	0.56	-0.079	0.011	-0.036
intercept	-0.4	0.56	1	-0.035	0.012	-0.022
v	-0.047	-0.079	-0.035	1	-0.87	0.97
n	0.018	0.011	0.012	-0.87	1	-0.93
k	0.011	-0.036	-0.022	0.97	-0.93	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
lalpha	-1.80576	0.231567	-2.25962	-1.3519
rho	1.64217	0.134128	1.37929	1.90506
intercept	0.0414733	0.00916969	0.023501	0.0594456
v	7.13177	1.53276	4.12763	10.1359
n	1.5598	0.351807	0.870274	2.24933
k	27.2072	9.48702	8.61301	45.8014

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Est Mean	Obs Std Dev	Est Std Dev	Scaled Res.
0	10	0.041	0.0415	0.0318	0.0297	-0.0504
9.375	10	1.16	1.18	0.45	0.464	-0.137
18.75	10	2.72	2.6	1.09	0.888	0.437
37.5	10	4.37	4.48	0.757	1.39	-0.256
75	10	5.95	5.96	2.25	1.75	-0.0105

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = exp(lalpha + rho*ln(Mu(i)))
Model A3 uses any fixed variance parameters that were specified by the user

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	# Param's	AIC
A1	-30.863270	6	73.726539
A2	13.937796	10	-7.875592
A3	10.783003	7	-7.566006
fitted	10.109497	6	-8.218994
R	-68.956119	2	141.912237

Explanation of Tests

Test 1: Do responses and/or variances differ among Dose levels? (A2 vs. R)
Test 2: Are Variances Homogeneous? (A1 vs A2)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)
(Note: When rho=0 the results of Test 3 and Test 2 will be the same.)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	165.788	8	<.0001
Test 2	89.6021	4	<.0001
Test 3	6.30959	3	0.09748
Test 4	1.34701	1	0.2458

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels
It seems appropriate to model the data

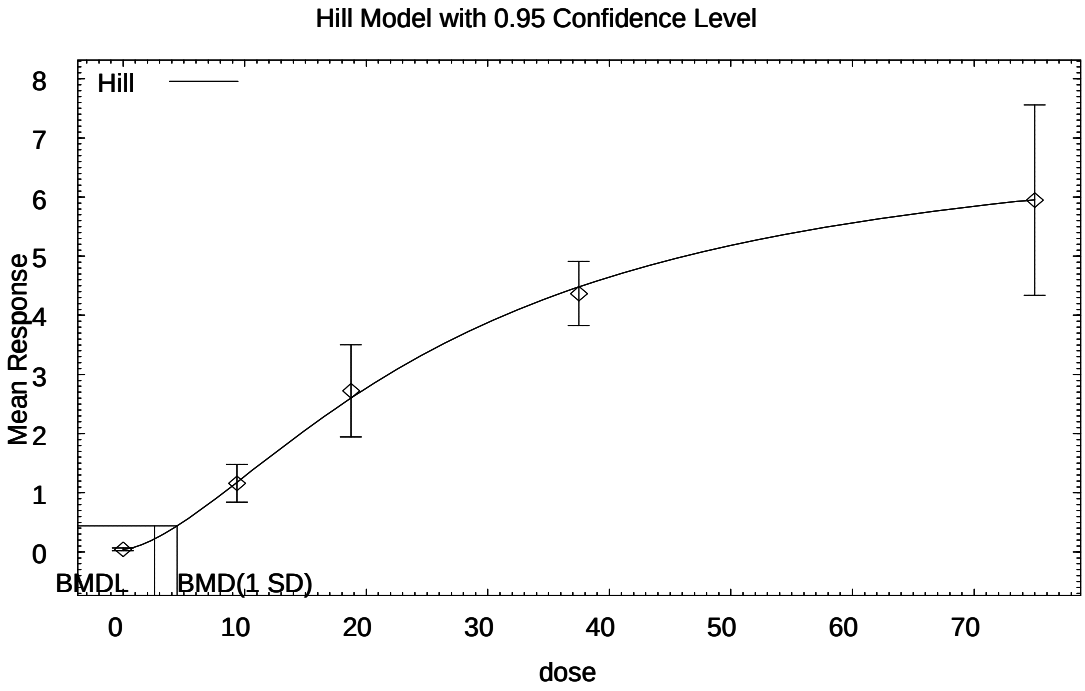
The p-value for Test 2 is less than .1. A non-homogeneous variance model appears to be appropriate

The p-value for Test 3 is less than .1. You may want to consider a different variance model

The p-value for Test 4 is greater than .1. The model chosen seems to adequately describe the data

Benchmark Dose Computation

Specified effect = 0.4	Specified effect = 1	Specified effect = 0.8
Risk Type = Absolute risk	Risk Type = Point risk	Risk Type = Absolute risk
Confidence level = 0.95	Confidence level = 0.95	Confidence level = 0.95
BMD = 4.4529	BMD = 8.24303	BMD = 7.22256
BMDL = 2.56818	BMDL = 6.34379	BMDL = 5.2537


Hill Model with 0.95 Confidence Level
Hill
Mean Response
8
7
6
5
4
3
2
1
0
BMDL
BMD(1 SD)
0
10
20
30
40
50
60
70
dose

B-1.2.5. Male F344 rat reticulocytes

Homogenous variance ($p = 0.43$).

Adequate fit ($p > 0.1$) to means with linear model.

Model fit to means	df	*p* Value for model fit	AIC for fitted model	BMD (mg/kg-day)	BMDL (mg/kg-day)
Linear and higher order polynomial models; power model	**3**	**0.16**	**2.52**	**9.43**	**7.94**
Hill model	3	0.10	4.08	9.39	6.07

OUTPUT V. Male F344 rat reticulocytes

```
=================================================================
         Polynomial Model. (Version: 2.11;  Date: 01/18/2007)
         Input Data File: G:\_BMDS\UNSAVED1.(d)
         Gnuplot Plotting File:  G:\_BMDS\UNSAVED1.plt
                                                 Tue Feb 12 17:30:59 2008
 =================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = COLUMN1
   rho is set to 0
   Signs of the polynomial coefficients are not restricted
   A constant variance model is fit

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =      0.34428
                            rho =            0   Specified
                         beta_0 =       2.2845
                         beta_1 =    0.0621333


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -rho
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )

                   alpha       beta_0       beta_1

     alpha             1    -7.3e-012    -1.8e-012

    beta_0    -7.3e-012            1        -0.73

    beta_1    -1.8e-012        -0.73            1


                             Parameter Estimates
```

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
alpha	0.343138	0.0686276	0.20863	0.477646
beta_0	2.2845	0.120762	2.04781	2.52119
beta_1	0.0621333	0.00312416	0.0560101	0.0682566

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Est Mean	Obs Std Dev	Est Std Dev	Scaled Res.
0	10	2.23	2.28	0.44	0.586	-0.294
9.375	10	2.62	2.87	0.45	0.586	-1.33
18.75	10	3.72	3.45	0.65	0.586	1.46
37.5	10	4.75	4.61	0.62	0.586	0.731
75	10	6.84	6.94	0.72	0.586	-0.564

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2
Model A3 uses any fixed variance parameters that were specified by the user

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	# Param's	AIC
A1	4.291513	6	3.416974
A2	6.202123	10	7.595754
A3	4.291513	6	3.416974
fitted	1.740565	3	2.518871
R	-52.940659	2	109.881318

Explanation of Tests

Test 1: Do responses and/or variances differ among Dose levels? (A2 vs. R)
Test 2: Are Variances Homogeneous? (A1 vs A2)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)
(Note: When rho=0 the results of Test 3 and Test 2 will be the same.)

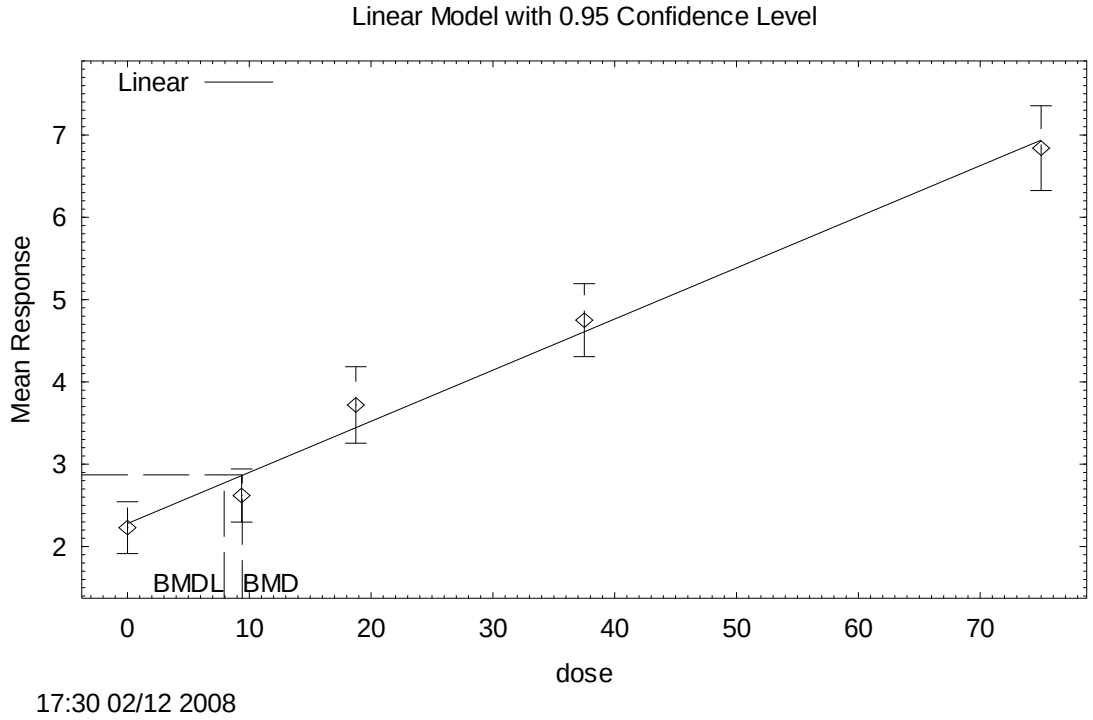
Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	118.286	8	<.0001
Test 2	3.82122	4	0.4307
Test 3	3.82122	4	0.4307
Test 4	5.1019	3	0.1645

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels
It seems appropriate to model the data

The p-value for Test 2 is greater than .1. A homogeneous variance model appears to be appropriate here

```
The p-value for Test 3 is greater than .1.  The modeled variance appears
 to be appropriate here

The p-value for Test 4 is greater than .1.  The model chosen seems
to adequately describe the data


             Benchmark Dose Computation

Specified effect =             1

Risk Type        =     Estimated standard deviations from the control mean

Confidence level =          0.95

             BMD =        9.42779

            BMDL =        7.93875
```



B-1.2.6. Female F344 rat reticulocytes

Nonhomogenous variance; BMDS variance model had adequate fit.

BMR = 1 SD = 0.3%.

Model fit to means	df	*p* Value for variance model	*p* Value for model fit	AIC for fitted model	BMD (mg/kg)	BMDL (mg/kg)
Linear	4	0.29	<0.01	77.30	2.36	2.86
4 Degree polynomial (betas ≥0)	1	0.29	<0.01	55.59	4.68	3.15
Power (power ≥1)	3	0.29	<0.01	76.82	3.50	2.17
Hill	2	<0.01	0.96	38.2	6.8	4.9
Dropped high-dose group						
Linear, polynomial	3	0.354	0.05	19.2	3.65	2.62
Hill[a]	**1**	**0.354**	**0.58**	**15.77**	**2.69**	**1.80**

[a]Note that, in order to obtain an adequate fit from this model, starting parameters were adjusted from those supplied by the software package (see "User Inputs Initial Parameter Values" in the output below).

OUTPUT VI. Female F344 rat reticulocytes

```
 ================================================================
         Hill Model. (Version: 2.12;  Date: 02/20/2007)
         Input Data File: C:\BMDS\UNSAVED1.(d)
         Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                                 Wed Feb 20 21:45:52 2008
 ================================================================

 BMDS MODEL RUN
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   The form of the response function is:

   Y[dose] = intercept + v*dose^n/(k^n + dose^n)


   Dependent variable = MEAN
   Independent variable = mg_kg_d
   Power parameter restricted to be greater than 1
   The variance is to be modeled as Var(i) = exp(lalpha  + rho * ln(mean(i)))

   Total number of dose groups = 5
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008


                  User Inputs Initial Parameter Values
                          lalpha =            2
                             rho =            1
                       intercept =          0.1
                               v =            7
                               n =            2
                               k =           50


           Asymptotic Correlation Matrix of Parameter Estimates

                 lalpha          rho    intercept            v            n            k

    lalpha            1        -0.96        -0.17        0.092       -0.076        0.082
```

rho	-0.96	1	0.15	-0.099	0.078	-0.086
intercept	-0.17	0.15	1	-0.14	0.24	-0.13
v	0.092	-0.099	-0.14	1	-0.95	1
n	-0.076	0.078	0.24	-0.95	1	-0.97
k	0.082	-0.086	-0.13	1	-0.97	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
lalpha	-4.74651	0.737447	-6.19188	-3.30114
rho	2.44281	0.45382	1.55334	3.33228
intercept	2.61278	0.0947709	2.42703	2.79853
v	14.6759	11.8681	-8.58515	37.9369
n	1.06017	0.27084	0.52933	1.591
k	103.143	136.737	-164.857	371.142

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Est Mean	Obs Std Dev	Est Std Dev	Scaled Res.
0	10	2.6	2.61	0.37	0.301	-0.134
9.375	10	3.69	3.68	0.32	0.458	0.0464
18.75	10	4.75	4.68	0.68	0.614	0.354
37.5	10	6.28	6.35	0.9	0.892	-0.261
75	10	8.72	8.72	1.49	1.31	-0.00728

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = exp(lalpha + rho*ln(Mu(i)))
Model A3 uses any fixed variance parameters that were specified by the user

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	# Param's	AIC
A1	-15.052307	6	42.104613
A2	-0.106653	10	20.213306
A3	-1.734001	7	17.468001
fitted	-1.885026	6	15.770053
R	-66.367642	2	136.735284

Explanation of Tests

Test 1: Do responses and/or variances differ among Dose levels? (A2 vs. R)
Test 2: Are Variances Homogeneous? (A1 vs A2)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)
(Note: When rho=0 the results of Test 3 and Test 2 will be the same.)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	132.522	8	<.0001
Test 2	29.8913	4	<.0001
Test 3	3.2547	3	0.354
Test 4	0.302052	1	0.5826

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels
It seems appropriate to model the data

The p-value for Test 2 is less than .1. A non-homogeneous variance model appears to be appropriate

The p-value for Test 3 is greater than .1. The modeled variance appears to be appropriate here

The p-value for Test 4 is greater than .1. The model chosen seems to adequately describe the data

Benchmark Dose Computation

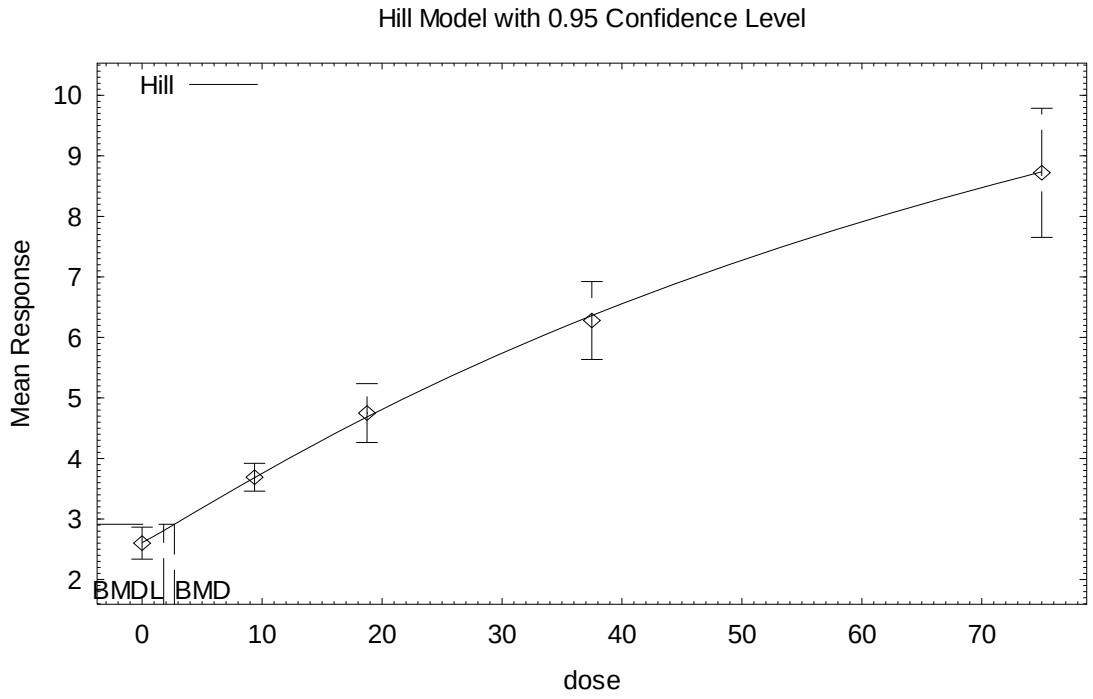
Specified effect = 1

Risk Type = Estimated standard deviations from the control mean

Confidence level = 0.95

BMD = 2.69076

BMDL = 1.79887



APPENDIX B–2: Dose-Response Modeling for Derivation of an RfC for Nitrobenzene

B-2.1. METHODS

Data considered were exposure-related increases in olfactory degeneration and alveolar bronchiolization following 2-year exposure in mice (Cattley et al., 1994; CIIT, 1993). See section 5.2 for the data and more information on endpoint selection; the data are also repeated in the model outputs in section B-2.3. See section B-1.1 for additional information regarding modeling methods.

B-2.2. RESULTS

The BMD modeling results for olfactory epithelium degeneration are summarized in Table B-2.1, and for bronchiolization are summarized in Table B-2.2. Detailed model output for both endpoints is in Section B-2.3.

Table B-2.1. Modeling results for olfactory degeneration in mice; bioassay data from Cattley et al. (1994) and CIIT (1993)

Sex	Model	*p* Value	BMC_{10} (ppm)	$BMCL_{10}$ (ppm)
Males[a]	Probit	0.38	12.3	10.0
Females[a]	Gamma, multistage (1-stage), Weibull	0.50	1.75	1.42

[a]High-dose group excluded.

Table B-2.2. Modeling results for bronchiolization in mice; bioassay data from Cattley et al. (1994) and CIIT (1993)

Sex	Model	*p* Value	AIC	BMC_{10} (ppm)	$BMCL_{10}$ (ppm)
Males	Log-logistic[a]	0.0276	105.1	0.134	0.083
Females	Log-probit	0.12	49.4	0.40	0.278
	Log-logistic	0.80	49.4	0.18	0.022

[a]High-dose group excluded.

B-2.2.1. Olfactory degeneration in male mice following chronic nitrobenzene inhalation

None of the models fit ($p < 0.05$). Dropping the high dose left only one dose level with a response greater than control, at approximately 50%. All models fit ($p > 0.1$), with all $BMDL_{10}$s ≧8 ppm. Output from the best-fitting model (lowest AIC), probit, is provided below.

```
 ======================================================================
         Probit Model. (Version: 2.9;  Date: 09/23/2007)
         Input Data File: C:\BMDS\NB_CIIT_MICE_RFC_WO_GP4.(d)
         Gnuplot Plotting File:  C:\BMDS\NB_CIIT_MICE_RFC_WO_GP4.plt
                                                 Fri Feb 22 15:43:37 2008
 ======================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = CumNorm(Intercept+Slope*Dose),

   where CumNorm(.) is the cumulative normal distribution function


   Dependent variable = olf_degen_m
   Independent variable = ppm
   Slope parameter is not restricted

   Total number of observations = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial (and Specified) Parameter Values
                     background =            0   Specified
                      intercept =     -2.34241
                          slope =     0.090894


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -background
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )

              intercept        slope

 intercept            1         -0.9

     slope         -0.9            1


                                Parameter Estimates

                                                        95.0% Wald Confidence Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf. Limit
      intercept          -2.4247         0.316052           -3.04415            -1.80525
          slope        0.0958901        0.0144674          0.0675344            0.124246
```

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-55.4261	3			
Fitted model	-55.7859	2	0.719573	1	0.3963
Reduced model	-90.8025	1	70.7528	2	<.0001

AIC: 115.572

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0077	0.513	1	67	0.682
5.0000	0.0259	1.708	1	66	-0.549
25.0000	0.4891	31.788	32	65	0.053

Chi^2 = 0.77 d.f. = 1 P-value = 0.3806

Benchmark Dose Computation

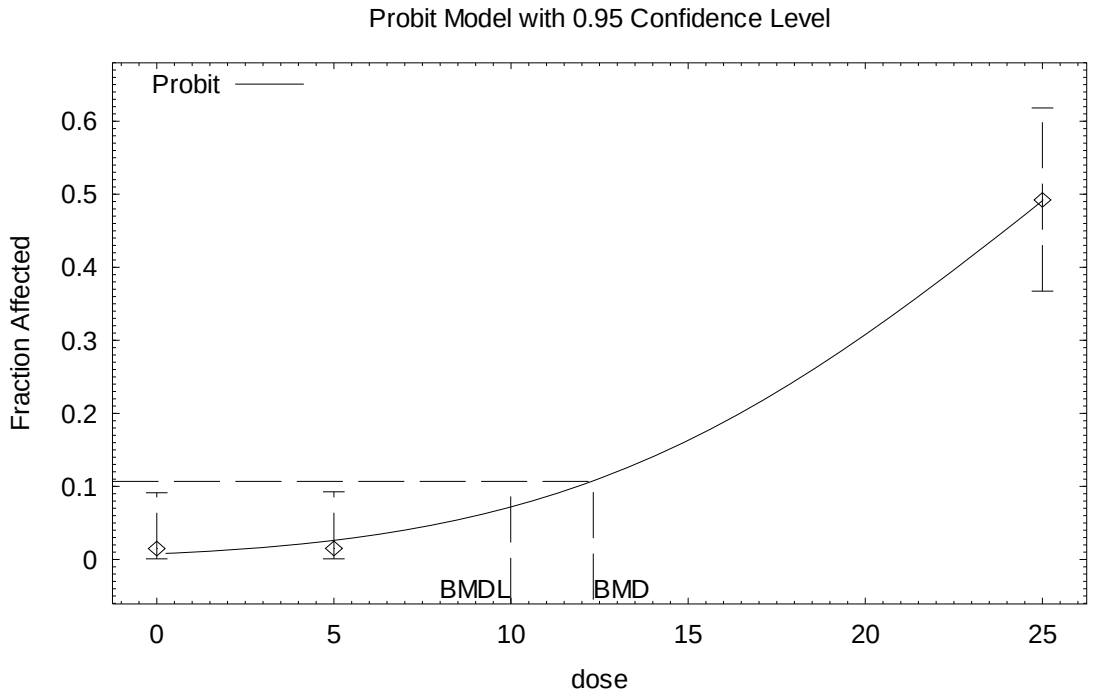
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 12.3212

BMDL = 9.99197



B-2.2.2. Olfactory degeneration in female mice following chronic nitrobenzene inhalation

Gamma, multistage, and Weibull models shared the lowest AIC; multistage output is provided below.

BMC_{10} = 1.75 ppm
$BMCL_{10}$ = 1.42 ppm

Model used[a]	*p* Value	AIC	BMC_{10} (ppm)	$BMCL_{10}$ (ppm)
Gamma	0.50	149.64	**1.75**	**1.42**
Multistage, 1-stage	0.50	149.64		
Weibull	0.50	149.64		
Log-probit	0.13	152.06	2.82	2.29
Log-logistic	1.00	150.32	1.44	0.79

[a]High-dose group excluded.

```
 ======================================================================
          Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
          Input Data File: C:\BMDS\UNSAVED1.(d)
          Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                                  Fri Jun 16 12:34:52 2006
 ======================================================================

 BMDS MODEL RUN
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Observation # < parameter # for Multistage model.
   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = Incidence
   Independent variable = Concentration

 Total number of observations = 3
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

Background =	0
Beta(1) =	0.0814877
Beta(2) =	0
Beta(3) =	0

Asymptotic Correlation Matrix of Parameter Estimates

(*** The model parameter(s) -Background -Beta(2) -Beta(3)
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	Beta(1)
Beta(1)	1

Parameter Estimates

Variable	Estimate	Std. Err.
Background	0	NA
Beta(1)	0.0602508	0.0101428
Beta(2)	0	NA
Beta(3)	0	NA

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-73.159			
Fitted model	-73.8205	1.3229	2	0.5161
Reduced model	-115.963	85.6088	2	<.0001

AIC: 149.641

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Chi^2 Res.
i: 1					
0.0000	0.0000	0.000	0	52	0.000
i: 2					
5.0000	0.2601	15.607	19	60	0.294
i: 3					
25.0000	0.7783	49.031	47	63	-0.187

Chi-square = 1.38 DF = 2 P-value = 0.5025

Benchmark Dose Computation

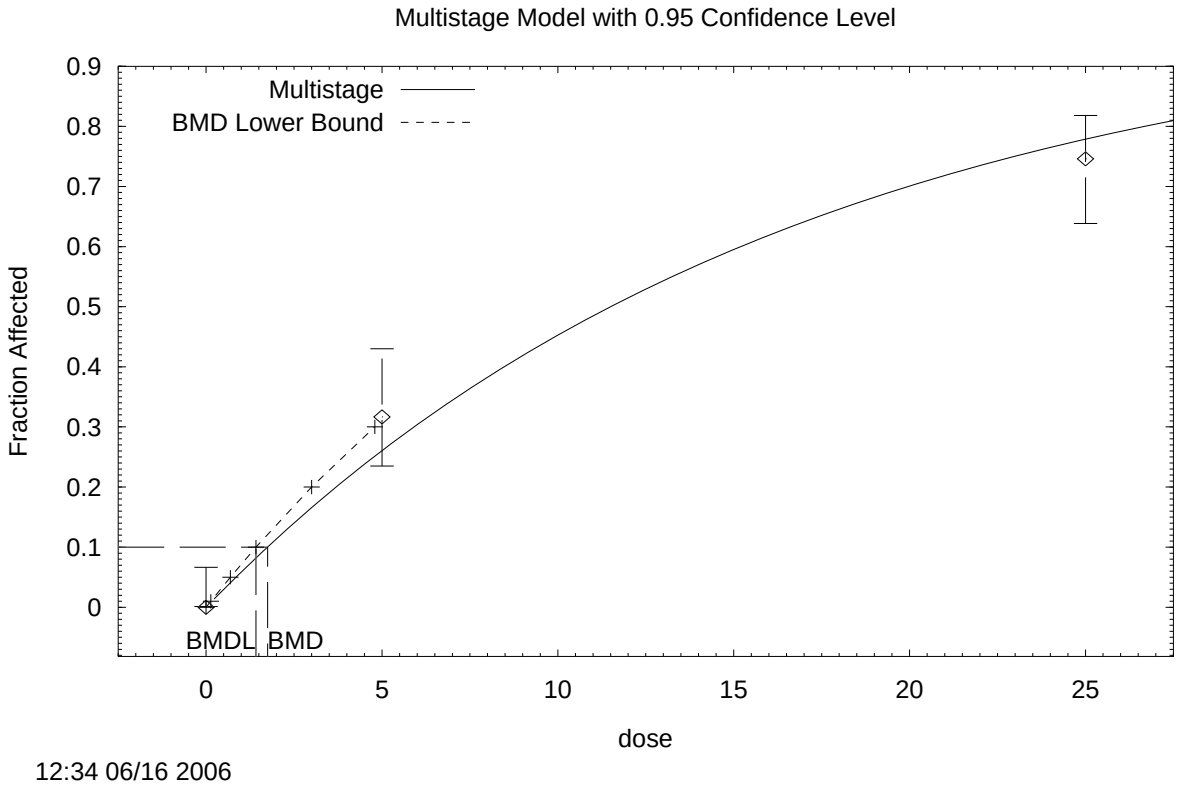
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 1.7487

BMDL = 1.41665



B-2.2.3. Alveolar bronchiolization in male mice

The best fitting model for each subset considered below did not meet the usual $p > 0.1$ goodness-of-fit criterion; only the model results for the best fitting model in each case is reported (AICs are not shown because they are not comparable between nonidentical datasets.)

The fit for the subset excluding the high dose was considered best overall because it came closest to fitting the low dose response.

Conditions	Model	*p* Value	BMC_{10}	$BMCL_{10}$
All dose groups	Log-logistic[a]	0.0245	0.156	0.102
High-dose group dropped	Log-logistic[a]	0.0276	**0.134**	**0.083**
Highest two groups dropped	Multistage, 1°	–[b]	0.262	0.204

[a]All other models had goodness-of-fit *p* values <0.01.
[b]Fit is "perfect" because there were only two points; all other models were unsuitable, with more parameters than this reduced data set could support.

```
================================================================
         Logistic Model. (Version: 2.8; Date: 01/18/2007)
         Input Data File: G:\_BMDS\NB_CIIT_M_MICE_WO4TH_GP.(d)
         Gnuplot Plotting File:  G:\_BMDS\NB_CIIT_M_MICE_WO4TH_GP.plt
                                                Thu Mar 06 13:14:52 2008
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background+(1-background)/[1+EXP(-intercept-slope*Log(dose))]


   Dependent variable = bronch
   Independent variable = ppm
   Slope parameter is restricted as slope >= 1

   Total number of observations = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


                  Default Initial Parameter Values
                     background =            0
                      intercept =    -0.519431
                          slope =            1


          Asymptotic Correlation Matrix of Parameter Estimates

          ( *** The model parameter(s)  -background    -slope
                have been estimated at a boundary point, or have been specified by the user,
                and do not appear in the correlation matrix )

             intercept

 intercept           1
```

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
background	0	*	*	*
intercept	-0.186463	*	*	*
slope	1	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-48.6418	3			
Fitted model	-51.5597	1	5.83582	2	0.05405
Reduced model	-136.058	1	174.833	2	<.0001

AIC: 105.119

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	68	0.000
5.0000	0.8058	53.989	58	67	1.239
25.0000	0.9540	62.011	58	65	-2.375

Chi^2 = 7.18 d.f. = 2 P-value = 0.0276

Benchmark Dose Computation

Specified effect = 0.1

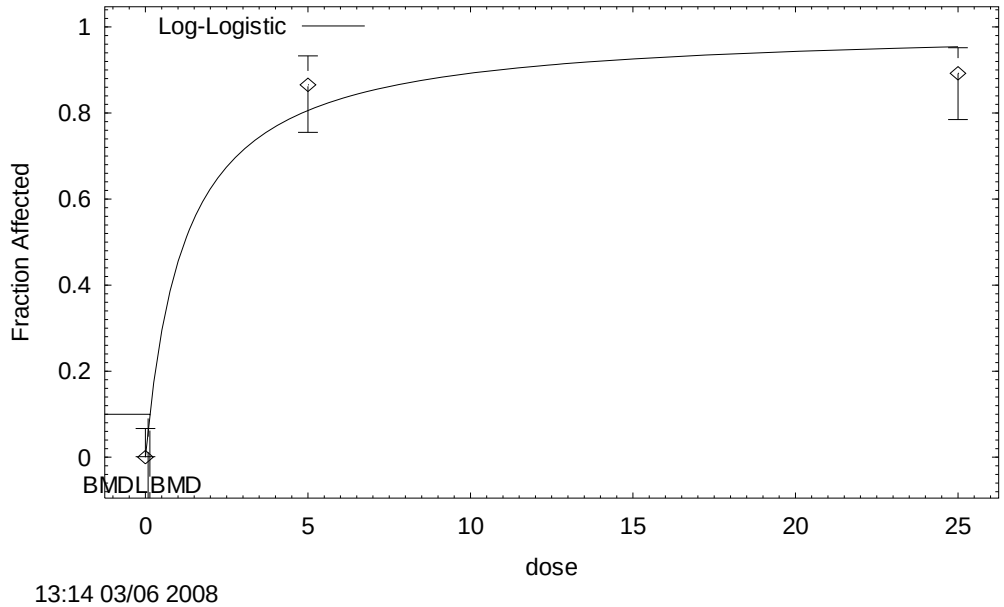
Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.133887

BMDL = 0.083223

Log-Logistic Model with 0.95 Confidence Level



13:14 03/06 2008

B-2.2.4. Alveolar bronchiolization in female mice

All models had *p* values <0.01 except the log-logistic and log-probit models.

Model	*p* value	AIC	BMC_{10}	$BMCL_{10}$
Log-logistic	0.80	49.4	0.18	0.022
Log-probit	0.12	49.4	0.40	0.278

The log-logistic model had a smaller chi-squared residual at the low exposure.

```
================================================================
         Logistic Model. (Version: 2.8; Date: 01/18/2007)
         Input Data File: M:\_BMDS\NB_CIIT_F_MICE.(d)
         Gnuplot Plotting File:  M:\_BMDS\NB_CIIT_F_MICE.plt
                                              Wed Oct 01 15:35:13 2008
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background+(1-background)/[1+EXP(-intercept-slope*Log(dose))]


   Dependent variable = bronch
   Independent variable = ppm
   Slope parameter is restricted as slope >= 1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


                 Default Initial Parameter Values
                    background =            0
                     intercept =     0.699779
                         slope =       1.0606


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -background
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )

              intercept        slope

 intercept            1        -0.94

     slope        -0.94            1


                                 Parameter Estimates

                                                          95.0% Wald Confidence Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf. Limit
     background                0            *               *                   *
      intercept         0.170022            *               *                   *
          slope          1.37009            *               *                   *

* - Indicates that this value is not calculated.


                        Analysis of Deviance Table

       Model      Log(likelihood)  # Param's  Deviance  Test d.f.   P-value
     Full model        -22.3612         4
   Fitted model        -22.6898         2      0.657286      2          0.7199
  Reduced model        -133.568         1       222.413      3         <.0001
```

AIC: 49.3797

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	53	0.000
5.0000	0.9149	54.895	55	60	0.049
25.0000	0.9899	63.350	63	64	-0.437
50.0000	0.9960	61.755	62	62	0.496

Chi^2 = 0.44 d.f. = 2 P-value = 0.8028

Benchmark Dose Computation

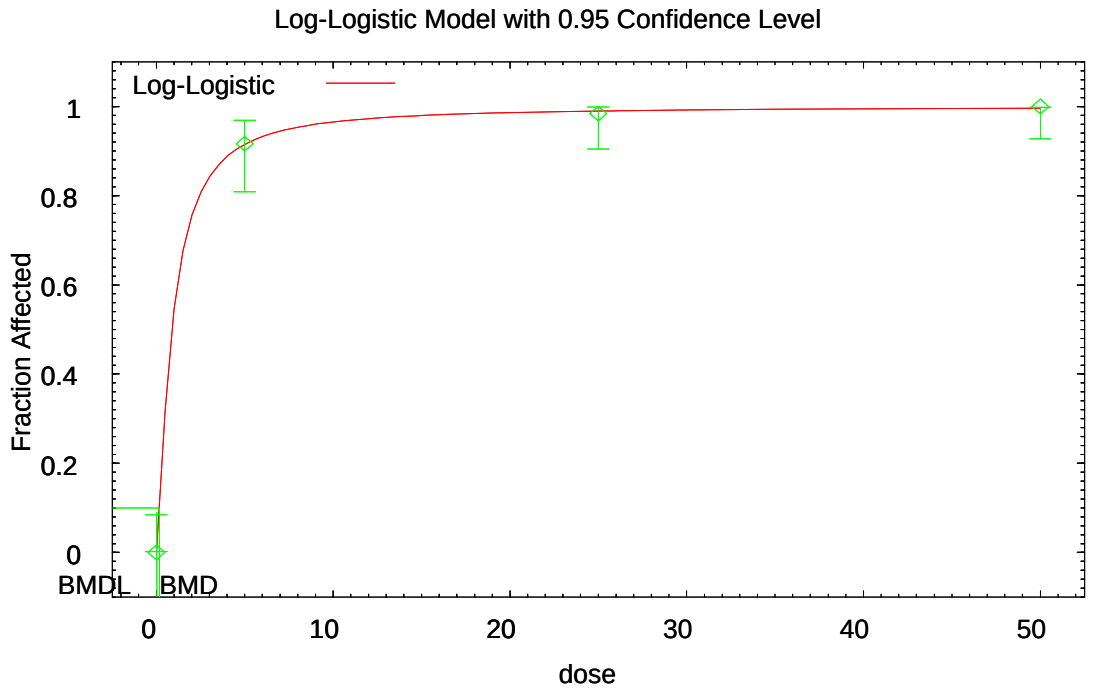
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

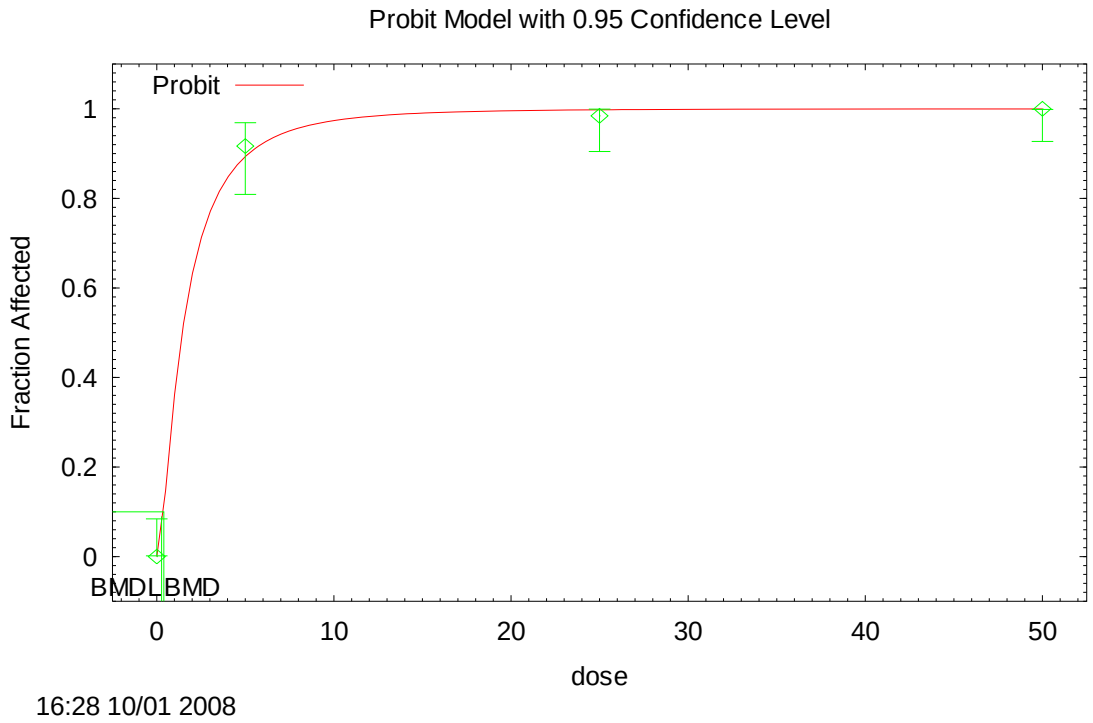
BMD = 0.177674

BMDL = 0.0221528



```
================================================================
         Probit Model. (Version: 2.7;  Date: 01/18/2007)
         Input Data File: M:\_BMDS\NB_CIIT_F_MICE.(d)
         Gnuplot Plotting File:  M:\_BMDS\NB_CIIT_F_MICE.plt
                                                Wed Oct 01 15:39:45 2008
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = Background
               + (1-Background) * CumNorm(Intercept+Slope*Log(Dose)),

   where CumNorm(.) is the cumulative normal distribution function


   Dependent variable = bronch
   Independent variable = ppm
   Slope parameter is restricted as slope >= 1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


                 Default Initial (and Specified) Parameter Values
                    background =            0
                     intercept =     -1.09733
                         slope =            1


          Asymptotic Correlation Matrix of Parameter Estimates

          ( *** The model parameter(s)  -background    -slope
                have been estimated at a boundary point, or have been specified by the user,
                and do not appear in the correlation matrix )

             intercept

 intercept           1


                                Parameter Estimates

                                                        95.0% Wald Confidence Interval
      Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf. Limit
    background                0              NA
     intercept        -0.363696        0.209203           -0.773726           0.0463335
         slope                1              NA

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.


                         Analysis of Deviance Table

      Model      Log(likelihood)  # Param's  Deviance  Test d.f.   P-value
    Full model         -22.3612         4
  Fitted model         -23.6802         1       2.63796      3          0.4509
 Reduced model         -133.568         1       222.413      3         <.0001

          AIC:         49.3603


                                  Goodness  of  Fit
                                                                    Scaled
    Dose     Est._Prob.    Expected    Observed     Size       Residual
  ----------------------------------------------------------------------
   0.0000     0.0000         0.000         0          53        0.000
   5.0000     0.8936        53.614        55          60        0.580
  25.0000     0.9978        63.862        63          64       -2.327
  50.0000     0.9998        61.988        62          62        0.110

 Chi^2 = 5.76      d.f. = 3        P-value = 0.1237
```

```
   Benchmark Dose Computation
Specified effect =            0.1
Risk Type        =      Extra risk
Confidence level =           0.95
             BMD =       0.399375
            BMDL =       0.278169
```



APPENDIX B–3: Dose-Response Modeling of Carcinogenicity Data for Nitrobenzene

B–3.1. METHODS

Data sets in rats and mice exposed to nitrobenzene by inhalation for up to 2 years (Cattley et al., 1994; CIIT, 1993) showing at least a statistical trend for increased tumor incidence with increasing exposure were fit using the multistage model in EPA's BMDS (version 1.3.2) (U.S. EPA, 1999). The data modeled are shown in Table B-3.1. The exposure levels used were those reported in the study, not adjusted for duration of exposure or converted to HECs prior to modeling. Model parameters were restricted to be positive, consistent with a monotonic dose-response relationship. A BMR of 10% increase in extra risk was used for kidney adenomas and carcinomas, and a 5% increase in extra risk was used for liver and thyroid adenomas and carcinomas.

Table B–3.1. Tumorigenic responses in experimental animals exposed to nitrobenzene via inhalation for up to 2 years

Strain/species (sex) Site	Incidence of neoplasms			
	Concentration of nitrobenzene (ppm)			
	0	**1**	**5**	**25**
F-344 rats (male) Liver: hepatocellular adenoma or carcinoma	1/43[a]	4/50	5/47	16/46
Thyroid: follicular cell adenoma or adenocarcinoma	1/43[a]	1/50	5/47	8/46
Kidney: tubular adenoma and carcinoma	0/43[a]	0/50	0/47	6/46
	Concentration of nitrobenzene (ppm)			
	0	**5**	**25**	**50**
B6C3F1 mice (male) Lung: A/B adenoma or carcinoma	8/42[a]	16/44	20/45	21/48
Thyroid: follicular cell adenoma	0/41[a]	4/44	1/45	6/46

[a]Statistically significant positive exposure-related trend in incidence by Cochran-Armitage trend test ($p < 0.05$).

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

B-3.2. RESULTS

The BMD modeling results are summarized in Table B-3.2. This table shows the BMDs and BMDLs derived from each endpoint modeled. The BMDS outputs for all model runs are presented below.

Table B–3.2. Summary of BMD modeling results for nitrobenzene cancer data

Tumor	Number of stages	*p* Value for model fit	BMD_{10} (ppm)	$BMDL_{10}$ (ppm)
Male F344 rats				
Liver: hepatocellular adenoma or carcinoma	1	0.63	**6.76**	**4.46**
Thyroid: follicular cell adenoma or adenocarcinoma	1	0.38	13.64	7.78
Kidney: tubular cell adenoma or carcinoma	1[a]	0.99	22.81	16.78
Male B6C3F1 mice				
Lung: A/B adenoma or carcinoma	1	0.18	15.43	8.52
Thyroid: follicular cell adenoma	1[a]	0.056[b]	52.23	30.46

[a]Model was one-stage with a three-degree polynomial (coefficients for lower degrees were 0).
[b]While this data set had a statistically significant trend, the dose-response shape was not strictly monotonic (see Output B-3.2.V). Within the context of applying one model for the purpose of consistent low-dose linear extrapolation, the multistage model provides an adequate fit.

OUTPUT B-3.2.1. Male F344 rat liver tumors (hepatocellular adenoma or carcinoma)

```
 ================================================================
         Multistage Model. (Version: 2.8;  Date: 02/20/2007)
         Input Data File: C:\BMDS\UNSAVED1.(d)
         Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                                Fri Feb 22 10:39:11 2008
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
                 -beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = liver
   Independent variable = ppm

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     Background =    0.0425205
                        Beta(1) =    0.0148284
                        Beta(2) =            0
                        Beta(3) = 8.47487e-007


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Beta(2)    -Beta(3)
                 have been estimated at a boundary point, or have been specified by the user,
```

and do not appear in the correlation matrix)

	Background	Beta(1)
Background	1	-0.57
Beta(1)	-0.57	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0.0410129	*	*	*
Beta(1)	0.0155753	*	*	*
Beta(2)	0	*	*	*
Beta(3)	0	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-64.3357	4			
Fitted model	-64.7955	2	0.919545	2	0.6314
Reduced model	-75.2506	1	21.8296	3	<.0001

AIC: 133.591

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0410	1.764	1	43	-0.587
1.0000	0.0558	2.792	4	50	0.744
5.0000	0.1129	5.304	5	47	-0.140
25.0000	0.3503	16.114	16	46	-0.035

Chi^2 = 0.92 d.f. = 2 P-value = 0.6314

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

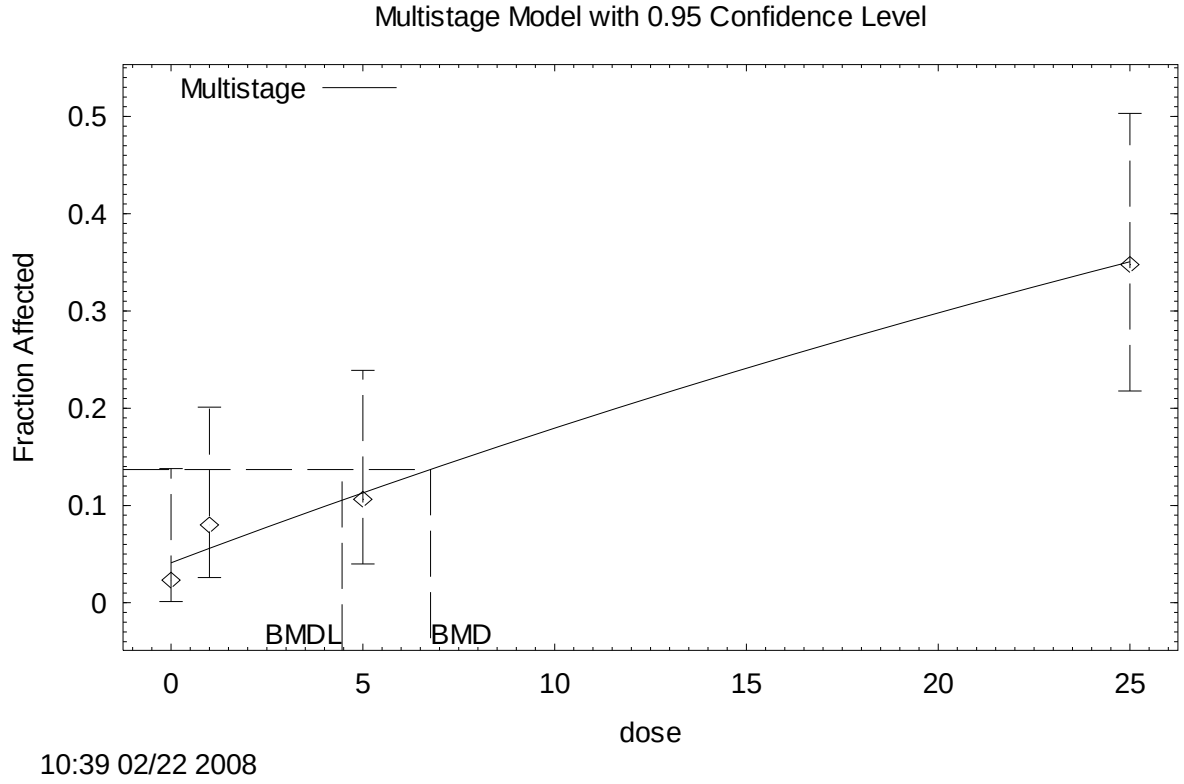
Confidence level = 0.95

BMD = 6.76461

BMDL = 4.46073

BMDU = 18.5642

Taken together, (4.46073, 18.5642) is a 90 % two-sided confidence interval for the BMD



OUTPUT B-3.2.2. Male F344 rat thyroid tumors (follicular cell adenoma or adenocarcinoma)

```
 ======================================================================
        Multistage Model. (Version: 2.8;  Date: 02/20/2007)
        Input Data File: C:\BMDS\UNSAVED1.(d)
        Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                                  Fri Feb 22 10:36:26 2008
 ======================================================================

 BMDS MODEL RUN
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   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
                -beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = thyroid
   Independent variable = ppm

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values
Background = 0.0358259
Beta(1) = 0.0064946
Beta(2) = 0
Beta(3) = 0

Asymptotic Correlation Matrix of Parameter Estimates

(*** The model parameter(s) -Beta(2) -Beta(3)
have been estimated at a boundary point, or have been specified by the user,
and do not appear in the correlation matrix)

	Background	Beta(1)
Background	1	-0.62
Beta(1)	-0.62	1

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
Background	0.0266522	*	*	*
Beta(1)	0.00772486	*	*	*
Beta(2)	0	*	*	*
Beta(3)	0	*	*	*

* - Indicates that this value is not calculated.

Analysis of Deviance Table

Model	Log(likelihood)	# Param's	Deviance	Test d.f.	P-value
Full model	-46.8328	4			
Fitted model	-47.7145	2	1.76351	2	0.4141
Reduced model	-52.1437	1	10.6218	3	0.01396

AIC: 99.429

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0267	1.146	1	43	-0.138
1.0000	0.0341	1.707	1	50	-0.551
5.0000	0.0635	2.986	5	47	1.204
25.0000	0.1976	9.089	8	46	-0.403

Chi^2 = 1.94 d.f. = 2 P-value = 0.3799

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

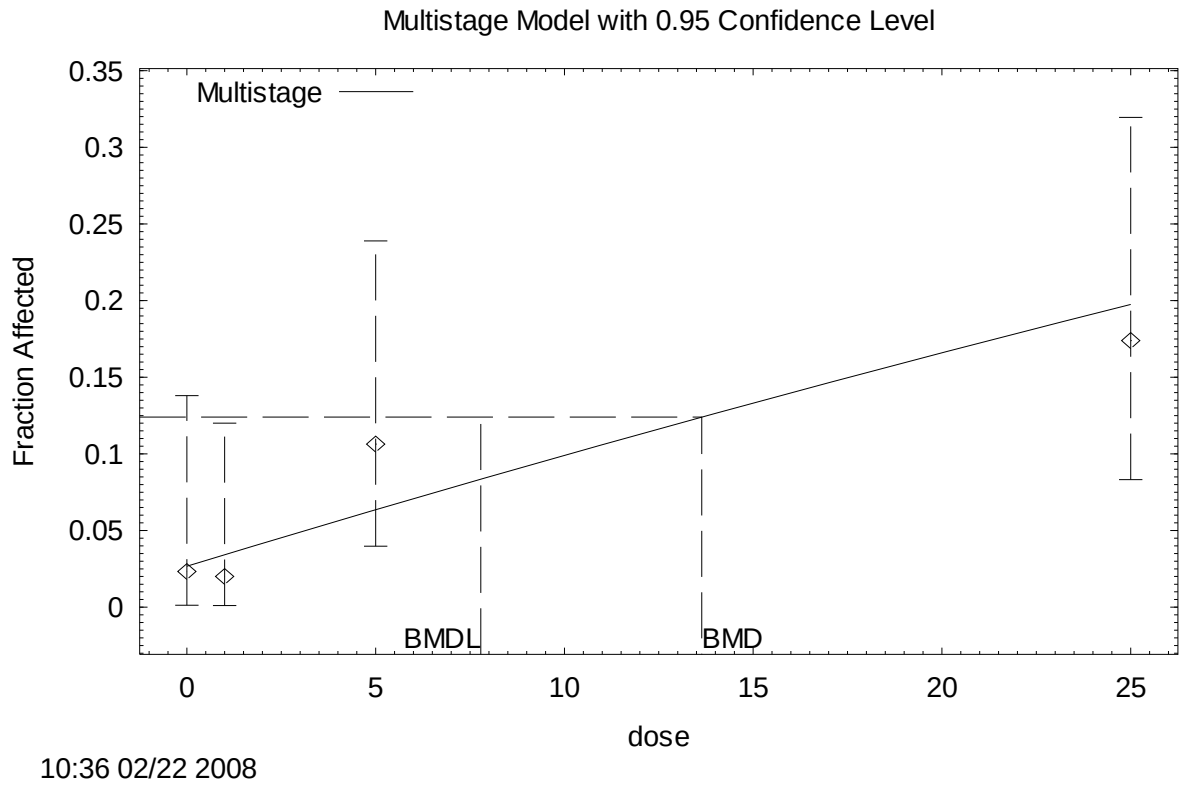
Confidence level = 0.95

BMD = 13.6391

BMDL = 7.78329

BMDU = 34.2184

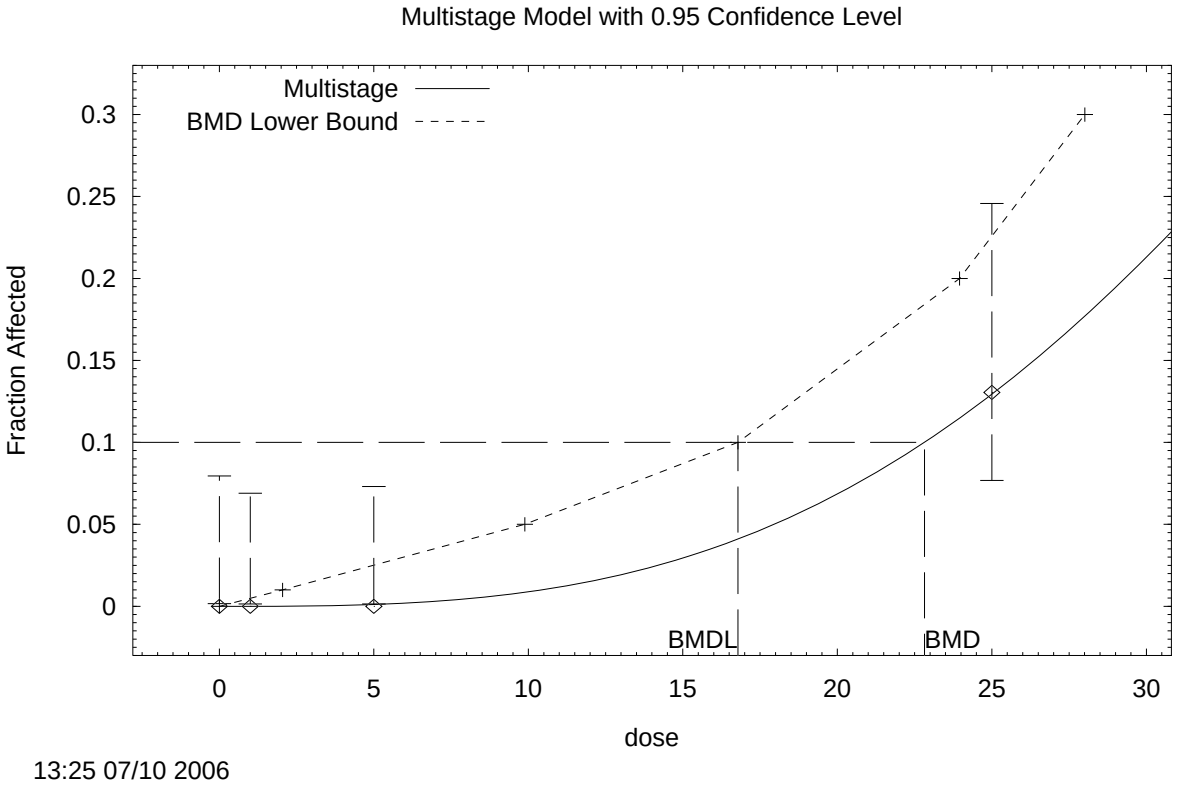
Taken together, (7.78329, 34.2184) is a 90 % two-sided confidence
interval for the BMD



OUTPUT B-3.2.3. Male F344 rat kidney tumors (tubular cell adenomas or carcinomas)

```
 ======================================================================
         Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
         Input Data File: C:\BMDS\UNSAVED1.(d)
         Gnuplot Plotting File:  C:\BMDS\UNSAVED1.plt
                                                    Mon Jul 10 13:25:30 2006
 ======================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = Incidence
   Independent variable = Concentration

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008


                  Default Initial Parameter Values
```

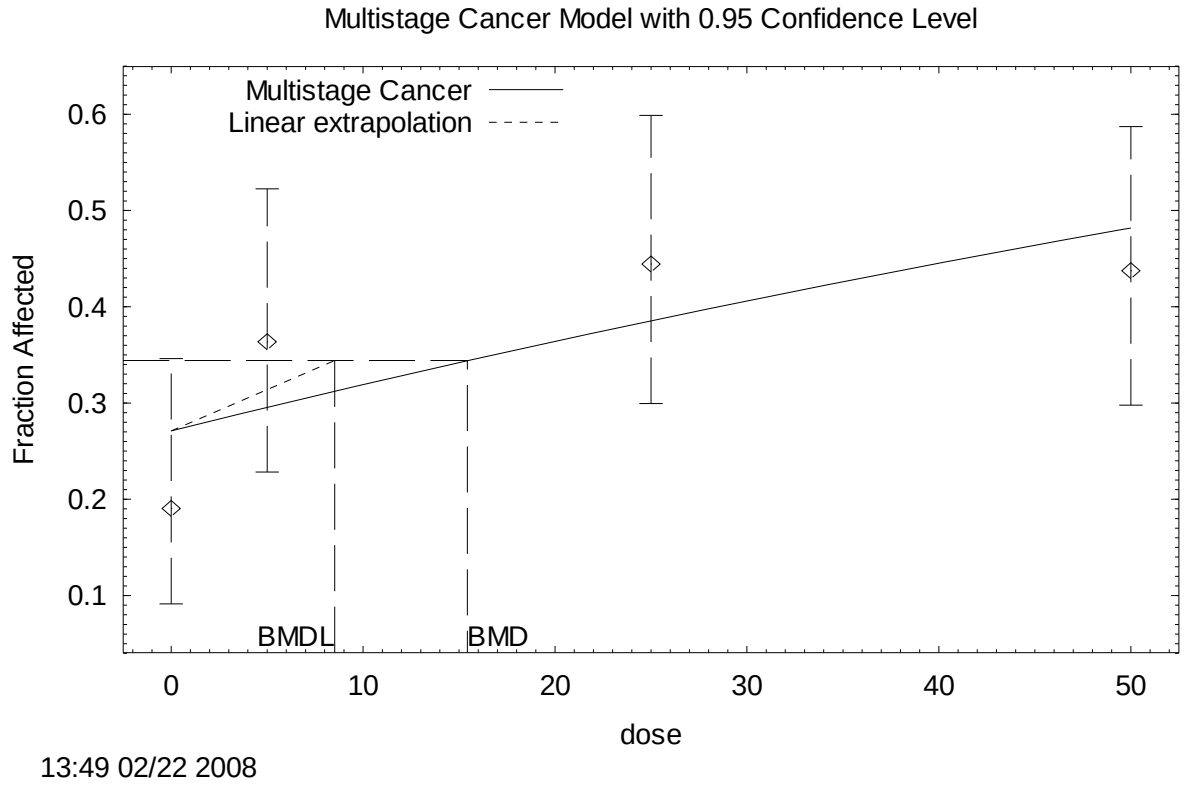
```
                    Background =           0
                       Beta(1) =           0
                       Beta(2) =           0
                       Beta(3) = 8.96836e-006


          Asymptotic Correlation Matrix of Parameter Estimates

          ( *** The model parameter(s)  -Background    -Beta(1)    -Beta(2)
                have been estimated at a boundary point, or have been specified by the user,
                and do not appear in the correlation matrix )

               Beta(3)

   Beta(3)           1



                         Parameter Estimates

       Variable           Estimate             Std. Err.
     Background                  0                NA
        Beta(1)                  0                NA
        Beta(2)                  0                NA
        Beta(3)       8.86633e-006       1.00717e-005

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.



                       Analysis of Deviance Table

       Model      Log(likelihood)  Deviance  Test DF     P-value
     Full model        -17.8118
   Fitted model        -17.8645     0.105529      3          0.9912
  Reduced model        -26.5061      17.3886      3       0.0005879

          AIC:         37.7291


                     Goodness  of  Fit

     Dose     Est._Prob.    Expected    Observed     Size     Chi^2 Res.
  ------------------------------------------------------------------------
i: 1
    0.0000     0.0000         0.000         0          43       0.000
i: 2
    1.0000     0.0000         0.000         0          50      -1.000
i: 3
    5.0000     0.0011         0.052         0          47      -1.001
i: 4
   25.0000     0.1294         5.951         6          46       0.009

 Chi-square =       0.05     DF = 3        P-value = 0.9968


   Benchmark Dose Computation

Specified effect =           0.1

Risk Type        =      Extra risk

Confidence level =          0.95

             BMD =        22.8198

            BMDL =        16.7833
```



OUTPUT B-3.2.4. Male B6C3F1 mouse lung tumors (A/B adenomas or carcinomas)

```
 ================================================================
         Multistage Cancer Model. (Version: 1.5;  Date: 02/20/2007)
         Input Data File: C:\BMDS\NB_CIIT_MFEMALE_RFC.(d)
         Gnuplot Plotting File:  C:\BMDS\NB_CIIT_MFEMALE_RFC.plt
                                                Fri Feb 22 13:49:01 2008
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
                -beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = thyroid
   Independent variable = ppm

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008


                 Default Initial Parameter Values
```

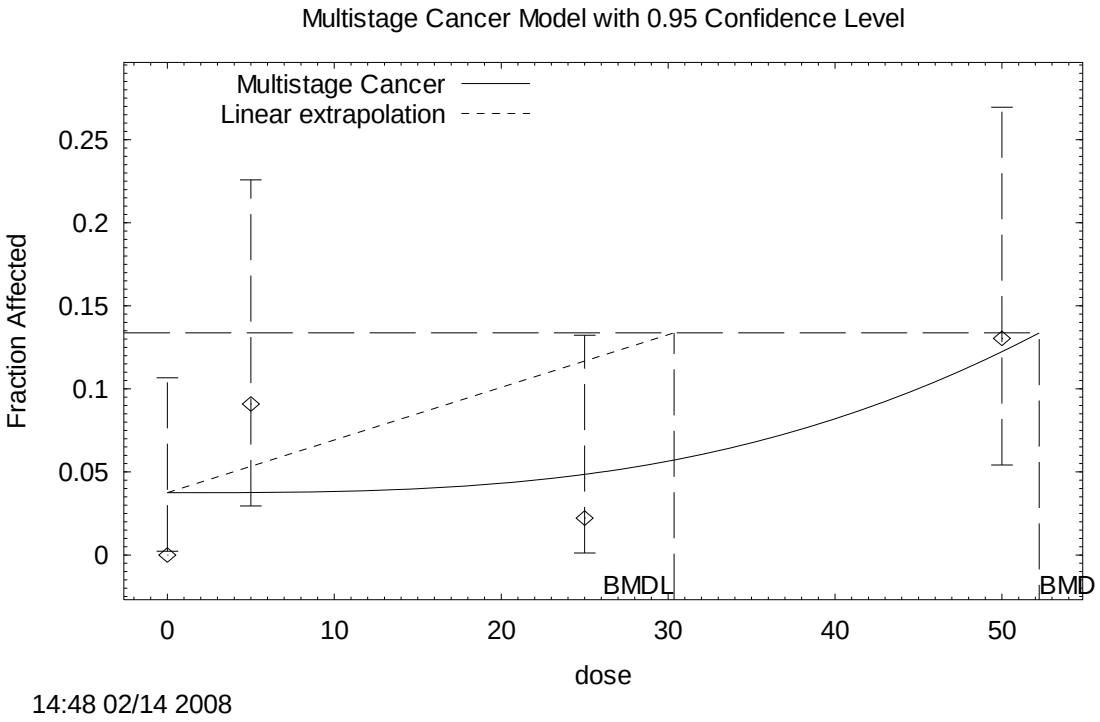
```
                    Background =     0.286791
                       Beta(1) =   0.00593155
                       Beta(2) =            0
                       Beta(3) =            0


          Asymptotic Correlation Matrix of Parameter Estimates

          ( *** The model parameter(s)  -Beta(2)    -Beta(3)
                have been estimated at a boundary point, or have been specified by the user,
                and do not appear in the correlation matrix )

            Background      Beta(1)

Background           1        -0.71

   Beta(1)       -0.71            1


                                Parameter Estimates

                                                          95.0% Wald Confidence Interval
      Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf. Limit
    Background         0.271318            *                 *                   *
       Beta(1)       0.00682817            *                 *                   *
       Beta(2)                0            *                 *                   *
       Beta(3)                0            *                 *                   *

* - Indicates that this value is not calculated.


                        Analysis of Deviance Table

      Model      Log(likelihood)  # Param's  Deviance  Test d.f.   P-value
    Full model          -113.1         4
   Fitted model        -114.829         2       3.45823      2          0.1774
  Reduced model         -117.28         1       8.36076      3         0.03912

          AIC:         233.658


                                Goodness  of  Fit
                                                                      Scaled
    Dose     Est._Prob.    Expected    Observed     Size       Residual
  ---------------------------------------------------------------------
   0.0000     0.2713        11.395         8          42       -1.178
   5.0000     0.2958        13.014        16          44        0.986
  25.0000     0.3857        17.355        20          45        0.810
  50.0000     0.4821        23.140        21          48       -0.618

 Chi^2 = 3.40      d.f. = 2        P-value = 0.1828


   Benchmark Dose Computation

Specified effect =           0.1

Risk Type        =      Extra risk

Confidence level =          0.95

             BMD =        15.4303

            BMDL =          8.523

            BMDU =        61.7258

Taken together, (8.523  , 61.7258) is a 90     % two-sided confidence
interval for the BMD

Multistage Cancer Slope Factor =      0.011733
```



OUTPUT B-3.2.5. Male B6C3F1 mouse thyroid follicular cell adenomas

```
=================================================================
         Multistage Cancer Model. (Version: 1.4;  Date: 01/18/2007)
         Input Data File: G:\_BMDS\NB_CIIT_M_MICE.(d)
         Gnuplot Plotting File:  G:\_BMDS\NB_CIIT_M_MICE.plt
                                                Thu Feb 14 14:48:52 2008
 =================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
                -beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = thyroid
   Independent variable = ppm

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008


                  Default Initial Parameter Values
```

```
                    Background =    0.0354703
                       Beta(1) =            0
                       Beta(2) =            0
                       Beta(3) = 8.03455e-007


          Asymptotic Correlation Matrix of Parameter Estimates

          ( *** The model parameter(s)  -Beta(1)    -Beta(2)
                have been estimated at a boundary point, or have been specified by the user,
                and do not appear in the correlation matrix )

            Background      Beta(3)

Background            1        -0.54

   Beta(3)        -0.54            1


                                Parameter Estimates

                                                          95.0% Wald Confidence Interval
      Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf. Limit
    Background         0.037499            *                 *                   *
       Beta(1)                0            *                 *                   *
       Beta(2)                0            *                 *                   *
       Beta(3)     7.39469e-007            *                 *                   *

* - Indicates that this value is not calculated.


                        Analysis of Deviance Table

       Model      Log(likelihood)  # Param's  Deviance  Test d.f.   P-value
     Full model        -36.0112         4
   Fitted model         -39.264         2       6.50555      2         0.03867
  Reduced model        -41.1473         1       10.2722      3         0.01639

          AIC:          82.528


                                Goodness  of  Fit
                                                                      Scaled
     Dose     Est._Prob.    Expected    Observed     Size       Residual
  ---------------------------------------------------------------------------
    0.0000     0.0375         1.537         0          41       -1.264
    5.0000     0.0376         1.654         4          44        1.860
   25.0000     0.0486         2.185         1          45       -0.822
   50.0000     0.1225         5.634         6          46        0.165

 Chi^2 = 5.76      d.f. = 2        P-value = 0.0562


   Benchmark Dose Computation

Specified effect =           0.1

Risk Type        =      Extra risk

Confidence level =          0.95

             BMD =        52.2299

            BMDL =        30.3586

            BMDU =        334.893

Taken together, (30.3586, 334.893) is a 90     % two-sided confidence
interval for the BMD

Multistage Cancer Slope Factor =    0.00329396
```


Multistage Cancer Model with 0.95 Confidence Level
Multistage Cancer
Linear extrapolation
Fraction Affected
0.25
0.2
0.15
0.1
0.05
0
BMDL
BMD
0
10
20
30
40
50
dose
14:48 02/14 2008

B-3.3. Estimation of Overall Cancer Risk from Inhalation Exposure to Nitrobenzene by Combining Risk Estimates Across Multiple Tumor Sites

In the CIIT (1993) bioassay that was selected for use in the cancer dose-response modeling of nitrobenzene, increased tumor incidences were observed at multiple sites in the male rat following inhalation exposure to nitrobenzene (i.e., in the kidney, thyroid, and liver). Given the multiplicity of tumor sites, basing the unit risk on one tumor site may underestimate the carcinogenic potential of nitrobenzene. In addition, application of one model to a composite data set does not accommodate biologically relevant information that may vary across sites or may only be available for a subset of sites. Following the recommendations of NRC (1994) and the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), a statistically appropriate upper bound on total risk was estimated in order to gain some understanding of the total risk from multiple tumor sites in the selected data set. Note that this estimate of overall risk describes the risk of developing any combination of the tumor types considered, not just the risk of developing all simultaneously.

NRC (1994) stated that an approach based on counts of animals with one or more tumors (or tumor-bearing animals) would tend to underestimate overall risk when tumor types occur independently and that an approach based on combining the risk estimates from each separate tumor type should be used. This assessment considers both types of approach for estimating total risk.

Separate dose-response fits for each site. Following the NRC (1994) recommendation, there are several ways to address combining the risk estimates from each separate tumor type. For individual tumor sites modeled using the multistage model,

$$P(d \mid \boldsymbol{q}) = 1 - exp[-(q_0 + q_1 d + q_2 d^2 + ... + q_k d^k)]$$

the model for the combined tumor risk would still be multistage, with a functional form that has the sum of stage-specific multistage coefficients as the corresponding multistage coefficient

$$P_c(d \mid \boldsymbol{q}) = 1 - exp[-(\Sigma q_{0i} + \Sigma q_{1i} d + \Sigma q_{2i} d^2 + ... + \Sigma q_{ki} d^k)]$$

The resulting equation for fixed extra risk (BMR) is polynomial in dose (when logarithms of both sides are taken) and can be straightforwardly solved for combined BMD. But confidence bounds (based on the profile likelihood method) for that BMD are not provided in available software.

A Bayesian approach to finding a confidence bound on the combined BMD was implemented by using WinBugs (Spiegelhalter et al., 2003). WinBugs software is freely available and it implements Markov chain Monte Carlo computations. Use of WinBugs to derive a distribution of BMD for a single multistage model (Kopylev et al., 2007) was

straightforwardly generalized to derive a distribution of BMD for combined tumor risk, according to the NRC (1994) methodology described above. The advantage of the Bayesian approach is that it produces a distribution of the BMD, allowing better characterization of uncertainty than would calculation of only the expected risk.

A diffuse (high variance or low tolerance) Gaussian prior restricted to be nonnegative was used. The posterior distribution is based on three chains with 50,000 burn-ins (50,000 initial simulations dropped) and thinning rate of 10, resulting in 150,000 simulations total. The mean and 5th percentile of the posterior distribution provide estimates of the mean BMD ("central estimate") and the BMDL for combined tumor risk, respectively. The WinBugs code for generating these distributions is provided at the end of this section.

Table B-3.3 presents the results of the Bayesian analysis. The upper bound on the combined risk estimate for nitrobenzene is 4×10^{-5} $(\mu g/m^3)^{-1}$, rounding the value derived above to one significant figure. This analysis, using combined tumor sites, suggests a higher potency than when considering only the most sensitive tumor site alone (i.e., liver tumors). In this case, however, there is no appreciable difference between the two approaches when the potencies are rounded to one significant figure. Regardless, neither of these risk estimates should be used with continuous lifetime nitrobenzene exposures greater than about 3×10^3 $\mu g/m^3$, the human equivalent of the POD defined for the male rat liver tumors, because the observed dose-response curve is not likely to be linear at higher doses.

Table B-3.3. Overall risk based on kidney, thyroid, or liver adenomas/carcinomas in F344 rats, using simulated data derived from observed incidences (Bayesian approach)

	Bioassay concentrations		Human equivalents			
Tumor site and type	**Median BMC (BMC_{10}), ppm**	**$BMCL_{10}$ (ppm)**	**$BMC_{10/HEC}$ ($\mu g/m^3$)[a]**	**$BMCL_{10/HEC}$ ($\mu g/m^3$)**	**Slope from BMC_{10} $(\mu g/m^3)^{-1}$ [b]**	**Upper bound risk estimate, $(\mu g/m^3)^{-1}$ [b]**
Kidney, adenoma or carcinoma	19.4[c]	14.2[c]	17,500	12,800	5.7×10^{-6}	7.8×10^{-6}
Thyroid, follicular cell adenoma or carcinoma	13.6	7.8	12,200	7,020	8.2×10^{-6}	1.4×10^{-5}
Liver, hepatocellular adenoma or carcinoma	6.7	4.4	6,030	4,000	1.7×10^{-5}	2.5×10^{-5}
Combined tumors	4.1	2.9	3,650	2,610	**2.7×10^{-5}**	**3.8×10^{-5}**

[a]$BMC(L)_{HEC} = BMC(L) \times 5.04$ mg/m^3 $\times$ 5/7 $\times$ 6/24, assuming ratio of animal to human air:blood partition coefficients is 1.

[b]The slope to background from the $BMC_{10/HEC} = 0.1/BMC_{10/HEC}$, and the upper bound risk estimate = $0.1/BMCL_{10/HEC}$, using the models developed and reported in section B-3.3.

[c]Note that the BMC_{10} and $BMCL_{10}$ for kidney tumors are not the same values estimated by using BMDS (which are 22.8 and 16.8 ppm, respectively; see Output, B-3.2.III). Alternate methodology does not generally provide identical results. In this case, the Bayesian results are expected to be more accurate. However, this tumor site is the least sensitive and with very little impact on the composite estimated risk.

Tumor-bearing animals. For comparison, dose-response analysis was carried out on the incidence of tumor-bearing animals. Tabulation of tumor-bearing animals is best carried out with histopathology results reported for individual animals. These results were not available in the CIIT (1993) report. Under the assumption that the three tumor types are independent of each other, the incidences of tumor-bearing animals in each group can be estimated using the following probability relationship:

$$P(A, B, \text{or } C) = P(A) + P(B) + P(C) - P(A)P(B) - P(A)P(C) - P(B)P(C) + P(A)P(B)P(C)$$

where A, B, and C represent the three tumor types, and P( ) denotes the proportion responding. This relationship sums the proportions responding across tumor types and adjusts for double counting. A summary of the application of this relationship to the male rat tumor incidence (in Table B-3.1) is provided in Table B-3.4. The estimated incidences in the control and low-dose groups are the same as just adding the raw incidences, while those in the mid- and high-dose groups are approximately 1 and 16% less than the sums of the raw incidences for those groups.

A one-stage multistage model provided an adequate fit to the estimated incidences of tumor-bearing animals (see Output, B-3.4.1), with a BMD_{10} of 3.8 ppm and a $BMDL_{10}$ of 2.7 ppm, very similar to the BMD_{10} and $BMDL_{10}$ for overall risk derived above using the Bayesian approach. The BMC_{HEC} was calculated as BMC × 5.04 mg/m^3 × 5/7 × 6/24 = 3,420 $\mu g/m^3$, assuming the ratio of animal to human air:blood partition coefficients is 1. The slope to background from the $BMC_{10/HEC} = 0.1/BMC_{10/HEC}$ or 3×10^{-5} $(\mu g/m^3)^{-1}$. The $BMCL_{HEC}$ was calculated as BMCL × 5.04 mg/m^3 ×5/7 × 6/24 = 2,430 $\mu g/m^3$. The upper bound risk estimate = $0.1/BMCL_{10/HEC} = 4 \times 10^{-5}$ $(\mu g/m^3)^{-1}$.

Table B-3.4. Estimated incidence of male F344 rats with liver, thyroid, or kidney tumors from separately tabulated site-specific incidences following nitrobenzene exposure for 2 years

Exposure (ppm)	Incidence of neoplasms and (%) responding			Estimated probability of an animal having at least one of the tumor types	Estimated incidence of tumor-bearing animals
	Liver	Thyroid	Kidney		
0	1/43 (2.3%)	1/43 (2.3%)	0/43 (0%)	0.046	2/43
1	4/50 (8.0%)	1/50 (2.0%)	0/50 (0%)	0.098	5/50
5	5/47 (10.6%)	5/47 (10.6%)	0/47 (0%)	0.201	9/47
25	16/46 (35%)	8/46 (17%)	6/46 (17%)	0.532	24/46

Although both approaches in this case lead to a similar result, it is not clear that the two approaches will generally agree. Further examination of the operating characteristics of the respective approaches is necessary for any such generalizations.

References

Kopylev, L., Chen, C., White, P. (2007). Towards quantitative uncertainty assessment for cancer risks: central estimates and probability distributions of risk in dose-response modeling. Regul Tox Pharmacol 49:203–207.

NRC (1994). Science and Judgment. National Academy Press. Washington, DC.

Spiegelhalter, D., Thomas, A., Best, N., (2003). WinBugs Version 1.4 User Manual. http://www.mrc-bsu.cam.ac.uk/bugs/winbugs/manual14.pdf

Computer Code for Bayesian Approach

WinBugs computer code that was used to derive posterior distribution of multistage parameters for individual tumors is provided below. The resulting distributions can be used to solve for distribution of individual and combined BMD as described above.

To run the following programs, one needs to have R and WinBugs (freeware) installed, and the R package RtoWinbugs should be installed and loaded in R. The next step is to create two text files in the directory that R uses:

sum.txt is a text file containing the following text:

```
model {
for( i in 1 : doses ) {
y1[i]~dbin(g1[i],n1[i])
log(g1[i])<- -b0-b1*x[i]
y2[i]~dbin(g2[i],n2[i])
log(g2[i])<- -c0-c1*x[i]-c2*pow(x[i],2)-c3*pow(x[i],3)
y3[i]~dbin(g3[i],n3[i])
log(g3[i])<- -d0-d1*x[i]
}
b0~dnorm(0,.0001)I(0,)
b1~dnorm(0,.0001)I(0,)
c0~dnorm(0,.0001)I(0,)
c1~dnorm(0,.0001)I(0,)
c2~dnorm(0,.0001)I(0,)
c3~dnorm(0,.0001)I(0,)
d0~dnorm(0,.0001)I(0,)
d1~dnorm(0,.0001)I(0,)
}
```

data.txt is a text file containing the following text:

```
list(doses=4,
n1=c(43, 50, 47, 46),
n2=c(43, 50, 47, 46),
n3=c(43, 50, 47, 46),
x=c(0, 1,5,25),
y1=c(42, 46, 42, 30),
y2=c(43, 50, 47, 40),
y3=c(42, 49, 42, 38)
)
```

The next step is to run three R commands:

```
inits<-function(){list(b0=runif(1,0,.1),b1=runif(1,0,.1),c0=runif(1,0,.1),c1=runif(1,0,.1),c2=runif(1,0,.1),c3=runif(1,0,.1),
d0=runif(1,0,.1),d1=runif(1,0,.1))}
parameters<-c("b1","c1","c2","c3,"d1")
NB.sim<-bugs("data.txt",inits,parameters,"sum.txt",n.chains=3,n.iter=550000,n.burnin=50000,n.thin=10)
```

The last step is to run the R command

```
attach(NB.sim)
```

that creates posterior distributions of the individual multistage parameters (here b1 for liver, d1 for thyroid and c1,c2,c3 for thyroid).

Output B-3.4.1.

```
 ======================================================================
         Multistage Model. (Version: 2.7;  Date: 01/18/2007)
         Input Data File: M:\_BMDS\NB_TBA_RM.(d)
         Gnuplot Plotting File:  M:\_BMDS\NB_TBA_RM.plt
                                                 Wed Mar 26 13:50:47 2008
 ======================================================================
 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
                -beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = tba
   Independent variable = ppm

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     Background =    0.0649281
                        Beta(1) =    0.0269233
                        Beta(2) =            0
                        Beta(3) =            0


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Beta(2)    -Beta(3)
                 have been estimated at a boundary point, or have been specified by the user,
                 and do not appear in the correlation matrix )

             Background      Beta(1)

Background            1        -0.54

   Beta(1)        -0.54            1

                                Parameter Estimates

                                                        95.0% Wald Confidence Interval
      Variable         Estimate        Std. Err.     Lower Conf. Limit   Upper Conf. Limit
    Background        0.0601791            *                *                   *
       Beta(1)        0.0278086            *                *                   *
       Beta(2)                0            *                *                   *
       Beta(3)                0            *                *                   *

* - Indicates that this value is not calculated.


                        Analysis of Deviance Table

       Model      Log(likelihood)  # Param's  Deviance  Test d.f.   P-value
     Full model        -79.1379         4
   Fitted model        -79.2961         2      0.316284      2          0.8537
  Reduced model        -96.8271         1       35.3784      3         <.0001
           AIC:         162.592
```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0602	2.588	2	43	-0.377
1.0000	0.0860	4.298	5	50	0.354
5.0000	0.1822	8.562	9	47	0.165
25.0000	0.5311	24.429	24	46	-0.127

Chi^2 = 0.31 d.f. = 2 P-value = 0.8560

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 3.78878

BMDL = 2.70515

BMDU = 10.7878

Taken together, (2.70515, 10.7878) is a 90 % two-sided confidence interval for the BMD