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Nitrobenzene Carcinogenicity
(CAS No. 98-95-3)

National Center for Environmental Assessment–Washington Office
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ABSTRACT

Nitrobenzene (NB, CAS No. 98-95-3) oxidizes to p-aminophenol and p-nitrophenol in animals and humans, while being reduced also to nitrosobenzene, phenylhydroxylamine, and aniline. The reductants are known to cause methemoglobinemia and anemia. NB is negative for mutagenicity systems in Salmonella, hepatocyte repair, or sister chromatid exchange assays.

No human studies cancer data are available, therefore this analysis is based on a Chemical Industry Institute of Toxicology (CIIT) NB inhalation study. The study involves both sexes of B6C3F1 mice (0, 5, 25, 50 ppm NB) and F344/N rats (0, 1, 5, 25 ppm NB), but only male Sprague-Dawley (CD strain) rats (0, 1, 5, 25 ppm NB). All are exposed at 6 hrs/day, 5 days/wk, for 104 wks. Mouse results show increased benign male tumors in the alveolus, bronchus, and thyroid, whereas female mice indicated mammary cancers. F344/N rats responded with male liver cancer and benign responses in the follicular thyroid and kidney, whereas females had benign endometrial polyps. Male CD rats had benign hepatocellular adenomas. Because NB causes tumorigenicity at 8 sites, 6 organs, both sexes, among 2 test species, NB is classified as a B2 carcinogen or a likely human carcinogen by any route according to the EPA Cancer Guidelines (U.S.EPA, 1986)

PREFACE

This hazard assessment of nitrobenzene (NB) carcinogenicity in rodents (rats and mice) was prepared by the National Center for Environmental Assessment, Washington office. NB ($C_6H_5NO_2$) is a nitroarene that is a potentially hazardous air pollutant (HAP) in the United States. It is listed as HAP in the 1990 Clean Air Act Amendment, Section 112b. This NB support document for carcinogenicity originally was developed to assist the U.S. EPA Office of Air.

NB has been presented in summary report form to the Carcinogen Risk Assessment Verification Endeavor (CRAVE) group (12/7/94) . The presentation (oral and written) was reviewed for inclusion into the Integrated Risk Information System (IRIS) database; comments were taken from all members. This support document was corrected and a final IRIS summary submission was sent to CRAVE on April 24, 1995, for submission to the IRIS database. The quantitative cancer unit cancer slope has been re-estimated (September 1995); the quantitative section is presented in the Appendix. The NB support document was reviewed again administratively by NCEA-W in February 1997, further revised, and finalized in April 1998.

The cancer classification of nitrobenzene will receive further review as a part of the IRIS process.

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1. INTRODUCTION

The purpose of this report is to characterize the carcinogenic hazard potential of nitrobenzene (NB, CAS No. 98-95-3). NB is a potentially hazardous air pollutant (HAP) listed in the 1990 Clean Air Act Amendment. Alternate NB chemical names are nitrobenzol and oil of mirbane.

The chemical formula of NB is $C_6H_5NO_2$ and the molecular weight is 123.11. The structural formula for NB is presented in figure 1. NB is a lipophilic bipolar compound that can be hydrolyzed various nitrophenols and phenol, or it can be reduced to aniline.

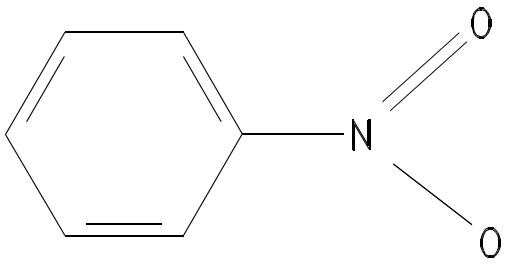

Figure 1. The chemical structure of the potentially hazardous air pollutant nitrobenzene. Nitrobenzene is chemically produced by treating benzene with H_2SO_4 and HNO_3.

NB is a colorless to pale yellow oily liquid that has the odor of almond oil. The flash point of NB = 89°C, melting point = 6°C, boiling point = 210°C, density = 1.2037, and vapor pressure = 1.22 mm Hg (at 25°C). NB volatility is presented in table 1 (number 6) and can be compared with the volatility of other reference compounds at 20°C. Some of the reference compounds in table 1 are themselves potential HAPs. The data in table 1 indicate that NB is volatile enough to be a potential inhalation toxicant wherever it is stored, handled, processed, or disposed.

NB is chemically stable and dissolves in aqueous solution at 0.2% (v/v). It can be manufactured by a batch process or by a continuous process in big plants. Nitronium ion ($+NO_2$) is generated from a sulfuric and nitric acid mixture. A nitronium ion attack on benzene forms nitrobenzene ($^{+}NO_2 + C_6H_6 \rightarrow C_6H_5NO_2$) among other nitrated benzenes such as the dinitro and trinitro products. The mononitrated product (mp = 6°C) is separated from the mixture of sulfuric and nitric acids and other nitrated benzenes (mp = 173°C) by cold crystallization, decanting, and distillation at 210°C.

The U.S. production volume of NB ranks in the top 75 chemicals at 1.4 billion pounds/year. At the present time, the bulk of NB production is used in making chemical intermediates, the most of which (98%) is used in aniline production. Aniline is manufactured

Table 1. Vapor pressure of comparable compounds (mm Hg)[a]

Number	Compound	Vapor pressure
1	Diphenylamine	0.011
2*	Phthalic anhydride	0.025
3*	Quinoline	0.181
4	Camphor	0.260
5*	Naphthalene	0.53
6*	***Nitrobenzene***	1.22
7*	Ethylene dibromide	10.1
8	Chlorobenzene	12.5
9	Acetic acid	15.7
10	Water	17.3
11*	Dichloroethane	60.6
12*	Benzene	74.6
13*	Carbon tetrachloride	76.4
14	Hexane	120.0
15	Ethyl ether	290.8
16*	Bromoethane	475.0
17*	Acetaldehyde	764.3
18*	Chloroethane	1002.3
19	Butane	1645.3
20	Propane	6677.8

[a]Approximate vapor pressures are presented and are calculated from data presented in *The Handbook of Chemistry and Physics*, 67th edition, CRC Press, 1986-1987, pp. D-212ff. The estimating formula for vapor pressure at 20°C is: $\log p = -1.78 \times 10^{-4} a + b$, where a and b are presented in the handbook. Compounds with asterisks are potential HAPs as presented by U.S. EPA's Office of Air. The above table compares HAP nitrobenzene volatility with the volatility of other representative compounds.

from NB in the gaseous state by reduction with H_2 and $FeCl_3$ or by reduction in solution with Fe^{+3} and HCl. In addition to being used as a chemical feeder stock, NB also is used to make pesticides, shoe polishes, analgesics, dyes, and pyroxylin compounds.

The oral acute LD_{50} of NB in the rat is $\approx$1 g/kg, which puts NB in the slightly toxic range of acute oral toxicity. Oral or inhalation exposures can cause red blood cell (RBC) damage as well as spleen swelling and engorgement and anemia. The toxicity of NB to humans can lead to death, especially in infants and children. Inhalation exposure of NB is considered more likely than oral exposure in the United States. Short inhalation exposures cause hepatic, splenic, and testicular lesions in B6C3F1 male mice. Testicular injury as a target tissue for NB involves necrosis of primary and secondary spermatocytes and development of giant multinucleated cells. The Registry for Toxic Effects of Chemical Substances (RTECS) lists rat testicular injury at a TC_{Lo} = 5 ppm of inhaled NB. RTECS lists rat fertility effects as occurring at 252 ppm of exposure. The Occupational Safety and Health Administration has recommended a human threshold limit of 1 ppm (5 mg/m^3). Nitrobenzene is a skin and eye irritant and is considered a dermal toxicant.

The current U.S. EPA reference dose (RfD) is 4.6 mg/kg/day based on a B6C3F1 mouse inhalation study (IRIS database, June 1997). This RfD is based on a 1984 subchronic inhalation study that included hematologic, adrenal, renal, and hepatic lesions (CIIT, 1993). After acute high NB exposure, liver injury includes bile stasis, fatty degeneration, centrilobular necrosis, and hepatocellular nucleolar enlargement. Excessive doses of inhaled NB can lead to central nervous system suppression, headache, nausea, methemoglobinemia, and liver, brain (e.g., malacia of the cerebellar peduncle), and testes injuries (ATSDR, 1990).

NB is readily absorbed by human or animal skin, oral, or inhalation exposures. At high inhaled doses, NB is metabolized in a few days to p-nitrophenol and p-aminophenol in human urine (Ikeda and Kita, 1964; Dorigan and Hushorn, 1976; Rickert, 1984), whereas at lower doses only p-nitrophenol is found in volunteer urines (Salmowa et al., 1963). A typical animal metabolism study (gavage, rabbit) at high NB dose suggests an array of NB metabolites in urine (figure 2) (Robinson et al., 1951; Parke, 1956). The generality of this NB metabolic response was demonstrated in other animals (rat, guinea pig) and by other routes of exposure (intravenous, dermal) (Piotrowski et al., 1975; Kiese, 1974; Eyer and Ascherl, 1987; Beauchamp et al., 1982).

Various NB resonance structures are possible due to the nitro group electron withdrawing properties (figure 2). Predictable P-450 mediated ring-hydroxylations occur at the meta position, and to a lesser extent at the *o*- and *p*- positions. Epoxidation followed by an isosceles hydrolytic-cleavage of the three-membered ring explains the presence of both *o*- and *p*-

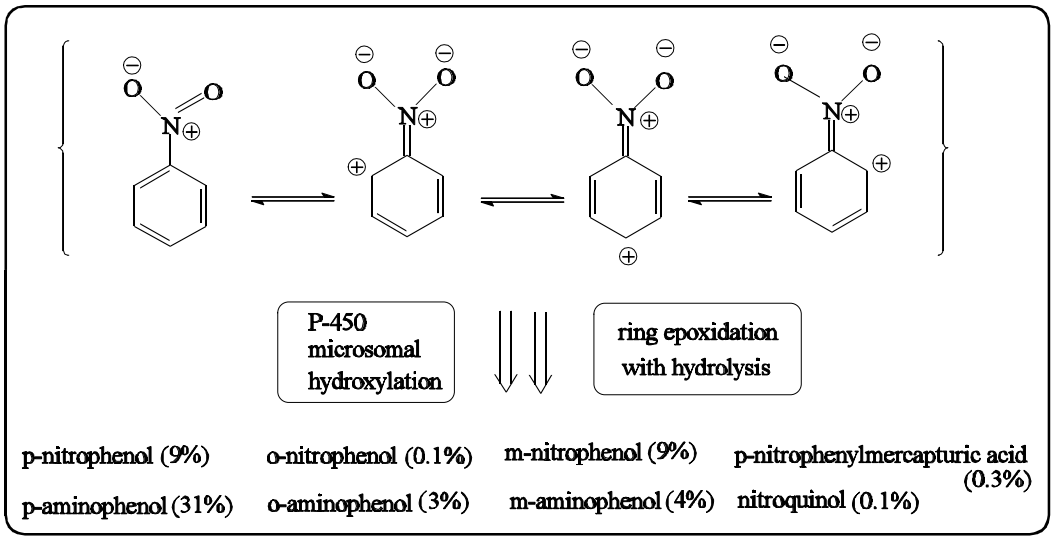

Figure 2. The ring hydroxylated metabolites of nitrobenzene. These metabolites account for most of the nitrobenzene converted to eliminated products in the excreta.

isomers (Tomaszewski et al., 1975). NB is catabolized by these microsomal oxygenations to form phenolic residues that usually conjugate with sulfates or glucuronides to reduce their toxicity and aid excretion (Beauchamp et al., 1982). These metabolic conversions generally are expected to be shared by man and animals (Dorigan and Hushorn, 1976; Beauchamp et al., 1982).

Considering its small molecular weight, volatility, and limited water solubility (0.2%), inhaled NB likely has free access to most compartments of the corpus (Mabey et al., 1982). NB is expected to deposit transiently in fat depots because of its lipophilic nature. However, NB would not be expected to be a lingering corporal contaminant due to its moderately low log (octanol/water) coefficient ($K_{o/w}$) of 1.87 (Mabey et al., 1982). This $K_{o/w}$ is similar to the $K_{o/w}$ for chloroform, thus demonstrating about the same lipophilicity.

The flora of the cecum of the large intestine can metabolically reduce NB to aniline ($C_6H_5NH_2$) (Ikeda and Kita, 1964; Rickert et al., 1983). It has been observed that aniline-caused methemoglobinemia does not occur in NB-exposed germ-free (axenic) animals but is present in normal animals. The normal animals have gut flora to convert NB to aniline by cecum reductases (Reddy et al., 1976). Presumably then, aniline is an essential part of the NB metabolic and toxicity profile. Aniline is already classified as a Category B2 carcinogen, *a likely human carcinogen*. This is based on the carcinogenicity in the spleen and body cavity in two strains of rat (U.S. EPA, 1995).

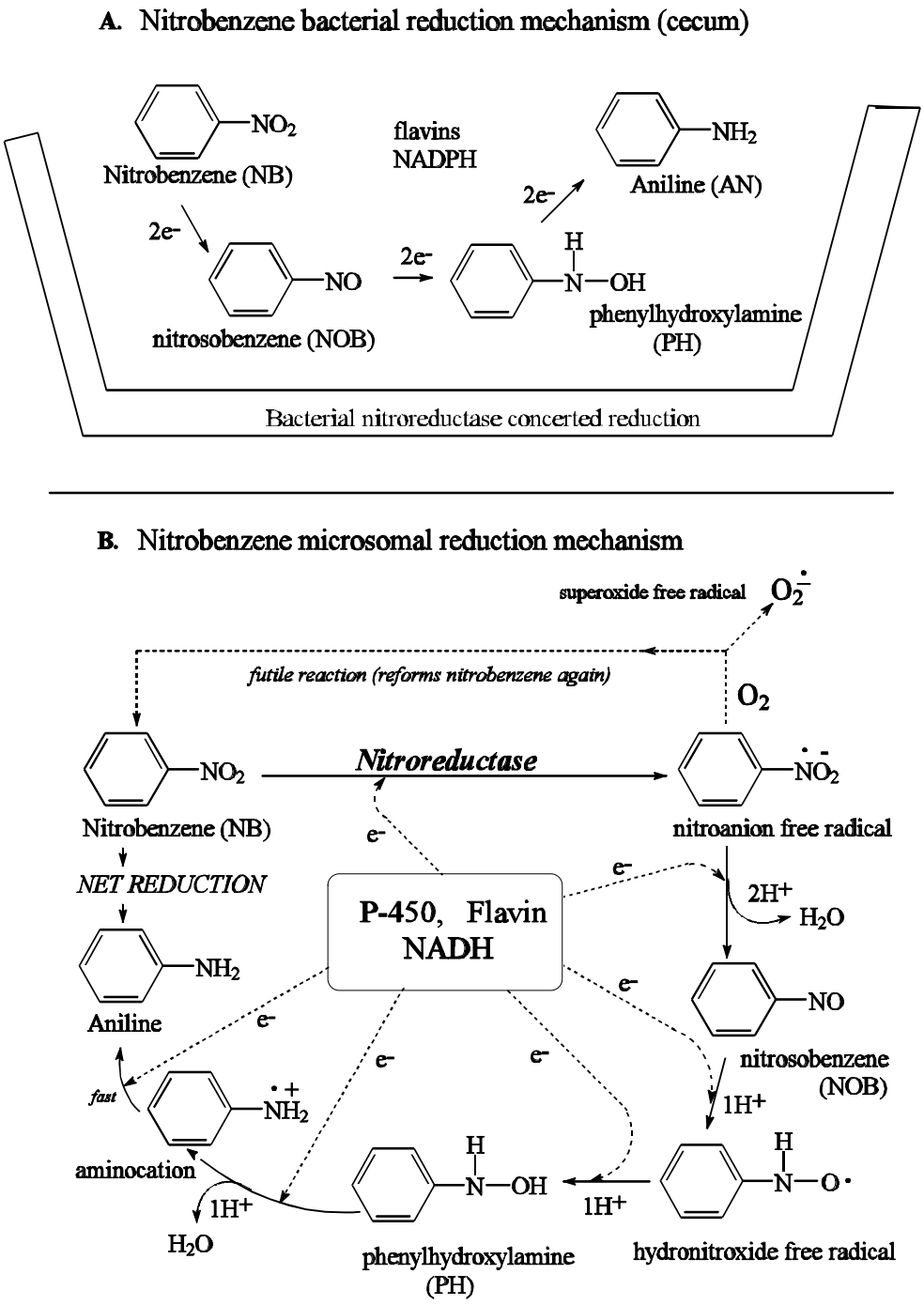

Figure 3. A. The cecal nitroreductase reduction of nitrobenzene by intestinal flora. B. The systemic reduction of nitrobenzene by P-450 enzymes to aniline. Note the production of the free radical intermediates and nitrosobenzene and phenyhydroxlamine.

The steps by which NB is reduced to aniline go through the intermediates nitrosobenzene and phenylhydroxylamine (Goldstein and Rickert, 1984) (see figure 3). The nitroanion free radical is formed by a 1*e* transfer and is a relatively stable free radical. If oxygen is sufficiently present in the tissue, then a reaction takes place that produces the superoxide free radical, that is, the nitroanion free radical reacts with O_2 reproducing NB, a futile reaction, plus creating the superoxide free radical $O_2^{\cdot-}$ (figure 3B) (Mason and Holtzman, 1975b; Sealy et al., 1978; Levin et al., 1982). The futile reaction can account for a number of the toxic and carcinogenic actions of NB based on the known carcinogenic properties of $O_2^{\cdot-}$ (Kensler et al., 1989a, b; Flohé et al., 1985; Guyton and Kensler, 1993; Feig et al., 1994; Cerutti, 1994; Dreher and Junod, 1996). In excess of antioxidant capacity, this radical can cause cancer. If the NB exposure escapes the intestinal reduction—as inhalation exposure does—then the P-450 system and possibly the mitochondrial systems reduce NB to aniline (Mason and Holtzman, 1975a). Nitrosobenzene and phenylhydroxylamine are produced (figure 3) as well as free radical intermediates of which the nitroanion free radical is the most stable.

These intermediates are known to bind to hemoglobin and are the direct mechanism of observed methemoglobinemia activity from NB exposure (Goldstein and Rickert, 1985). Because of their chemical reactivity, they likely bind to many other cellular components such as DNA. Almost all nitrosoamine and hydroxylamine compounds are thought to be potential carcinogens (Miller, 1970; Weisburger and Weisburger, 1973). However, a literature search did not find any carcinogenicity studies specifically on nitrosobenzene or phenylhydroxylamine. The amount of NB exposure determines if these potentially pernicious reduction intermediates exceed the metabolic capacity to remove them. If not quenched, the free radicals generated from NB exposure can cause tissue damage, which is also a dose-dependent process (Keher, 1993). That is, the free radicals could exceed the tissue's ability to quench them by antioxidants such as vitamins C and E (Netke et al., 1997; Kimmick et al., 1997; Primiano et al., 1997).

2. CARCINOGENICITY OF NITROBENZENE

NB mutagenicity was reviewed and was found to be negative in Salmonella test systems with reductase activation (Chiu et al., 1978; Suzuki et al., 1987) and in V79 cells (Kuroda, 1986). Negative results also were observed in primary hepatocyte repair assays and in vivo sister chromatid exchange assays. Thus, with negative mutagenicity data, no current cancer bioassay data, and no human carcinogenicity information, NB previously has been classified as a Category D carcinogen (IRIS database 1993). The purpose of this current review is to determine the carcinogenicity of NB based on the latest information available.

The Chemical Industry Institute of Toxicology (CIIT) began a 2-year NB inhalation carcinogenicity bioassay in 1983, and the results of NB bioassay data became available in 1993. The results of this bioassay were reported in full in January 1993 by James A. Popp, study director, and his colleagues (CIIT, 1993). The report provides the basis of this assessment of the carcinogenicity of NB. The inhalation study involved both sexes of B6C3F1 mice (0, 5, 25, 50 ppm NB), F344/N rats (0, 1, 5, 25 ppm), and Sprague-Dawley (CD strain) rats (0, 1, 5, 25 ppm) (1.0 ppm = 5.12 mg NB/m^3 or 1 mg/m^3 = 0.20 ppm). Exposures were 6 hours/day × 5 days/week × 104 weeks. The results were later published (Cattley et al., 1994 #52). NB was introduced into the dosing chamber as a vapor at >99% purity. The chamber concentration did not vary significantly in target concentrations. NB was chemically stable during the test.

At the study end, the tumor incidences in each treated group was pair-wise compared with tumor incidence of the vehicle air-dosed control animals to determine any change in tumorigenicity. A number of organs were examined for tumorigenicity. The necropsied tissues were nose (anatomical regions 1 to 4), adrenal glands, brain, ear canal, bronchial lymph nodes, clitoral or preputial gland, cecum, urinary bladder, esophagus, gallbladder, trachea, tissue masses with regional lymph nodes and any gross lesions, heart, thymus, thyroid, ileum, jejunum, rectum, kidneys, spleen, sternebrae, salivary glands, larynx, liver, lungs, bronchi, mammary gland, mandibular lymph nodes, snout, pancreas, parathyroid glands, pituitary, prostate, testes, epididymis or ovaries, and bone. Tumor discovery was either at autopsy after adventitial death or death by other pathologic means or at planned mid- or terminal-necropsy.

In some cases there were no changes in body weights, and because the remainder body weights decreased only 4% to 8% compared with controls, the mouse and rat body weights were only minimally affected by NB during the 2-year dosing. The maximum tolerated dose (MTD) was achieved in this study in both species based on the toxic effects observed: nasal epithelial degeneration, induced methemoglobinemic anemia, and hepatic enlargement. All these toxicities were graded as significant effects. Compared with controls, the mouse and rat survivals were unaffected by NB inhalation (CIIT, 1993).

Inflammatory degeneration occurred in the nasal and nasal olfactory passages in all test animals due to chronic inspiration of NB. Decreased RBC counts and hematocrit and hemoglobin levels were observed as a result of NB exposure in mice and rats. Hematopoietic dysfunction also was noted in both B6C3F1 mice and F344/N rats based on RBC interaction and platelet parameter changes. NB caused methemoglobinemic anemia in all test species (both sexes).

Increases in bone marrow cells also suggested hemotoxic effects, but no erythroid stem cell carcinogenesis or leukemias were observed. Most of the hemotoxic effects caused by exposure can be explained by the hemotoxic actions of aniline, a NB metabolite. Nitrosobenzene and phenylhydroxylamine, both NB metabolites, also produce hemotoxic effects (Weisburger and Weisburger, 1973 #13; Kiese, 1974 #31; Beauchamp et al., 1982 #9; Kiese, 1966 #11). Enlarged and multinucleated hepatocytes were seen in male and female mice and in female rats. Testicular atrophy and epididymal hypospermia were viewed in male B6C3F1 mice and CD rats (Bond et al., 1981 #51; McLaren et al., 1993 #78; Levin et al., 1988 #71). It is known that Fischer rats always have old-age testicular effects and are not adequate to determine testicular cancer, so the CD-1 rats were added to this study to appropriately assess the carcinogenicity in testes.

3. CANCER BIOASSAY RESULTS

Increased tumor incidences were observed at various organ sites and are presented in tables 2 through 4. The statistical methods used are as follows. The adjusted incidences at risk are corrected according to Kaplan-Meier (K-M) methods. The life table method corrects for intercurrent mortality, and the incidence method corrects for tumor occurrence, assuming the tumors were only incidentally found and were not the cause of death. The Cochran-Armitage probability is the test for trend of tumor incidence versus dose. The Fisher exact test compares the paired increase of tumor incidence at each dose with the incidence of the concurrent control at that site. Probability values ≤ 0.05 and ≥ 0.01 are considered significant, and values <0.01 are considered quite significant.

Table 2 presents data on tumor incidence in male and female B6C3F1 mice. Significant tumor increases were observed in the alveolus and bronchus, thyroid (follicular cells), and mammary gland.

Table 3 presents data on tumor incidence in male and female F344/N rats. Significant tumor increases were observed in liver, thyroid (follicular cell), kidney, and endometrium.

Table 4 presents data on tumor incidence in male Sprague-Dawley (CD) rats. A significant tumor increase was observed in the liver of the Sprague-Dawley (CD) rat.

Table 2. Tumor incidence in B6C3F1 mice

Male B6C3F1 mouse lung (alveolar/bronchial) adenomas or carcinomas[a]				
Dose	**0 ppm**	**5 ppm**	**25 ppm**	**50 ppm**
Adenomas, A/B	7/68 (10)	12/67 (18)	15/65 (23)	18/66 (27)
Carcinomas, A/B	4/68 (6)	10/67 (15)	8/65 (12)	8/66 (12)
Total incidence	9/68 (13%)	21/67 (31%)	21/65 (32%)	23/66 (35%)
Adjusted incidence	20.5%	42.6%	45.6%	46.0%
First occurrence (days)	507	536	711	704
Life table	*0.059*	*0.017*	*0.015*	*0.011*
Incidental tumor	*0.035*	*0.031*	*0.017*	*0.010*
Cochran-Armitage	*0.017*	—	—	—
Fisher exact	*not applicable*	*0.010*	*0.007*	*0.003*

Table 2. Tumor incidence in B6C3F1 mice (continued)

Male B6C3F1 mouse thyroid follicular cell adenomas				
Dose	**0 ppm**	**5 ppm**	**25 ppm**	**50 ppm**
Overall incidence	0/65 (0%)	4/65 (6%)	1/65 (2%)	7/64 (11%)
Adjusted incidence	0%	9.1%	2.2%	14.7%
First occurrence (days)	—	724 T	724 T	659
Life table	*0.022*	*0.073*	*0.519*	*0.015*
Incidental tumor	*0.019*	*0.073*	*0.519*	*0.014*
Cochran-Armitage	*0.015*	—	—	—
Fisher exact	*not applicable*	*0.060*	*0.500*	*0.006*
Female B6C3F1 mouse mammary gland adenocarcinomas				
Dose[b]	**0 ppm**	**5 ppm**	**25 ppm**	**50 ppm**
Overall incidence	0/48 (0%)	not examined	not examined	5/60 (8%)
Adjusted incidence	0%	—	—	11.9%
First occurrence (days)	—	—	—	609
Life table	—	—	—	*0.048*
Incidental tumor	—	—	—	*0.069*
Cochran-Armitage	—	—	—	—
Fisher exact	*not applicable*	—	—	*0.049*

[a] Tumor incidence, K-M adjusted tumor incidence, and first occurrence of tumor at a particular site are presented above the double line for each tumor site showing carcinogenicity. Below the double line, statistics are presented in italics; the *p*-values below the zero dose are for the trend test for each accounting method of tumor observation. The other *p*-values are Fisher exact tests compared with the zero dose concurrent control.

[b] Only mammary gland control and high dose examined. Low and mid doses were not examined, and there was no rationale presented why these groups were not analyzed for carcinogenicity. Because this is a two-dose examination, no trend can be assessed by the Cochran-Armitage method.

Source: CIIT, 1993.

Table 3. Tumor incidence in F344/N rats

Male F344/N rat hepatocellular adenomas/carcinomas[a]				
Dose	**0 ppm**	**1 ppm**	**5 ppm**	**25 ppm**
Adenomas, liver	1/69 (1)	3/69 (4)	3/70 (4)	15/70 (21)
Carcinomas, liver	0/69 (0)	1/69 (1)	2/70 (3)	4/70 (6)
Total incidence	1/69 (0%)	4/69 (6%)	5/70 (7%)	16/70 (23%)
Adjusted incidence	2.3%	8.0%	10.6%	34.8%
First occurrence (days)	714 T	714 T	714 T	714 T
Life table	*<0.001*	*0.228*	*0.125*	*<0.001*
Incidental tumor	*<0.001*	*0.228*	*0.125*	*<0.001*
Cochran-Armitage	*0.001*	—	—	—
Fisher exact	*not applicable*	*0.183*	*0.108*	*<0.001*

Male F344/N rat thyroid follicular cell adenomas/adenocarcinomas				
Dose	**0 ppm**	**1 ppm**	**5 ppm**	**25 ppm**
Total incidence	2/69 (3%)	1/69 (1%)	5/70 (7%)	8/70 (11%)
Adjusted incidence	4.1%	2.0%	10.6%	17.4%
First occurrence (days)	563	714 T	714 T	714 T
Life table	*0.009*	*0.463 N*	*0.249*	*0.060*
Incidental tumor	*0.011*	*0.491 N*	*0.220*	*0.067*
Cochran-Armitage	*0.010*	—	—	—
Fisher exact	*not applicable*	*0.500 N*	*0.226*	*0.051*

Table 3. Tumor incidence in F344/N rats (continued)

Male F344/N rat kidney tubular adenomas/adenocarcinomas				
Dose	**0 ppm**	**1 ppm**	**5 ppm**	**25 ppm**
Adenomas, kidney	0/69 (0)	0/68 (0)	0/70 (0)	5/70 (7)
Carcinomas, kidney	0/69 (0)	0/68 (0)	0/70 (0)	1/70 (1)
Total incidence	0/69 (0%)	0/68 (0%)	0/70 (0%)	6/70 (9%)
Adjusted incidence	0%	0%	0%	13.0%
First occurrence (days)	—	—	—	714 T
Life table	*<0.001*	—	—	*0.022*
Incidental tumor	*<0.001*	—	—	*0.022*
Cochran-Armitage	*<0.001*	—	—	*0.022*
Fisher exact	*not applicable*	—	—	*0.015*

Female F344/N rat endometrial polyps				
Dose	**0 ppm**	**1 ppm**	**5 ppm**	**25 ppm**
Overall incidence	11/69 (16%)	17/65 (26%)	15/65 (23%)	25/69 (36%)
Adjusted incidence	21.7%	32.6%	29.1%	44.5%
First occurrence (days)	611	459 I (interim)	459 I	459 I
Life table	*0.006*	*0.166*	*0.287*	*0.008*
Incidental tumor	*0.008*	*0.102*	*0.201*	*0.007*
Cochran-Armitage	*0.010*	—	—	—
Fisher exact	*not applicable*	*0.107*	*0.205*	*0.006*

[a] Tumor incidence, K-M adjusted tumor incidence, and first occurrence of tumor at a particular site are presented above the double line for each tumor site. Below the double line, statistics are presented in italics; the *p*-values below the zero dose are for the trend test for each accounting method of tumor observation. The other *p*-values are Fisher exact tests compared with the zero dose concurrent control.

Source: CIIT, 1993.

Table 4. Tumor incidence in Sprague-Dawley (CD) rats

Male Sprague-Dawley (CD) rat hepatocellular adenomas or carcinomas[a]				
Dose	**0 ppm**	**1 ppm**	**5 ppm**	**25 ppm**
Overall incidence	2/63 (3%)	1/67 (1%)	4/70 (6%)	9/65 (14%)
Adjusted incidence	5.3%	3.3%	9.5%	30.8%
First occurrence (days)	592	696	457 I	609
Life table	*0.002*	*0.449 N*	*0.409*	*0.036*
Incidental tumor	*0.003*	*0.446 N*	*0.370*	*0.046*
Cochran-Armitage	*0.002*	—	—	—
Fisher exact	*not applicable*	*0.447 N*	*0.391*	*0.031*

[a] Tumor incidence, K-M adjusted tumor incidence, and first occurrence of tumor at a particular site are presented above the double line for each tumor site showing carcinogenicity. Below the double line, statistics are presented in italics; the *p*-values below the zero dose are for the trend test for each accounting method of tumor observation. The other *p*-values are Fisher exact tests compared with the zero dose concurrent control.

Source: CIIT, 1993.

4. DISCUSSION

The CIIT study was started in November 1983 but was not finalized as a full report until January 1993 (CIIT, 1993) because of a backlog in the CIIT histopathology laboratory. Subsequent to the drafting of this EPA report, the CIIT study scientists and sponsors involved with the bioassay published the results of their study (Cattley et al., 1994). The hazard evaluation analysis presented in the published paper closely agrees with the independent hazard evaluation work reported here.

The CIIT study was apparently well conducted, used good laboratory practices, and was well reported. The report has adequate statistical analysis except that historical control averages and variations were not presented for all significantly increased tumor sites. The MTD was obtained in this study showing respiratory, testicular, and hemopoietic effects. Even though the rodent fur/skin was exposed in the chambers with NB for 2 years, no skin cancer resulted.

Hyperplasia of the alveolus and bronchus was observed in males but not in females. This could be a predisposing factor in the lung epithelial tumor causation in male mice, and in fact, increases in B6C3F1 mouse *alveolar and bronchial (A/B) tumors* were observed in male but not in female mice (table 2). The increased lung incidence was significant by all statistical measures: life table analysis, incidental analysis, Cochran-Armitage trend, and Fisher pairwise comparisons of treated incidences versus control incidence. The effect is driven by the adenomas, that is, there is a trend with adenomas but not with carcinomas. This makes the effect tumorigenic rather than carcinogenic. Because there is no indication that carcinomas (evidence of cancer) were increased with NB in the pool of increased hyperplastic cells and adenomas in 2 years of exposure, this A/B tumor evidence is benign and therefore limited.[1]

B6C3F1 mouse *thyroid follicular cell* adenomas increased also (table 2). This effect too was tumorigenic, not carcinogenic. The thyroid effect was statistically increased by all measures of statistical analysis (listed above). This thyroid adenoma evidence is a benign response in 2 years of exposure. Because each *male* B6C3F1 mouse response demonstrated limited evidence, the male mouse response is not sufficient by itself for a determination of carcinogenicity. The male B6C3F1 mouse cancer responses, however, do factor into the overall determination of NB carcinogenicity (see below).

In the female B6C3F1 mouse, increases were observed in malignant mammary adenocarcinomas (table 2), with some having squamous metaplasia. Unfortunately, only the high-

[1] A "limited" response is not a sufficient response; it is something less. A sufficient response is a carcinogenicity dose response that is qualitatively applicable from animals to humans and statistically significant by trend and pair-wise comparisons (FEXT). Limited responses can occur in various degrees of relevance and concern in carcinogenicity considerations. Expert judgment is necessary to factor in the relative weights of each respective limited response.

dose group and the control groups were examined histologically. This is a data gap. The high-dose incidence of cancers was 5/60 (8%) (corrected incidence of 11.9%) versus none in controls. The first tumor of this kind was discovered late in the study at 609 days. The historical control average (n = 1,791) is 1.76% with standard deviation = 2.6%. This makes the observed response between the 2σ and 3σ limits (6.95% < 8.0% < 9.55%). The whole observed historical control range for mammary adenocarcinomas incidence is 0% to 12%, which may include outliers (Haseman et al., 1985). These references suggest that the incidence magnitude is marginal and *could* be in the population of normally occurring tumors of the mammary gland. The corrected incidence of 11.9% is almost out of the range of experience for control animals. Moreover, the observed incidence of 8% is sufficiently greater than the historical control average (1.8%) to suggest but not prove a positive effect. When no additional information is known, as in this case, the concurrent control (0/48 = 0%) is taken as the valid reference. Therefore, this effect in the mouse mammary gland is determined to be positive and marginally sufficient (see footnote 1) to determine carcinogenicity.

In the male F344/N rat, a cancer response was observed in the liver (table 3). The cancer response was mostly adenomas (trend $p < 0.001$), with a modest increase in hepatocellular carcinomas (trend $p = 0.040$). This suggests that the adenomas may serve as a pool for developing into carcinomas, making this a malignant effect. The combination of these tumors (benign and malignant) is increased by all statistical measures (vide ante). This carcinogenic effect was discovered only at terminal sacrifice and therefore did not cause these animals to die early because of the liver tumors. This liver cancer effect in male F344/N rats is sufficient to determine carcinogenicity.

Significant increases of thyroid adenomas or adenocarcinomas were observed in male F344/N rats. These results are presented in table 3. It is notable that the thyroid follicular cells were affected in both the male F344/N rat and the male mouse (see above). Because there were more carcinoma-bearing male rats than benign follicular cell adenoma-bearing male rats, the rat thyroid response is considered carcinogenic rather than tumorigenic. There was a trend (Cochran-Armitage) of adenomas and adenocarcinomas with increasing NB dose ($p = 0.01$), but the other statistical measures were not significant. Therefore, the certainty of the cancer response is similar for male F344/N rats and male B6C3F1 mice: both show limited evidence for carcinogenicity.

The male rat kidney showed increased tubular adenomas and adenocarcinomas (table 3). It was a high-dose effect only. Of the 6 tumors observed in 70 male F344/N rats, 5 were adenomas and 1 was an adenocarcinoma. Thus, this is primarily a benign tumorigenic effect. The tumors were observed at day 714, which was the terminal sacrifice; the tumors were not life threatening and were statistically increased by all statistical measures (vide ante). This kidney

response shows some evidence of carcinogenicity.

The female F344/N rats had an endometrial polyp response to NB (table 3). This benign response is statistically increased, at the high dose only, over a high background concurrent control response of 11/69 (16%). These tumors were first discovered at day 611 or 86% through the study. This benign endometrial response provides limited evidence for carcinogenicity.

A second rat species, the Sprague-Dawley (CD) rat, was tested for NB carcinogenicity because the F344 rat shows high testicular tumors normally in old age. Therefore, Sprague-Dawley (CD) rats were added to cover this tissue. No tumor increases were observed in testes, but as shown in table 4, there were increases in hepatocellular adenomas or adenocarcinomas. Liver is the only site in Sprague-Dawley (CD) rats for which tumor incidence increased. Although the response was flat for liver carcinomas (2/63, 0/67, 2/70, 2/65), the adenomas showed an increased trend (1/63, 1/67, 2/70, 7/65) that was determined to be significant (trend p = 0.003). The combination of adenomas and carcinomas is quite significant by all statistical measures (table 4). Because this is a mostly benign response, it is determined that Sprague-Dawley (CD) rats have some evidence for carcinogenicity.

In summary, there were eight tumorigenic or carcinogenic rodent organ sites responding to NB exposure in the CIIT inhalation study. Significant oncogenic increases in organ sites are summarized in table 5.

NB inhalation exposure produces an array of tumor types, some of which could be influenced by the toxicity caused by chronic NB exposure for 2 years. Of the eight sites covered in tables 2 through 4, significant carcinogenicity evidence exists for one site: liver carcinogenicity in the male F344/N rat. Also, limited rat responses occur in the male rat kidney and thyroid and the female F344 rat endometrium. These rat responses collectively suggest a sufficient response in the F344 rat. The evidence for carcinogenicity in the B6C3F1 mouse is not as clear as in the rat. The weight of evidence of the three mouse sites—male lung/bronchus and thyroid follicular cell and female mammary gland—suggests collectively a sufficient response in the B6C3F1 mouse. The Sprague-Dawley (CD) rats show a benign, limited liver response but at a site concordant with the other rat species tested (F344/N). The Sprague-Dawley (CD) rat does not respond with testicular tumors, although there are known toxic effects of NB with male reproduction and sperm production.

Table 5. Summary of nitrobenzene carcinogenicity results

Site of increased tumorigenicity	Sex	Evidence of carcinogenicity	Comments
B6C3F1 Mouse			
Lung: Alveolus and bronchus	M	Limited	Benign tumor increase only; carcinomas spread evenly over dose groups (no trend)
Thyroid: Follicular cell	M	Limited	Benign tumors only with dose trend
Mammary gland	F	Marginally sufficient	Historical and concurrent controls suggest malignant but low-level cancer incidence in a two-dose examination (0 and 50 ppm dose groups).
F344/N Rat			
Liver: Hepatocellular	M	Sufficient	Clear evidence of malignancy and exposure related to cancer incidence in the liver
Thyroid: Follicular cell	M	Limited	Another thyroid follicular response; marginal statistics: only a trend with dose and only a suggestion of malignancy
Kidney: Tubular cell	M	Limited	High dose only; benign tumorigenic response
Endometrial polyp	F	Limited	Benign response
Sprague-Dawley (CD) rat			
Liver: Hepatocellular	M	Limited	Benign response (unlike the male F344/N rat)

NB is metabolically related to aniline by nitroreduction, that is, aniline can be made from NB in the gastrointestinal tract by resident flora nitroreductases (Mirsalis et al., 1982; Ikeda and Kita, 1964; Rickert et al., 1983; Reddy et al., 1976). After NB oral ingestion and after NB passes through the acid stomach, aniline forms in the cecum (figure 3A). This bacterial nitroreductase activity is found in rodents as well as in humans. Aniline already is classified by EPA as a Category B2 carcinogen. A compound such as NB that generates aniline also should be considered a B2 carcinogen *if* comparable exposure and metabolic conversion conditions occur.

In the CIIT study exposure is by inhalation. The aniline metabolite may not be formed in the nasal passage or lungs of CIIT rodents because the nasal pH is neutral in the nasal passages, and there is a lack of nitroreduction flora in the passageways. However, rodents consistently groom each other, and because the fur most likely has bound NB residues from the chronic inhalation exposure, rodents can obtain oral doses of the parent compound (NB) from licking the fur during grooming. Therefore, aniline can be formed from the licked and ingested NB parent compound. The amount of aniline produced by this mechanism is uncertain. It is clear that NB exposure also can be metabolized systemically, as other exocylic nitroaromatics, to the amine (in this case aniline) passing through reactive free radicals and nitrosoamine and phenylhydroxylamine (figure 3B). These are likely carcinogenic compounds participating in the cancer mechanism of NB.

Dinitrobenzene is a chemical analogue of NB. However, there is inadequate cancer information for the *ortho* or *para* isomers of the chemical analogue dinitrobenzene; these isomers are classified as Category D carcinogens. Benzene is a rather remote chemical analogue of NB and is a Category A carcinogen. Also, in tissues where oxygen is sufficiently available superoxide $O_2^{\cdot-}$ can form, and $O_2^{\cdot-}$ is thought to be carcinogenic (Dreher and Junod, 1997; Kensler et al., 1989a; b; Guyton and Kensler, 1993). Therefore, based on the potential presence of the above reactive metabolites, NB may be considered carcinogenic based on metabolic mechanisms. The mutagenicity of NB is not positive and thus does not support the carcinogenicity classification by a *direct* DNA-altering mechanism. It is not certain whether NB or NB/metabolite combinations cause the multisite and two species carcinogenesis.

In conclusion, inhaled NB is toxic, causing anemia and methemoglobinemia, at a number of noncarcinogenic sites (Cattley et al., 1994). Whether this type of toxicity occurs at carcinogenic sites and is related to the mechanisms of carcinogenicity is unknown; nonetheless, NB is carcinogenic in rats and in mice, a fact that is likely related to the redox reactions of NB. In the male F344/N rat, evidence for liver carcinogenicity is sufficient, and limited carcinogenicity occurs at rat thyroid, kidney, and endometrium sites. There also is limited liver tumorigenic evidence in a second rat strain, Sprague-Dawley (CD). In the mouse, NB is sufficiently carcinogenic in the mammary gland at the high dose (lower and mid doses were not histologically

examined). There are two additional limited mouse sites, thyroid and lung. The systemic excessive tumors, compared with no-dose controls, suggest that inhaled NB causes internal cancer effects that stem from absorbed NB and/or its metabolites (Cattley et al., 1994). The NB parent, aniline, nitrosobenzene, and phenylhydroxylamine are all carcinogenic candidate causal agents, and it could be a combination of these agents—in the proper organ deposition—that actuates the initiation process followed by tumor promotion and, in the F344/N rat liver, tumor progression to malignancy. This mixture could be responsible for the spectra of oncogenicity both in various organ sites and degree of tumorigenicity severity. Furthermore, aniline, nitrosobenzene, and phenylhydroxylamine can cause methemoglobinemic anemia. Methemoglobinemic anemia was observed in all three species tested in the CIIT study (1993) and has been seen in humans too (Kiese, 1974). Therefore, the presence of methemoglobinemic anemia suggests the corporal presence of these metabolites of NB in NB exposures.

It is presumed that due to similarity in metabolism between animals and man that both species are at risk by either inhalation, dermal, or ingestion routes of exposure. It is expected, however, that the inhalation and dermal routes are the primary means by which humans could be exposed to NB in the United States. The weight of evidence for NB carcinogenicity is based on an adequate CIIT inhalation study (1993) that indicates, according to the 1986 Guidelines for Cancer Risk Assessment (U.S. EPA, 1986), that NB is a Category B2 carcinogen: *NB is probably carcinogenic to humans*. The B2 categorization is supported by (1) metabolism and (2) structure-activity relationships, but not by the mutagenicity information available. This report recommends the upgrading of the current carcinogenicity Category D (memorandum from J. Holder to V. Dellarco, Office of Health and Environmental Assessment, dated September 28, 1992) to a B2.

In summary, there were eight NB-caused tumorigenic organ sites spread over different species, sexes, and doses. The specific mechanism of NB carcinogenicity is still not completely understood but likely proceeds by redox mechanisms in various tissues. NB is classified by *any human route* as Category B2 according to the 1986 cancer guidelines (U.S. EPA, 1986); according to the April 23, 1996, proposed cancer guidelines (U.S. EPA, 1996), NB is classified as *a likely human carcinogen by any route of human exposure*.

NB should be examined further for human metabolic and cancer results. A literature search did not produce any relevant epidemiologic carcinogenicity studies on NB that could substantiate the results of the animal studies presented here in support of the carcinogenicity classification of NB.

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6. APPENDIX

This section is abstracted from the 1995 CRAVE presentation as submitted to the Integrated Risk Information System (IRIS) committee. It has been approved for IRIS. Questions concerning these calculations should be addressed to Jennifer Jinot, NCEA-Washington office.

QUANTITATIVE ESTIMATE OF CARCINOGENIC RISK FROM INHALATION EXPOSURE

1. SUMMARY OF RISK ESTIMATES

- Inhalation unit risk: 3E-5 per $\mu g/m^3$
- Inhalation slope factor: 1E-1 per mg/kg/d
- Extrapolation method: linearized multistage (GLOBAL86), extra risk
- ED10: 1 mg/kg/d (5E+3 $\mu g/m^3$)
- Air concentrations at specified risk levels:

Risk Level		Concentration
E-4 (1 in 10,000)	=	3 $\mu g/m^3$
E-5 (1 in 100,000)	=	0.3 $\mu g/m^3$
E-6 (1 in 1,000,000)	=	0.03 $\mu g/m^3$

2. DOSE-RESPONSE DATA

- Tumor type: hepatocellular, thyroid follicular cell, or kidney tubular cell adenomas or carcinomas
- Test animal: male F344/N rat
- Route: inhalation
- Reference: A chronic inhalation toxicity study of nitrobenzene in B6C3F1 mice, Fischer 344 rats, and Sprague-Dawley rats (CIIT, 1-22-93)

Administered	Human equivalent	Adenomas + carcinomas		
exposure (ppm)	exp (µg/cu.m.)	liver	thyroid	kidney
0	0	1/60	2/60	0/60
1	9.1E+2	4/60	1/60	0/60
5	4.6E+3	5/60	5/60	0/60
25	2.3E+4	16/60	8/60	6/60

3. ADDITIONAL COMMENTS

- The unit risk is considered to be a plausible upper bound on the increased cancer risk from lifetime inhalation of nitrobenzene.
- The human equivalent exposures were based on an assumption of ppm equivalence in exposure across species and were calculated by converting ppm nitrobenzene to $\mu g/m^3$ (1 ppm = 5.12 mg/m^3) and adjusting for partial exposure 6 hours/day, 5 days/week.
- The risk estimates were derived for the proportions of tumor-bearing animals, that is, those animals with any individual tumor or combination of tumors from the three significant tumor sites (liver, thyroid, and kidney), as determined from the individual animal data provided by CIIT. The proportions of tumor-bearing animals were 3/60, 5/60, 10/60, and 26/60 for 0, 1, 5, and 25 ppm exposure, respectively.
- The tumor incidence denominators reflect the original number of animals per dose group (excluding those for interim sacrifice). No adjustments were made for the number of animals at risk at the time of the first tumor because these were late-occurring tumors (the first tumor at one of these sites was a thyroid tumor at day 563), and there was no substantial early mortality (only one rat died in the first year).