CHAPTER 3. TOXICOKINETICS, SUSCEPTIBLE POPULATIONS, BIOMARKERS, CHEMICAL INTERACTIONS

3.1 TOXICOKINETICS

Case reports and experimental exposure studies in humans provide limited information on the absorption, metabolism, and excretion of nitrobenzene. Studies in rats, mice, and rabbits exposed primarily by oral administration provide further details on the toxicokinetics. An overview of the information is provided below.

- Nitrobenzene is systemically absorbed in humans and animals after inhalation, oral, and dermal exposure, as evidenced by measurements of this compound in blood and by measurements of radioactivity in animals exposed to radiolabelled nitrobenzene. The rate of absorption has not been quantified. The extent of oral absorption has been estimated to be at least 35-65% based on urinary excretion of radioactivity, which may underestimate total absorption.

- Nitrobenzene is preferentially distributed to adipose tissue, from where it may be redistributed into systemic circulation. Based on measurement of radioactivity in animals exposed orally, nitrobenzene or its metabolites may also be distributed to liver, kidneys, and lungs.

- Nitrobenzene may be metabolized by oxidative or reductive pathways, but the reductive pathways appear to yield the ultimate toxicants. Reduction of nitrobenzene occurs in the gastrointestinal tract via oxygen-insensitive nitroreductase enzymes produced by gut microflora. Nitrobenzene reduction may also occur in hepatic microsomes and in erythrocytes via oxygen-sensitive nitroreductases.

- Redox cycling of nitrobenzene metabolites, especially nitrosobenzene and phenylhydroxylamine, is believed to be responsible for conversion of hemoglobin to methemoglobin. In addition, metabolism of nitrobenzene yields free radicals at several steps in the reductive pathways.

- Species differences in metabolism of nitrobenzene have been observed and may correlate to differences in susceptibility to nitrobenzene hematotoxicity.

- Urinary metabolites of nitrobenzene in humans and mammals include nitrophenols and aminophenols, and animal studies have also detected p-hydroxyacetanilide. The urinary metabolites are often conjugated with sulfate or glucuronide.

- The primary route of elimination of nitrobenzene is via urinary excretion of metabolites.

3.1.1 Absorption

Nitrobenzene is absorbed across the respiratory tract, as demonstrated indirectly in case reports of symptoms after inhalation exposure (Ikeda and Kita 1964; Lee et al. 2013) and directly in studies of experimental human exposures (Piotrowski 1967; Salmowa et al. 1963). The absorption of inhaled

nitrobenzene has been demonstrated qualitatively in a case report of elevated methemoglobin in a 37-year-old man who was exposed to nitrobenzene via inhalation while cleaning a nitrobenzene pump (Lee et al. 2013). Similarly, Ikeda and Kita (1964) reported methemoglobinemia in a 47-year-old woman whose job for the prior 1.5 years involved painting pan lids with a product that contained nitrobenzene as a solvent. The study authors detected metabolites of nitrobenzene (p-nitrophenol and p-aminophenol) in the subject's urine. The levels of these metabolites in urine declined over time in parallel with decreases in the patient's methemoglobin levels (Ikeda and Kita 1964).

Salmowa et al. (1963) estimated the lung retention of nitrobenzene in a study of seven male volunteers who were exposed for 6 hours to concentrations of 1–6 ppm. The subjects were exposed through a gas mask to prevent dermal absorption. Expired air was collected and analyzed for nitrobenzene, and absorption was estimated as the difference between the mass of nitrobenzene inhaled and the mass exhaled. Using this method, the study authors estimated that ~80% of the nitrobenzene vapor was retained in the lungs. Piotrowski (1967) conducted an experiment with male volunteers exposed to the same concentration range for repeated exposures (6 hours/day for ≥4 days). The detection of the nitrobenzene metabolite, p-nitrophenol, in the urine of the exposed subjects demonstrated absorption of inhaled nitrobenzene.

Oral absorption of nitrobenzene in humans has been shown indirectly in case reports of human poisoning after ingestion of this compound. Only one case report (Martínez et al. 2003) reported measurement of nitrobenzene in blood or tissues. Martínez et al. (2003) reported that the concentration of nitrobenzene in the blood was 3.2 μg/mL in an 82-year-old man 48 hours after he ingested about 250 mL of nitrobenzene. Myslak et al. (1971) described a case in which a 19-year-old female consumed about 50 mL of nitrobenzene; high levels of the nitrobenzene metabolites, p- aminophenol and p-nitrophenol, were detected in the patient's urine. Several other fatal human poisonings have been reported where the ingested substance is known to be nitrobenzene (Gupta et al. 2000, 2012; Kumar et al. 2017). In addition, numerous cases of methemoglobinemia in humans have been reported due to oral exposure to nitrobenzene (Agrawal et al. 2011; Balwani et al. 2017; Boukobza et al. 2015; Chongtham et al. 1997; D'sa et al. 2014; Kumar et al. 1990; Perera et al. 2009; Saxena and Prakash Saxena 2010). These case reports demonstrate absorption of nitrobenzene from the gastrointestinal tract. No quantitative estimates of oral absorption in humans were located.

Studies of rats, mice, and rabbits exposed to radiolabelled [^{14}C]-nitrobenzene suggest oral absorption estimates of 35–65% based on urinary excretion of radioactivity over ≥3 days after dosing (Albrecht and

Neumann 1985; Levin and Dent 1982; Parke 1956; Rickert et al. 1983; Robinson et al. 1951). Estimates of oral absorption based only on urinary excretion may underestimate the total uptake of nitrobenzene. Rabbits were shown to excrete small amounts of radioactivity (~1% of administered dose) as CO_2 and aniline in expired air (Parke 1956), and rats excreted between 2 and 4% of the administered dose in the bile (Rickert et al. 1983). In addition, Rickert et al. (1983) observed comparable amounts of radioactivity in the feces of rats given nitrobenzene via oral and intraperitoneal administration, suggesting that fecal excretion may not reflect unabsorbed nitrobenzene.

Rickert et al. (1983) investigated species differences and dose dependence of the oral absorption of nitrobenzene. In this study, mice exhibited lower total urinary excretion of radioactivity than rats given the same dose (35 versus ~60% in rats over 3 days after a dose of 225 mg/kg). However, the total recovery of radioactivity was also lower in mice (54 versus ~75–80% in rats). Dose did not markedly alter oral absorption estimates. In both F344 and CD rats, a 10-fold difference in oral dose (22.5 versus 225 mg/kg) did not change the percentage of dose excreted in urine (Rickert et al. 1983), suggesting little effect of dose on absorption in this dose range.

A portion of the radioactivity absorbed via the gastrointestinal tract is likely already in the form of nitrobenzene metabolites, as there is evidence for metabolism of nitrobenzene by gut microflora. This was demonstrated in a study showing that antibiotic-treated rats did not develop methemoglobinemia after oral exposure to nitrobenzene (Levin and Dent 1982). After a gavage dose of 225 mg/kg ^{14}C-nitrobenzene, antibiotic-treated rats excreted similar levels of most urinary metabolites, but significantly less p-hydroxy-acetanilide than control rats (0.9% of administered dose versus 16.2% in controls). In agreement with this finding, the antibiotic-treated rats had normal methemoglobin concentrations (2–3%), while the control rats still showed elevated methemoglobin (20%) 96 hours after exposure (Levin and Dent 1982).

A case report of poisoning in an infant exposed to nitrobenzene via dermal contact provides qualitative evidence of dermal absorption. Mallouh and Sarette (1993) reported that a 2-month-old infant whose mother had treated his skin with hair oil containing 1% nitrobenzene developed methemoglobinemia (31.5% methemoglobin).

Feldmann and Maibach (1970) evaluated dermal uptake of liquid nitrobenzene in volunteers. The subjects received a dermal application of ^{14}C-labelled nitrobenzene in acetone on the forearm (4 μg/cm^2 applied to a 13-cm^2 area, unoccluded) and were instructed not to wash the site for 24 hours. Based on the

amount of radioactivity in urine collected over the 5 days following application, the study authors estimated dermal absorption of about 1.53% of the applied dose. The absorption rate, estimated for each hour after application, was highest in the first 24 hours (0.022% per hour) and declined to 0.006% per hour in the final 24 hours of urine collection (Feldmann and Maibach 1970). The study authors suggested, however, that the continued excretion of radiolabel may have reflected redistribution from adipose tissue instead of absorption. Dermal absorption of liquid ^{14}C-nitrobenzene was measured in monkeys exposed on shaved abdominal skin at a dose of 4 μg/cm^2 (Bronaugh and Maibach 1985). Based on urinary excretion over 5 days (corrected for excretion via other routes), the study authors estimated that 4.2% of the applied dose was absorbed (Bronaugh and Maibach 1985).

Dermal absorption of nitrobenzene vapor was evaluated in a study of male volunteers (Piotrowski 1967). The volunteers were exposed to concentrations of 5–30 μg/L nitrobenzene (6 hours/day for ≥4 days) in an exposure chamber but were supplied clean air to breathe. The subjects were exposed either clothed or naked to assess the degree of protection afforded by clothes. The rate of absorption of nitrobenzene vapor across the skin was estimated from urinary excretion of p-nitrophenol. The rates of dermal absorption estimated were between 0.23 and 0.3 mg/hour/μg/L for the range of exposure concentrations. Based on these data, the study author estimated that normal clothes reduced absorption by a small amount (20–30%) (Piotrowski 1967).

The percutaneous uptake of ^{14}C-labelled nitrobenzene and related compounds across human and monkey skin *in vitro* was measured using flow-through diffusion cells with the tops covered with parafilm to prevent volatilization. Bronaugh and Maibach (1985) estimated that 7.8% of the applied dose of 4 μg/cm^2 nitrobenzene permeated across dermatomed human skin over 24 hours, and a similar absorption fraction was reported for monkey skin (6.2%) under the same conditions. These absorption estimates are higher than the *in vivo* estimates (1.5% for humans and 4.2% in monkeys). The study authors indicated that the use of occlusion in the *in vitro* experiments likely increased skin penetration by limiting evaporation of nitrobenzene.

3.1.2 Distribution

There are few data on the distribution of nitrobenzene to tissues in humans or animals. As noted in Section 3.1, nitrobenzene was detected in the blood of an elderly man who had ingested nitrobenzene (Martínez et al. 2003). Nitrobenzene was found in the stomach, liver, brain, and blood during autopsies of five patients who died from nitrobenzene poisoning. The highest concentrations were in the liver

(0.124 mg/kg tissue) and brain (0.164 mg/kg tissue) (Wirtschafter and Wolpaw 1944). In female rats exposed to 25 mg/kg ^{14}C-nitrobenzene by gavage, radioactivity was widely distributed in the tissues sampled, with the highest levels in the blood and kidneys (see Table 3-1). When rabbits were given ^{14}C-nitrobenzene (250 mg/kg), the highest levels of radioactivity in tissues sampled 8 days after dosing were in the intestinal and renal adipose tissue (Parke 1956). No information was located on tissue distribution after inhalation or dermal exposure in humans or animals.

Table 3-1. Distribution of Nitrobenzene-Derived Radioactivity in Tissues of Female Wistar Rats after Oral Exposure

	Radioactivity in tissue (pmol/mg)/dose (μmol/kg)	
Tissue	1 day after dosing	7 days after dosing
Blood	229±48[a]	134±19
Kidney	204±27	48±2.4
Liver	129±9.5	26.5±3.5
Lung	62±14	29±4.1

[a]Mean±standard deviation of three animals given 0.2 mmol/kg nitrobenzene.

Source: Albrecht and Neumann 1985

Nitrobenzene may be redistributed from adipose or other tissue into the bloodstream. Patel et al. (2008) reported the case of a 20-year-old male who had ingested about 75 mL of nitrobenzene. The patient exhibited very high methemoglobin (66.7%) and was treated with methylene blue, which decreased methemoglobin to 5.4% within an hour. However, 1 day later, his methemoglobin level rose again to 15.8% before declining again. The study authors suggested that the secondary rise in methemoglobin may have resulted from redistribution of nitrobenzene from tissues.

In blood, nitrobenzene metabolites are bound to hemoglobin and, to a lesser extent, plasma proteins. Albrecht and Neumann (1985) compared the binding of nitrobenzene and acetanilide to hemoglobin in blood from female Wistar rats exposed by gavage. Results showed that nitrobenzene had higher affinity for hemoglobin (~10-fold higher binding index and specific binding) compared with acetanilide. At 1 and 7 days after a gavage dose of 25 mg/kg ^{14}C-nitrobenzene in female Wistar rats, the hemoglobin binding indices were 72.8 and 70 mmol/mol Hb/dose (mmol/kg), and specific binding values were 1,030 and 1,024 pmol/mg/dose (mmol/kg). In contrast, hemoglobin binding indices and specific binding of acetanilide were in the range of 7–12 and 102–177, respectively. Specific binding of nitrobenzene to

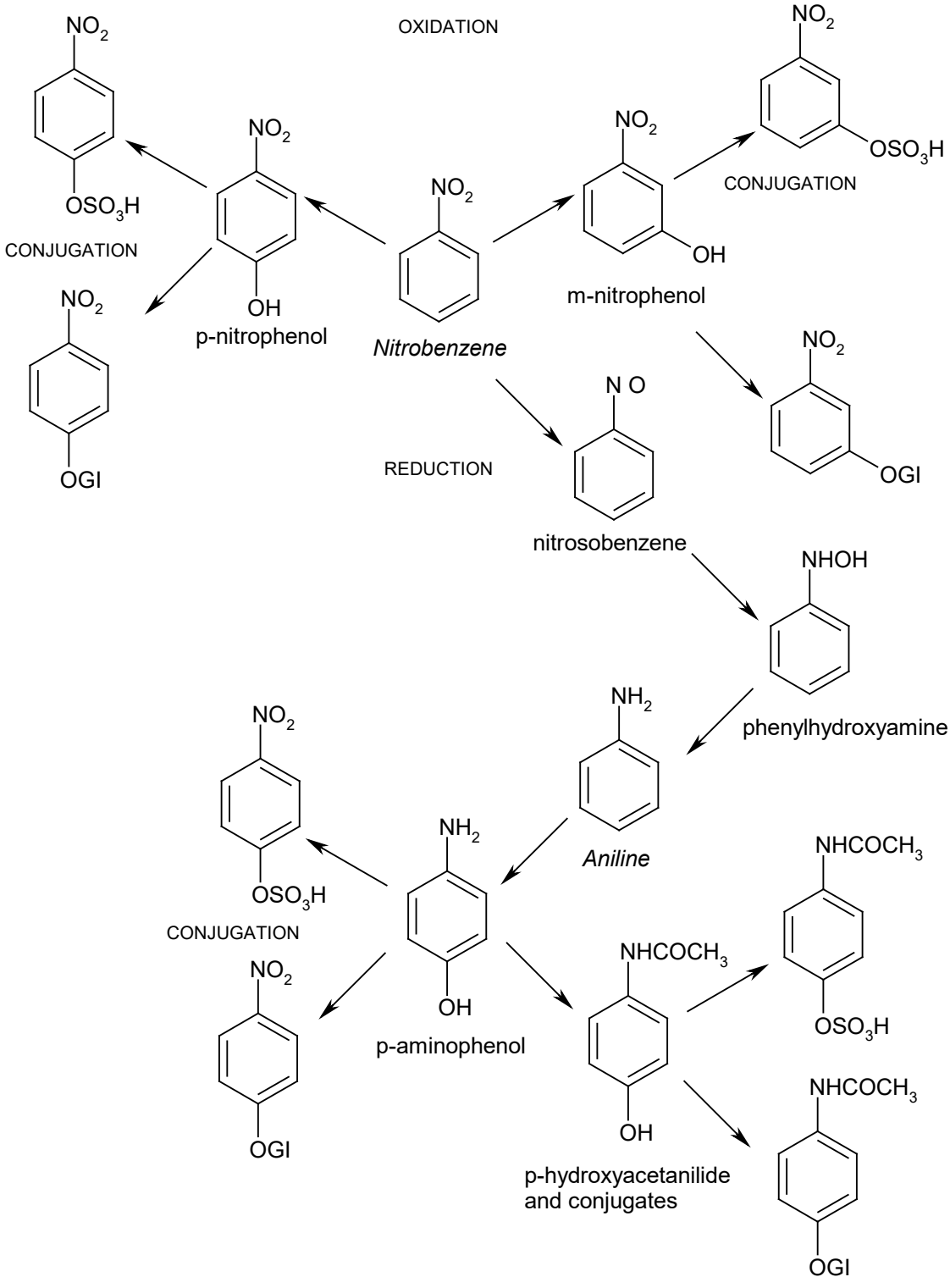
plasma proteins was comparable to that of acetanilide 1 day after dosing, and about 10-fold higher 7 days after dosing.

Goldstein and Rickert (1984) observed marked differences between rats and mice in the distribution of nitrobenzene-derived radiolabel to red blood cells and spleen. After single oral doses of 75–200 mg/kg ^{14}C-nitrobenzene, total and bound radiolabels in erythrocytes were 6–13-fold higher in rats than in mice. In the spleen, total and bound radiolabel levels were also higher in rats than in mice. In animals receiving the highest dose, peak binding in rats occurred 24 hours after dosing, while the peak occurred 10 hours after dosing in mice. At the peak, bound radiolabel in the erythrocytes and spleen were 429 and 74 nmol equivalents ^{14}C-nitrobenzene/g tissue in rats; in mice, bound radiolabel in erythrocytes peaked at 76 nmol equivalents ^{14}C-nitrobenzene/g tissue and no significant increase in bound radiolabel was detected in spleen. Goldstein and Rickert (1984) used sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to separate the compounds in blood and spleen that were covalently bound to the nitrobenzene-derived radiolabel. The study authors showed that most of the radiolabel in erythrocytes was bound to hemoglobin, and most of the radiolabel in the spleen was bound to methemoglobin and another, unidentified component.

3.1.3 Metabolism

Overview. Nitrobenzene metabolism may proceed through oxidative or reductive pathways. The reductive metabolism of nitrobenzene is the toxicologically more important pathway in that the intermediates produced are believed to be responsible for the hematopoietic and carcinogenic action of this compound. Cytochrome P450 enzymes in hepatic microsomes can catalyze oxidation of nitrobenzene, forming p- and m-nitrophenols and derivative aminophenols, which are subsequently conjugated with sulfate or glucuronide prior to excretion. Nitrobenzene reduction may proceed via oxygen-insensitive (Type I) or oxygen-sensitive (Type II) pathways, and yields p-aminophenol through a series of intermediates including nitrosobenzene, phenylhydroxylamine, and aniline. As with the oxidative metabolites, p-aminophenol may be conjugated with sulfate or glucuronide or may be acetylated at the nitrogen to p-hydroxyacetanilide and subsequently conjugated with sulfate or glucuronide. Figure 3-1 displays a schematic of the metabolism of nitrobenzene.

Figure 3-1. Metabolism of Nitrobenzene



Source: EPA 2009a

Importance of Gut Microflora. Reduction of nitrobenzene has been shown to be catalyzed by Type I (oxygen-insensitive) nitroreductase and/or by Type II nitroreductase (oxygen sensitive). In Sprague-Dawley rats, both nitroreductase enzymes are found in gut microflora, but also in microsomes from the liver, brain, lung, heart, and kidney (Ask et al. 2004). The importance of nitrobenzene metabolism by microbes in the gut was demonstrated most clearly by Levin and Dent (1982), who showed that, unlike control rats, antibiotic-treated rats did not develop methemoglobinemia after oral nitrobenzene exposure. The urinary metabolites of nitrobenzene were comparable between the control and antibiotic-treated rats, except that the antibiotic-treated rats produced much less p-hydroxyacetanilide (0.9% of total dose excreted, compared with 16.2% in controls) and an unidentified metabolite (0.5 versus 3.7% in controls).

Experiments by Reddy et al. (1976) provide support for the importance of microflora. The study authors measured formation of aniline from nitrobenzene in homogenized liver, kidney, gut wall, and gut contents obtained from control rats, germ-free rats, or germ-free rats acclimatized to room air for 7 days prior to sacrifice. The formation of aniline was higher in the gut contents of germ-free and control rats (15.2 and 11.1 nmol/mg protein/hour) than the other tissues (0.7–3.3 nmol/mg/protein/hour). In the germ-free rats, very little aniline was formed in the gut contents (0.2 nmol/mg protein/hour) or other tissues (0.5–2 nmol/mg protein/hour), demonstrating the importance of metabolism by microbes in the gastrointestinal tract.

Urinary Metabolites. In humans exposed to nitrobenzene by inhalation, urinary metabolites include p-nitrophenol and p-aminophenol (Ikeda and Kita 1964; Salmowa et al. 1963). Rickert et al. (1983) compared the urinary metabolites produced by male F344 rats, CD rats, and B6C3F1 mice given an oral dose of 225 mg/kg nitrobenzene. Table 3-2 summarizes the results. As the table shows, the urinary metabolites differed between rats and mice, and even between the two strains of rat. Only mice excreted p-aminophenol. In addition, the excretions (as a percent of administered dose) of p- and m-nitrophenol and p-hydroxyacetanilide were much higher in rats than in mice. The reduced levels of p-hydroxyacetanilide excreted in mice may partly explain why this species is less sensitive to methemoglobinemia induced by nitrobenzene; as noted above, Levin and Dent (1982) showed the importance of this metabolite in the mechanism for methemoglobinemia.

Table 3-2. Metabolites Excreted in Urine by Male Rats and Mice within 72 Hours after an Oral Dose of 225 mg/kg Nitrobenzene

	Percent of dose excreted		
Metabolite	F344 rat[a]	CD rat[b]	B6C3F1 mouse[b]
p-Nitrophenol	19.9–22.4	13.0	7.2
m-Nitrophenol	10.2–11.4	7.9	6.2
p-Aminophenol	ND	ND	9.7
p-Hydroxyacetanilide	16.2–19.0	8.8	3.9
Unidentified (I)	4.5–9.8	25.3	4.8
Unidentified (II)	ND–3.7	5.7	2.6

[a]Levin and Dent (1982) and Rickert et al. (1983).
[b]Rickert et al. (1983).

ND = not detected

Rickert et al. (1983) also measured the metabolite levels in urine before and after enzyme hydrolysis to assess species and strain differences in conjugation. In F344 rats, nitrobenzene metabolites were excreted exclusively as sulfates. In CD rats, the largest fractions of each metabolite were excreted as sulfates, with smaller fractions excreted as glucuronides or free (unconjugated) metabolites. Finally, in B6C3F1 mice, p-hydroxyacetanilide was excreted primarily as the glucuronide conjugate, while the sulfate conjugate was most important for p-aminophenol, p-nitrophenol, and m-nitrophenol (i.e., there was little to no glucuronidation). Small amounts of free metabolites were also detected in the urine of mice (Rickert et al. 1983).

In rabbits and guinea pigs exposed to nitrobenzene by gavage, the primary urinary metabolite was reported to be p-aminophenol, followed by p- and m-nitrophenol and o- and m-aminophenol (Parke 1956). However, Rickert (1987) noted that Parke (1956) used an acid hydrolysis procedure to cleave conjugates in urine, which would have resulted in conversion of p-hydroxyacetanilide (if present) to p-aminophenol. Therefore, the primary metabolite in rabbits and guinea pigs is not known but is probably either p-hydroxyacetanilide or p-aminophenol.

Metabolic Mechanisms. The mechanism by which nitrobenzene induces methemoglobinemia and related effects has been reviewed extensively (EPA 2009a; Holder 1999; Rickert 1987). The mechanism begins with its reductive metabolism, which can occur via oxygen-insensitive or oxygen-sensitive pathways. As discussed above, microflora in the gut reduce nitrobenzene to aniline via a three-step process that includes nitrosobenzene and phenylhydroxylamine intermediates. The reaction is catalyzed by an oxygen-insensitive nitroreductase (nicotine adenine dinucleotide phosphate [NADPH] dehydrogenase) that is

present at highest levels in the microflora of the gut but is also present at lower levels in tissues including the liver, heart, brain, lung, and kidney (Ask et al. 2004). Once absorbed, the products of microbial metabolism (nitrophenols, aminophenols, and aniline) may be further metabolized to form reactive intermediates including nitrosobenzene, phenylhydroxylamine, and the benzene nitronium ion (EPA 2009a).

Hepatic microsomes and erythrocytes can also reduce nitrobenzene, but these reactions are catalyzed by oxygen-sensitive nitroreductases (NADH dehydrogenase and the mitochondrial form, ubiquinone). Reduction of nitrobenzene through this pathway yields a number of toxic and reactive intermediates, including nitrosobenzene, phenylhydroxylamine, and several free radicals (superoxide, nitroanion, and hydronitroxide) (EPA 2009a; Holder 1999).

Reduction of nitrobenzene by intestinal microflora appears to be the most important metabolic pathway for induction of methemoglobin, indicating that the metabolites from this pathway are responsible for this effect. Available data indicate that the oxygen-insensitive reduction of nitrobenzene by gut bacteria proceeds much more rapidly than the oxygen-sensitive pathway occurring in liver microsomes (EPA 2009a). In addition, *in vitro* incubation of erythrocytes with nitrobenzene does not trigger conversion of hemoglobin to methemoglobin (Facchini and Griffiths 1981). Finally, as discussed above, the importance of the bacterial reduction of nitrobenzene to its induction of methemoglobinemia was demonstrated when germ-free animals were shown to be resistant to this effect (Levin and Dent 1982).

Holder (1999) postulated that metabolic cycling between the nitrosobenzene and phenylhydroxylamine products of nitrobenzene reduction is responsible for the conversion of hemoglobin to methemoglobin. As proposed by Holder (1999), nitrosobenzene is converted to phenylhydroxylamine via oxidation of NADPH, and the latter converts hemoglobin to methemoglobin (via a phenylhydronitroxide intermediate) during its reversion to nitrosobenzene. Nitrosobenzene may also bind to cysteine groups on intact hemoglobin, resulting in denaturation of the globin moiety and dissociation of the heme group (Holder 1999; Smith and McHale 2018). Once dissociated from the globin chain, free heme can injure erythrocytes and tissues via induction of oxidative stress. In addition, ferric iron from heme may complex with chloride to form the hydrophobic compound hemin, which may accumulate in the membrane of erythrocytes and cause their lysis (Smith and McHale 2018). Erythrocyte damage may also result from lipid peroxidation via free radicals formed during the metabolism of nitrobenzene and its metabolites.

Damaged erythrocytes are removed from circulation by splenic macrophages. Splenic effects seen in animals exposed to nitrobenzene, including increases in spleen weight, sinusoidal congestion, and hemosiderosis, result from accumulation of the products of erythrocyte destruction. Further, breakdown of heme in the liver induces hemosiderosis there. In addition, release of hemoglobin from injured red blood cells can trigger renal effects via formation of nephrotoxic hemoglobin dimers (Smith and McHale 2018).

In summary, metabolites of nitrobenzene convert hemoglobin to methemoglobin and destroy erythrocytes through a variety of molecular events, leading to splenic, liver, and kidney effects such as congestion and hemosiderosis. In addition, several steps in the metabolic pathways for nitrobenzene release free radicals and/or reactive intermediates that can cause lipid peroxidation and injury to a variety of tissues.

3.1.4 Excretion

The major route of nitrobenzene elimination in humans and animals is urinary excretion of metabolites. After a 17-month occupational exposure to nitrobenzene (via inhalation), a 47-year-old woman was admitted to the hospital, where urinary analysis showed excretion of p-nitrophenol and p-aminophenol in the urine that gradually declined over 2 weeks (Ikeda and Kita 1964). In experimental studies of humans exposed to nitrobenzene by inhalation, excretion of p-nitrophenol in urine was initially rapid but reached a steady-state rate of excretion around the 4^{th} day (Piotrowski 1967; Salmowa et al. 1963). Piotrowski (1967) estimated that about 16% of the absorbed dose excreted in the urine as p-nitrophenol during the steady-state phase. Myslak et al. (1971) reported extensive excretion of those metabolites in the urine of a subject who ingested about 50 mL of nitrobenzene. The levels of nitrobenzene metabolites in urine reached their maximum peak on days 2 (198 mg/day for p-aminophenol) and 3 (512 mg/day for p-nitrophenol). Piotrowski (1967) examined the rates of urinary p-nitrophenol excretion in a volunteer after intake of a single oral dose of 5 mg p-nitrophenol or 30 mg nitrobenzene. The excretion of p-nitrophenol was very rapid; in contrast, excretion was slow after exposure to nitrobenzene, probably due to the slow rate of nitrobenzene metabolism to p-nitrophenol. The initial half-time of elimination of p-nitrophenol after exposure to nitrobenzene was around 5 hours, with a late-phase half-time of >20 hours (Piotrowski 1967). All p-nitrophenol was eliminated by 8 hours when subjects were exposed to nitrophenol directly (Piotrowski 1967). In humans exposed to nitrobenzene vapor through the skin, about 20% was excreted as p-nitrophenol in the urine the first day (Piotrowski 1967). Feldmann and Maibach (1970) applied [^{14}C]-labeled nitrobenzene dissolved in acetone to the forearm skin of six subjects. The cumulative urinary excretion of radiolabel over the five days following application was estimated to be

1.5±0.84% of the applied dose or about 58% of the absorbed dose. After intravenous administration of [^{14}C]-nitrobenzene, the study authors observed 60.5% of the radioactive label in the urine and estimated a half-life of 20 hours (Feldmann and Maibach 1970).

In laboratory animals, nitrobenzene is primarily excreted via the urine as metabolites, as shown by studies of animals exposed orally to radiolabelled nitrobenzene. Table 3-3 summarizes the distribution of radioactivity in excreta collected over 3–7 days from rats, mice, and rabbits given single gavage doses of 22.5–250 mg/kg.

Table 3-3. Excretion of Radioactivity in Animals Treated Orally with ^{14}C-Nitrobenzene

			Percent of administered dose excreted in				
Strain and species (sex)	Dose mg/kg	Duration of collection	Urine	Feces	Expired air	Total recovery	Reference
F344 rat (M)	225	3 days	58.4	16.4	2.3	77.1	Levin and Dent (1982)
F344 rat (M)	225	3 days	63.2	14.2	1.6	79	Rickert et al. (1983)
F344 rat (M)	22.5	3 days	65.8	21.4	1.0	88.2	Rickert et al. (1983)
CD rat (M)	225	3 days	60.8	11.8	2.5	75.1	Rickert et al. (1983)
CD rat (M)	22.5	3 days	64.5	11.5	0.8	76.8	Rickert et al. (1983)
Wistar rat (F)	25	7 days	65	15.5	NR	NR	Albrecht and Neumann (1985)
B6C3F1 mouse (M)	225	3 days	34.7	18.8	0.8	54.3	Rickert et al. (1983)
Rabbit (NS)	250	4–5 days	58	9	1.6[a]	70	Parke 1956
Giant chinchilla rabbit (F)	150–200	2 days	~55	NR	NR	NR	Robinson et al. 1951

[a]In first 30 hours after dosing; not measured subsequently.

M = male; F = female; NS = not specified; NR = not reported

The available studies did not suggest substantial dose-related differences in patterns of nitrobenzene excretion in rats over the dose range of 22.5–225 mg/kg (Rickert et al. 1983). In addition, experiments in F344 rats comparing oral and intraperitoneal exposure routes showed similar excretory patterns (Rickert et al. 1983). However, species differences were evident. As the table shows, mice excreted less radioactivity (in total) and lower amounts in urine than rats of either strain exposed to the same dose of nitrobenzene; higher fecal excretion was seen in the mice as well (Rickert et al. 1983). The excretory patterns of rabbits were more similar to those of rats than mice (Parke 1956; Rickert et al. 1983; Robinson et al. 1951) (see Table 3-3).

3.1.5 Physiologically Based Pharmacokinetic (PBPK)/Pharmacodynamic (PD) Models

PBPK models use mathematical descriptions of the uptake and disposition of chemical substances to quantitatively describe the relationships among critical biological processes (Krishnan et al. 1994). PBPK models are also called biologically based tissue dosimetry models. PBPK models are increasingly used in risk assessments, primarily to predict the concentration of potentially toxic moieties of a chemical that will be delivered to any given target tissue following various combinations of route, dose level, and test species (Clewell and Andersen 1985). Physiologically based pharmacodynamic (PBPD) models use mathematical descriptions of the dose-response function to quantitatively describe the relationship between target tissue dose and toxic endpoints.

No PBPK models of nitrobenzene were located in the available literature.

3.1.6 Animal-to-Human Extrapolations

Nitrobenzene toxicity results from its metabolism (see Section 3.1.3 *Metabolic Mechanisms*), and there is evidence for species differences in metabolism that correspond to differences in toxicity. For example, mice appear to be somewhat less sensitive than rats to the induction of methemoglobin in several studies (Cattley et al. 1994, 1995; CIIT 1993; Hamm et al. 1984; NTP 1982), and mice have been shown to excrete less of the p-hydroxyacetanilide urinary metabolite that is important to the mechanism of action (Rickert et al. 1983). In addition, there are species differences in the level and/or activity of methemoglobin reductases, the enzymes that convert methemoglobin back to hemoglobin. For example, when erythrocytes from several species were exposed to nitrite at the same concentration, methemoglobin was produced by all species, but declined more rapidly in erythrocytes from mice and rabbits than in erythrocytes from humans, cats, dogs (Stolk and Smith 1966), or rats (Smith et al. 1967). Lo and Agar (1986) measured the activity of NADH-dependent methemoglobin reductase in erythrocytes from a variety of species and observed that mice and rabbits had the highest activity, followed by rats, with the lowest activity in humans. In *in vitro* studies with monomethylhydrazine, methemoglobin levels in human blood were ~4 times higher than levels in rat blood at the same concentration of monomethylhydrazine (Clark and De La Garza 1967). These data suggest that sensitivity to methemoglobinemia increases (low to high) from mice ≈ rabbits < rats < humans (i.e., humans may be more sensitive than rodents).

3.2 CHILDREN AND OTHER POPULATIONS THAT ARE UNUSUALLY SUSCEPTIBLE

This section discusses potential health effects from exposures during the period from conception to maturity at 18 years of age in humans. Potential effects on offspring resulting from exposures of parental germ cells are considered, as well as any indirect effects on the fetus and neonate resulting from maternal exposure during gestation and lactation. Children may be more or less susceptible than adults to health effects from exposure to hazardous substances and the relationship may change with developmental age.

This section also discusses unusually susceptible populations. A susceptible population may exhibit different or enhanced responses to certain chemicals than most persons exposed to the same level of these chemicals in the environment. Factors involved with increased susceptibility may include genetic makeup, age, health and nutritional status, and exposure to other toxic substances (e.g., cigarette smoke). These parameters can reduce detoxification or excretion or compromise organ function.

Populations at greater exposure risk to unusually high exposure levels to nitrobenzene are discussed in Section 5.7, Populations with Potentially High Exposures.

Populations that are considered unusually susceptible to nitrobenzene toxicity are those groups that are susceptible to methemoglobinemia. For example, the first 6 months of postnatal life is a period of increased susceptibility to methemoglobinemia (termed infantile methemoglobinemia or blue baby syndrome) due to a number of factors (Goldstein et al. 1969; Greer and Shannon 2005; Von Oettingen 1941):

- Fetal hemoglobin, which remains in the blood for some time after birth, is more prone to conversion to methemoglobin than is adult hemoglobin.
- Levels of NADH-dependent methemoglobin reductase (the major enzyme responsible for reduction of methemoglobin to normal hemoglobin) in the newborn increase approximately 2-fold during the first 4 months of postnatal life to reach adult levels.
- Umbilical cord blood is deficient in the enzyme glucose-6-phosphate dehydrogenase and thus cannot readily convert the methemoglobin that is formed “naturally” back to hemoglobin as is readily done in adults.

Additionally, a condition described as “hereditary methemoglobinemia” may result from a genetic defect (Goldstein et al. 1969). The enzyme, methemoglobin reductase, is absent and persons are hypersensitive to any substances such as nitrite or aniline derivatives capable of producing methemoglobinemia. The trait is

inherited as an autosomal recessive allele. Thus, either sex may exhibit the trait, which is ordinarily detected by the presence of cyanosis at birth. Such individuals would be extremely sensitive to the effects of nitrobenzene.

A more common genetic defect was also described in which the enzyme, glucose-6-phosphate, dehydrogenase has decreased activity (Goldstein et al. 1969). The pattern of inheritance of this trait is linked to one of several alleles on the X chromosome. The phenotype is expressed as an incomplete dominant trait. Thus, female heterozygotes are not known to have severely depressed enzyme levels and males may have a wide range of activity. These phenotypes express a wide range of levels of glucose-6-phosphate dehydrogenase enzyme in the red blood cell. This defect is ordinarily without adverse effects. It is only when these individuals are challenged with compounds that oxidatively stress erythrocytes (such as primaquine) that there is a hemolytic response. Reactors to primaquine (and fava beans) are found predominantly among groups that live in or trace their ancestry to malaria-hyperendemic areas such as the Mediterranean region or Africa. The incidence of "primaquine sensitivity" among Kurds, a Middle Eastern population, is 53%. Among black Americans, the incidence is 13%. Thus, individuals already exhibiting primaquine sensitivity would be expected to be more vulnerable to the additional hemolytic crisis that often follows 5–6 days after nitrobenzene exposure (Gosselin et al. 1984; Von Oettingen 1941).

People who have preexisting or underlying diseases such as anemia, cardiovascular disease, lung disease, sepsis, or abnormal hemoglobin species (e.g., carboxyhemoglobin, sulfhemoglobin, or sickle cell hemoglobin), maybe at greater risk of developing the chemically induced methemoglobinemia at much lower levels of exposure to nitrobenzene (Goldfrank et al. 1998).

In addition, external factors such as medications and exposure to xenobiotics from the environment can also cause methemoglobinemia. Nitrite-based medications, which are widely used to treat angina and other cardiac-related problems, can cause methemoglobinemia and are reported as a complication of the therapeutic use of these drugs (Bojar et al. 1987; Marshall and Ecklund 1980). Self-administration of local anesthetic drugs like benzocaine have also been known to cause this condition (Nappe et al. 2015).

Dapsone, a commonly used anti-inflammatory for treating infections has severe side effects, including methemoglobinemia, and patients are often recommended to use a pulse oximeter (Ashurst et al. 2010; Mahmood et al. 2019) or co-oximeter (Toker et al. 2015) to monitor blood oxygen levels regularly, with

the latter providing more accuracy. Acquired methemoglobinemia can also be caused by malaria medication (Kudale et al. 2014).

3.3 BIOMARKERS OF EXPOSURE AND EFFECT

Biomarkers are broadly defined as indicators signaling events in biologic systems or samples. They have been classified as biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility (NAS/NRC 1989).

A biomarker of exposure is a xenobiotic substance or its metabolite(s) or the product of an interaction between a xenobiotic agent and some target molecule(s) or cell(s) that is measured within a compartment of an organism (NAS/NRC 1989). The preferred biomarkers of exposure are generally the substance itself, substance-specific metabolites in readily obtainable body fluid(s), or excreta. Biomarkers of exposure to nitrobenzene are discussed in Section 3.3.1. The National Report on Human Exposure to Environmental Chemicals provides an ongoing assessment of the exposure of a generalizable sample of the U.S. population to environmental chemicals using biomonitoring (see http://www.cdc.gov/exposurereport/). If available, biomonitoring data for nitrobenzene from this report are discussed in Section 5.6, General Population Exposure.

Biomarkers of effect are defined as any measurable biochemical, physiologic, or other alteration within an organism that (depending on magnitude) can be recognized as an established or potential health impairment or disease (NAS/NRC 1989). This definition encompasses biochemical or cellular signals of tissue dysfunction (e.g., increased liver enzyme activity or pathologic changes in female genital epithelial cells), as well as physiologic signs of dysfunction such as increased blood pressure or decreased lung capacity. Note that these markers are not often substance specific. They also may not be directly adverse, but can indicate potential health impairment (e.g., DNA adducts). Biomarkers of effect caused by nitrobenzene are discussed in Section 3.3.2.

A biomarker of susceptibility is an indicator of an inherent or acquired limitation of an organism's ability to respond to the challenge of exposure to a specific xenobiotic substance. It can be an intrinsic genetic or other characteristic or a preexisting disease that results in an increase in absorbed dose, a decrease in the biologically effective dose, or a target tissue response. If biomarkers of susceptibility exist, they are discussed in Section 3.2, Children and Other Populations that are Unusually Susceptible.

3.3.1 Biomarkers of Exposure

Levels of nitrobenzene in blood reflect recent exposure. Metabolism of nitrobenzene to p-nitrophenol and p-aminophenol in humans is well-known, and the presence of metabolites in the urine can be used to indicate exposure to nitrobenzene (Ikeda and Kita 1964; Piotrowski 1967). About 10–20% of a dose is eliminated in the urine as p-nitrophenol, which is used in biological monitoring of occupational exposures. A smaller fraction of a dose is eliminated in urine as p-aminophenol (Astier 1992; IARC 1996). Urinary levels of p-nitrophenol and aminophenol also reflect recent exposure to nitrobenzene. In addition, neither of these metabolites is specific to nitrobenzene exposure. p-Nitrophenol is also a metabolite of other insecticides such as methyl parathion, ethyl parathion, and O-ethyl-O-(4-nitrophenyl) phenylphosphonothioate (Barr et al. 2002; CDC 2021a; Kissel et al. 2005; McCann et al. 2002; Rubin et al. 2002). p-Aminophenol is a urinary metabolite of aniline (Kao et al. 1978; McCarthy et al. 1985; Parke 1956; Robinson et al. 1951) and N-acetyl p-aminophenol (Newton et al. 1982; Chang et al. 1993). Measurement of p-nitrophenol and p-aminophenol, therefore, should not be used to quantify nitrobenzene exposure.

The nitrobenzene metabolites, nitrosobenzene and phenylhydroxylamine, have been found to bind with hemoglobin in the blood of orally exposed mice and rats and could be used as biomarkers of exposure (Goldstein and Rickert 1984). The presence of these hemoglobin adducts in human blood may also serve as a potential biomarker of exposure to nitrobenzene.

3.3.2 Biomarkers of Effect

The presence of methemoglobinemia can indicate effects of nitrobenzene exposure, but is also not specific, as methemoglobinemia can result from genetic defects as well as exposure to some medicines and many other toxic substances.

3.4 INTERACTIONS WITH OTHER CHEMICALS

Smyth et al. (1969) demonstrated synergism between orally administered nitrobenzene and six other common industrial compounds in rat studies using death (oral LD_{50}) as the endpoint. The combinations of chemicals showed increased lethality that varied from 20 to 47%. The compounds were: formalin, 20%; butyl ether, 28%; aniline, 32%; dioxane, 39%; acetone, 47%; and carbon tetrachloride, 47% (Smyth et al. 1969).

Alcohol also has the potential for enhancing the toxicity of nitrobenzene; however, the toxicokinetic mechanism is not known. It is clear, however, that alcohol does not simply enhance the absorption of nitrobenzene. When alcohol was given orally and nitrobenzene was given intravenously, there was increased toxicity in rabbits compared with nitrobenzene alone. Alcohol also enhanced the neural toxicity of nitrobenzene in rabbits when nitrobenzene was applied to the skin (Matsumara and Yoshida 1959).

In addition, there are several other chemicals that operate through a similar mechanism of action in causing increases in methemoglobin such as nitrates and nitrites. Exposure to multiple agents that induce methemoglobinemia would likely increase the risk of an adverse outcome.