FINAL

Report on Carcinogens Background Document for

Diazoaminobenzene

November 22, 2002

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Public Health Service
National Toxicology Program
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Contract Number N01-ES-85421

FOREWORD

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

Nominations for listing in or delisting from the RoC are reviewed by a formal process that includes a multi-phased, scientific peer review and multiple opportunities for public comment. The review groups evaluate each nomination according to specific RoC listing criteria. This Background Document was prepared to assist in the review of the nomination of diazoaminobenzene. The scientific information in this document comes from publicly available, peer reviewed sources. Any interpretive conclusions, comments or statistical calculations, etc made by the authors of this document that are not contained in the original citation are identified in brackets [ ]. If any member(s) of the scientific peer review groups feel this Background Document does not adequately capture and present the relevant information they will be asked to write a commentary for this Background Document that will be included as an addendum to the document. In addition, a meeting summary that contains a brief discussion of the respective review group's review and recommendation for the nomination will be added to the Background Document, also as an addendum.

A detailed description of the RoC nomination review process and a list of all nominations under consideration for listing in or delisting from the RoC can be obtained by accessing the NTP Home Page at http://ntp-server.niehs.nih.gov. The most recent RoC, the 9th Edition, was published in May, 2000 and may be obtained by contacting the NIEHS Environmental Health Information Service (EHIS) at http://ehis.niehs.nih.gov (800-315-3010).

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Support to the National Toxicology Program for the preparation of this background document was provided by Technology Planning and Management Corporation through NIEHS Contract Number NO1-ES-85421

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

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

Known to be Human Carcinogens:

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

Reasonably Anticipated to be Human Carcinogens:

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

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

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

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

Executive Summary

Introduction
Diazoaminobenzene (DAAB) is a triazene that is used as an intermediate in chemical synthesis, as a complexing agent, and as a polymer additive. DAAB was nominated by the National Institute for Environmental Health Sciences (NIEHS) for possible listing in the Report on Carcinogens based on the results of short-term toxicity and metabolism studies by the National Toxicology Program which found that DAAB is metabolized to the known human carcinogen benzene and to the animal carcinogen aniline.

Human Exposure
Use. DAAB is used as a chemical intermediate, complexing agent, and polymer additive. It has uses in organic synthesis and manufacture of dyes and insecticides and as a dopant for laser ablation of polymethylmethacrylate. DAAB has been identified as a contaminant in the dyes D&C Red No. 33, FD&C Yellow No. 5 (tartrazine), and FD&C Yellow No. 6; all are permitted for use in drugs and cosmetics, and the latter two are permitted in food.

Production. DAAB may be produced by reaction of aniline with isoamyl nitrate or by diazotization of aniline dissolved in hydrochloric acid with sodium nitrite, followed by addition of sodium acetate. No data were found on U.S. production of DAAB, but it was available from seven U.S. suppliers in 2001. U.S. imports of DAAB and *p*-aminoazobenzenedisulfonic acid (combined category) totaled 94,237 lb (42,835 kg) from January through October 2001.

Environmental Exposure. Environmental exposure to DAAB may occur from oral consumption of or dermal exposure to dyes and colorants (such as FD&C Yellow No. 5). A study conducted by the National Academy of Sciences in 1977 reported that average daily intakes were 43 mg of FD&C Yellow No. 5 and 37 mg of FD&C Yellow No. 6. Based on these intakes and the maximum allowable levels of DAAB in colorants under U.S. Food and Drug Administration (FDA) regulations, the theoretical maximum daily exposures to DAAB are approximately 1.7 ng for FD&C Yellow No. 5 and 1.5 ng for FD&C Yellow No. 6.

Occupational exposure. Occupational exposure to DAAB could occur from its use as a chemical intermediate and polymer additive. DAAB is explosive and is harmful to the respiratory tract, skin, and eyes through inhalation or dermal contact.

Regulations. No DAAB-specific regulations were found. The FDA regulates FD&C Yellow No. 5 and FD&C Yellow No. 6 for use as color additives in foods, drugs, and cosmetics and D&C Red No. 33 for use as a color additive in drugs and cosmetics.

Human Cancer Studies
No human studies were identified in which exposure to DAAB is specifically mentioned. DAAB is predicted to be a carcinogen because it is metabolized to benzene and aniline. Benzene is classified by the Report on Carcinogens as *known to be a human carcinogen* and by the International Agency for Research on Cancer (IARC) as carcinogenic to

humans (Group 1). Numerous epidemiological studies have shown a causal relationship between benzene exposure and leukemia. Aniline was listed by IARC in 1987 as not classifiable as to its carcinogenicity to humans (Group 3) based on limited evidence of carcinogenicity in animals and inadequate evidence in humans. Although an excess of bladder cancer has been observed in aniline-dye workers, these workers were also exposed to other bladder carcinogens. Studies of aniline-dye workers not exposed to other carcinogens found no increased risk of cancer but had methodological problems that limited their interpretation.

Studies of Cancer in Experimental Animals

In carcinogenicity studies conducted in the late 1940s, dermal exposure to DAAB resulted in skin and lung tumors in some mice. DAAB is metabolized to benzene and aniline, both of which are carcinogenic in laboratory animals. Oral exposure to benzene induced multiple tumors at multiple sites in rats (Zymbal gland, oral cavity, and skin) and mice (lymphoma, Zymbal gland, lung, Harderian gland, and preputial gland) of both sexes. In addition, Tg.AC transgenic mice, which carry a v-Ha-ras oncogene, developed skin tumors and leukemia following dermal exposure to benzene. Rats exposed to aniline in the diet developed sarcoma of the spleen and other body organs. Therefore, DAAB has been concluded to be carcinogenic in animals, based on its metabolism to benzene and aniline.

Genotoxicity

DAAB is mutagenic in bacteria with metabolic activation, and it induces chromosomal aberrations in plants and micronuclei in the bone marrow cells of mice exposed *in vivo*. Benzene, a major metabolite of DAAB, has a pattern of genotoxicity consistent with patterns observed for other leukemia-inducing chemicals; it is effective in damaging chromosomes but relatively ineffective in inducing point mutations. Mutagenicity studies in bacteria have given mixed results without metabolic activation and generally negative results with metabolic activation. In mammalian in *vitro* assays, benzene induced chromosomal recombination and DNA adducts in human tissue, and it induced DNA phosphorylation and sister chromatid exchange and inhibited RNA synthesis in animal cells. In *in vivo* studies, benzene induced chromosomal aberrations, DNA damage, and micronuclei in mice and rats. Chromosomal aberrations have been detected in the bone morrow and lymphocytes of humans exposed occupationally to benzene; the most common damage observed is chromosomal breakage, but deletions and other alterations also have been reported. Aniline does not induce mutations in bacteria. In mammalian *in vitro* assays, aniline induced sister chromatid exchange and chromosomal aberrations, but not DNA damage. In *in vivo* animal studies, aniline induced sister chromatid exchange and micronuclei.

Other Relevant Data

Absorption, distribution, and metabolism. In disposition studies in rats and mice, DAAB was almost completely absorbed from the gastrointestinal tract but sparingly absorbed from skin. Following administration by each route, the absorbed portion of the dose was rapidly metabolized and excreted primarily in urine. Metabolites identified in blood (of rats) or urine (of rats and mice) were benzene, aniline, and their metabolites. Benzene was exhaled by rats and mice after oral administration of DAAB. The metabolic

conversion of DAAB to metabolites of benzene is consistent with the metabolic pathway for benzene, in which formation of benzene oxide is the initial step. Metabolites of DAAB in the blood of rats and the urine of rats and mice included hydroquinone, muconic acid, and phenylmercapturic acid, which share benzene oxide as a common intermediate. Studies with human liver slices also demonstrated that DAAB could be cleaved to yield metabolites of benzene and aniline.

The proposed metabolic pathway for DAAB is that it is cleaved reductively by liver enzymes or gut flora to form aniline, benzene, and nitrogen. Benzene and aniline are subsequently metabolized by cytochrome P-450 and conjugating enzymes. DAAB metabolism also results in the formation of a reactive phenyl radical, which could account for an additional risk of toxicity or carcinogenicity. Evidence for the possible involvement of the phenyl radical in DAAB mutagenicity may be seen in the fact that DAAB is mutagenic in *Salmonella typhimurium* with metabolic activation, whereas benzene and aniline are not.

Toxicity studies. Symptoms observed in animals exposed to DAAB (dermally, but without protection of the application site, to allow for oral exposure via grooming) were similar to those that would be anticipated for animals exposed to benzene and/or aniline and included decreased thymus weights (rats and mice) and increases in the weights of the heart (rats and mice), spleen (rats), liver (rats) and kidney (male rats and female mice). DAAB induced hematologic effects in rats and mice, including chemical-related methemoglobinemia and Heinz-body formation. DAAB-related non-neoplastic lesions in both rats and mice included hyperplasia and inflammation of the skin, lymphoid atrophy of the thymus, atrophy of the lymph nodes, and hematopoietic cell proliferation in the spleen. Non-neoplastic lesions in the heart, kidney, and liver also were observed in mice. The erythrocytes, thymus, and lymphoid system are major targets of benzene, whereas symptoms of aniline exposure include methemoglobin formation, anemia, increased spleen weight, and regenerative hematopoiesis. DAAB also appeared to induce toxicity not observed for aniline or benzene, including skin lesions at the application site.

Potential mechanism of carcinogenicity. Studies have demonstrated that in rats and mice, DAAB is quantitatively metabolized to benzene, a known human and animal carcinogen, and to the rat carcinogen, aniline. Symptoms observed in animals administered DAAB in short-term studies are similar to those anticipated for animals exposed to benzene and/or aniline. DAAB is mutagenic in *S. typhimurium* and induces micronuclei and chromosomal aberrations in the bone marrow of rodents. Like DAAB, benzene and aniline induce micronuclei in mice; however, DAAB induced more micronuclei in mice than did equimolar doses of benzene or a mixture of benzene and aniline, an effect suggested to be due to the formation of the phenyl radical during metabolism of DAAB.

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1 Introduction

Diazoaminobenzene (DAAB) is a triazene that is used as an intermediate in chemical synthesis, as a complexing agent, and as a polymer additive. It has been used as a propellant for the molding of rubbers and plastics and as a coupler to promote adhesion of natural rubber to steel tire cords (Kirk-Othmer 1982). Occupational exposure to DAAB occurs from its use as an intermediate during organic synthesis and in the manufacture of dyes and insecticides (Lewis 1997). Other exposures to DAAB may occur through its presence in cosmetics and food products. It has been identified as a contaminant in several dyes that have been permitted for use in ingested and externally applied drugs and cosmetics (Bailey 1985, Palmer and Mathews 1986).

DAAB was nominated by the National Institute of Environmental Health Sciences (NIEHS) for possible listing in the Report on Carcinogens based on the results of National Toxicology Program (NTP) toxicity studies which concluded that DAAB is metabolized to the known human carcinogen benzene (see NTP 2002b, IARC 1982a, 1987a) and showed significant increases in micronuclei in the bone marrow of mice exposed to DAAB. DAAB is also metabolized to aniline that has been shown to be carcinogenic in rats. In addition, some toxic effects associated with benzene (atrophy of the lymphoid tissue) and aniline (methemoglobinemia and Heinz-body anemia) were identified in the toxicity studies of DAAB, which lends additional support to the prediction that DAAB has similar toxic effects to benzene and aniline. This Background Document is based in large part on the NTP (2002a) Report on the Metabolism, Toxicity, and Predicted Carcinogenicity of Diazoaminobenzene.

1.1 Chemical identification

Diazoaminobenzene ($C_{12}H_{11}N_3$, mol wt 197.24, CASRN 136-35-6) also is known as 1,3-diphenyl-1-triazene, DPT, anilinoazobenzene, benzeneazoanilide, benzeneazoaniline, DAAB, alpha-diazoamidobenzol, 1,3-diphenyltriazine, *N*-(phenylazo)aniline, 1,3-diphenyl-triazene, diazobenzeneanilide, and *p*-diazoaminobenzene (HSDB 2001a, NTP 2002a). [The validity of the reported synonym *p*-diazoaminobenzene is uncertain, because DAAB does not contain para bonds.] The RTECS number for DAAB is XY265000. The structure of DAAB is illustrated in Figure 1-1.

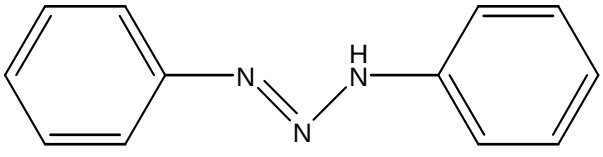


Figure 1-1. Chemical structure of DAAB

1.2 Physical-chemical properties

DAAB exists as small, golden-yellow crystals or an orange solid at room temperature. It melts at 98°C, decomposes at 130°C, and explodes at its boiling point of 150°C.

Decomposition products of DAAB include benzene, *o*- and *p*-aminodiphenyl, diphenylamine, and azobenzene. DAAB is soluble in ethyl alcohol, ethyl ether, benzene, pyridine, and hexane; it is insoluble in water. The physical and chemical properties of DAAB are summarized in Table 1-1.

Table 1-1. Physical and chemical properties of DAAB

Property	Information	References
Molecular weight	197.24	Budavari *et al.* 1996
Color	golden-yellow orange	Budavari *et al.* 1996 NTP 2002a
Physical state	crystals solid	Budavari *et al.* 1996 NTP 2002a
Melting point (°C)	98	Budavari *et al.* 1996
Boiling point (°C)	150	NTP 2002a
Vapor density	6.8	Lewis 2000
Solubility		
water at 22°C	< 1 mg/mL	NTP 2002a
acetone at 18°C	100 mg/mL	NTP 2001
benzene	soluble	NTP 2001
95% ethanol at 18°C	10–50 mg/mL	NTP 2001
ether	soluble	NTP 2001
Decomposition products	benzene, *o*- and *p*-aminodiphenyl, diphenylamine, and azobenzene	Mortimore *et al.* 1979

1.3 Identification of metabolites

DAAB is metabolized to benzene and aniline and to their metabolites. See Section 6.1.2 for further discussion of the metabolites of DAAB.

2 Human Exposure

2.1 Use

DAAB is used as a chemical intermediate, complexing agent, and polymer additive (Mathews and De Costa 1999). It also has uses in organic synthesis and manufacture of dyes and insecticides (Lewis 1997) and as a dopant for laser ablation of polymethylmethacrylate (Bolle *et al.* 1990). As a polymer additive, DAAB has been listed in a European patent as a chemical that may be used as a coupler to promote adhesion of natural rubber to steel tire cords (Shemenski and Starinshak 1982). It also is listed in a U.S. patent as a chemical that may be used as a blowing agent in the production of a foamed polymeric material having a camouflage appearance (Raad 1993).

DAAB has been identified as a contaminant in D&C Red No. 33, FD&C Yellow No. 5, and FD&C Yellow No. 6; all are permitted for use in drugs and cosmetics, and the latter two are permitted in food (FDA 2001, 21 CFR 74). D&C Red No. 33 is a color additive permitted for use in ingested and externally applied drugs, lipsticks, and cosmetics (Bailey 1985). Bailey (1985) identified DAAB in 9 of 11 samples of commercial D&C Red No. 33 at concentrations of up to 439 ppb, with an average of 99 ppb. DAAB also was identified at concentrations of 68 and 110 ppb in two "pharmacology samples" from the U.S. Food and Drug Administration's (FDA's) animal feeding studies to evaluate the toxicity of D&C Red No. 33. The common name for FD&C Yellow No. 5 is tartrazine, and this colorant is a common constituent of cosmetics, such as shampoos, hand and body lotions, bath and shower gels, facial cleansers, and sunless tanning products; medications, including pediatric antihistamines; and foods and beverages, including candy, baked goods, ice cream, and cereal. The FDA's risk evaluation of FD&C Yellow No. 5 concluded that the normal use of this colorant would not result in significant exposure to carcinogenic impurities, including DAAB (Palmer and Mathews 1986). FD&C Yellow No. 6 is commonly known as sunset yellow and is used in beverages, baked goods, desserts, and ice cream (Fraser 2002).

2.2 Production

DAAB may be produced by the rapid reaction of aniline with isoamyl nitrate, with a product yield of 67% (Smith and Ho 1990), or by diazotization of aniline dissolved in hydrochloric acid with sodium nitrite, followed by addition of a concentrated solution of sodium acetate (HSDB 2001). It also is formed as an intermediate during the preparation of iodobenzene from aniline and through the interaction of nitrous acid and an alcoholic solution of aniline (NTP 2002a). In a patented process, DAAB may be formed by the reaction of aniline with nitrogen oxides, which are produced via the oxidation of ammonia (Detrick 1977, Herkes 1977).

No data were found on U.S. production of DAAB. Chem Sources (2001) identified seven U.S. suppliers of DAAB. No import data for DAAB as an individual compound were available. However, U.S. imports of DAAB and *p*-aminoazobenzenedisulfonic acid combined totaled 94,237 lb (42,835 kg) from January through October 2001 (U.S. ITA 2001).

Although production data for DAAB could not be found in current literature, production data for dyes that contain DAAB as an impurity (D&C Red No. 33, FD&C Yellow No. 5, and FD&C Yellow No. 6) were found. The United States International Trade Commission (USITC) reported production of these dyes from 1980 to 1994, as summarized in Table 2-1.

Table 2-1. U.S. production of dyes that contain DAAB as an impurity (in pounds)

	Year[a]					
Dye	**1980**	**1986**	**1987**	**1992**	**1993**	**1994**
D&C Red No. 33	–[b]	5,000	7,000	12,000	NR	10,000
FD&C Yellow No. 5	1,527,000	1,569,000	1,618,000	NR	NR	NR
FD&C Yellow No. 6	1,206,000	1,528,000	1,153,000	NR	1,307,000	NR

Sources: USITC 1980, 1987, 1988, 1990, 1991, 1993, 1994a, 1994b, 1995.

[a]For the years 1989–1991, all three dyes reportedly were produced; however, specific production data were not provided for any of the three. NR = Production values not reported because of confidentiality issues.

[b]Although production of this dye was reported, specific production data were not provided.

The FDA regulates color additives for use in food, drugs, cosmetics, and medical devices in the United States. The FDA lists all approved color additives and requires domestic and foreign manufacturers of these colors to submit samples from each batch of color produced. FDA scientists test these samples to confirm that each batch is within established specifications that restrict the levels of impurities allowed in the color (FDA 2001). Table 2-2 summarizes total amounts of D&C Red No. 33, FD&C Yellow No. 5, and FD&C Yellow No. 6 certified by the FDA for 1997 through 2001.

2.3 Analysis

DAAB has been identified as a contaminant in 1-aryl-3,3-dialkyl triazine compounds by thin-layer chromatography. Solvent extraction and reversed-phase high-performance liquid chromatography (HPLC) were used to analyze for the compound in D&C Red No. 33 (Bailey 1985).

2.4 Environmental occurrence

No information was found regarding environmental occurrence of DAAB.

2.5 Environmental fate

No information was found regarding the environmental fate of DAAB.

Table 2-2. Total certification of color additives (in pounds)

Dye[b]	Year				
	1997	1998	1999	2000	2001
D&C Red No. 33					
Primary	24,568	21,027	30,661	42,928	25,940
Repacks	–[a]	–	–	1,513	15,689
Lakes	22,125	9,212	8,531	45,960	18,367
FD&C Yellow No. 5					
Primary	2,086,314	2,288,198	2,597,407	2,202,549	2,733,240
Repacks	–	–	–	3,455	8,262
Lakes	1,223,794	1,403,629	1,191,999	1,043,291	1,451,570
FD&C Yellow No. 6					
Primary	1,934,898	2,173,554	2,494,268	2,275,356	2,316,832
Repacks	–	–	–	–	27,784
Lakes	787,084	977,373	975,228	812,453	1,199,015

Source: FDA 2001.

[a]– = information not provided.

[b]Primary is defined as the color additive itself and is water soluble. Repacks refer to the packaging for a second time of a previously certified color additive. Lakes are the dye form attached to an aluminum or calcium substrate to make it insoluble.

2.6 Environmental exposure

The presence of DAAB as an impurity in dyes and colorants, such as FD&C Yellow No. 5, could result in very low-level consumer exposure by the oral and dermal routes. In 1977, the National Academy of Sciences surveyed the amount of certified FD&C colorants consumed by the U.S. population. For 12,000 persons over two years of age surveyed for 14 days, the results showed average daily intakes of 43 mg for FD&C Yellow No. 5 and 37 mg for FD&C Yellow No. 6 (Feingold 2002). [Although no data are available for exposure levels of DAAB in dyes and colorants, theoretical maximum daily exposures of 1.7 ng for FD&C Yellow No. 5 and 1.5 ng for FD&C Yellow No. 6 may be calculated, based on the average daily intakes calculated by Feingold (2002) and the maximum allowable levels of DAAB in colorants under the FDA regulations (see Table 2-3).]

2.7 Occupational exposure

Occupational exposure to DAAB could occur from its use as a chemical intermediate and polymer additive. DAAB is explosive and is harmful to the respiratory tract, skin, and eyes through inhalation or dermal contact (Aldrich Chemical 2002). DAAB was not listed in the National Occupational Exposure Survey conducted by the National Institute for Occupational Safety and Health between 1981 and 1983 (NTP 2002a).

2.8 Biological indices of exposure

No information was found regarding biological indices of exposure to DAAB.

2.9 Regulations

No DAAB-specific regulations were found. The FDA regulates FD&C Yellow No. 5 and FD&C Yellow No. 6 for use as color additives in foods, drugs, and cosmetics and D&C Red No. 33 for use as a color additive in drugs and cosmetics. Special labeling is required for drugs that contain FD&C Yellow No. 5 and FD&C Yellow No. 6. No regulations from other governmental agencies were located for D&C Red No. 33, FD&C Yellow No. 5, or FD&C Yellow No. 6. Table 2-3 summarizes the FDA regulations for these three dyes.

Table 2-3. FDA regulations

Regulatory action	Effect of regulation or other comments
21 CFR 74 – PART 74 – LISTING OF COLOR ADDITIVES SUBJECT TO CERTIFICATION. Promulgated: 42 FR 15654, 3/22/77. U.S. Codes: 21 U.S.C. 321, 341, 342, 343, 348, 351, 352, 355, 361, 362, 371, and 379e.	Lists color additives that are subject to certification in drugs, cosmetics, and medical devices. FD&C Yellow No. 5 and FD&C Yellow No. 6 may be safely used for coloring food, and D&C Red No. 33 may be safely used for coloring ingested drugs, other than mouthwashes and dentrifices, in amounts not to exceed 0.75 mg per daily dose of the drug. The maximum level of DAAB impurity in FD&C Yellow No. 5 is 40 ppb. The maximum level of DAAB impurity in FD&C Yellow No. 6 is 40 ppb. The maximum level of DAAB impurity in D&C Red No. 33 is 125 ppb.
21 CFR 82 – PART 82 – LISTING OF CERTIFIED PROVISIONALLY LISTED COLORS AND SPECIFICATIONS. Promulgated: FD&C Yellow No. 5: 51 FR 24519, 7/7/86; FD&C Yellow No. 6: 52 FR 21509, 6/8/87; D&C Red No. 33: 53 FR 33121, 8/30/88. U.S. Codes: 21 U.S.C. 371, 379e, and 379e note	General specifications are given for colors and certifiable mixtures that can be used in foods, drugs, and cosmetics. FD&C Yellow No. 5 and FD&C Yellow No. 6 are color additives that are allowed to be used in foods, drugs, and cosmetics. D&C Red No. 33 may be used in drugs and cosmetics.
21 CFR 201 – PART 201 – LABELING. Promulgated: 40 FR 13998, 3/27/75. U.S. Codes: 21 U.S.C. 321, 331, 351, 352, 353, 355, 358, 360, 371, 374, and 379e; 42 U.S.C. 216, 241, 262, and 264.	There must be labels for over-the-counter and prescription drugs containing FD&C Yellow No. 5 and FD&C Yellow No. 6 as color additives.

Source: The regulations in this table have been updated through the 2001 Code of Federal Regulations 21 CFR, December 31, 2001.

3 Human Cancer Studies

No human studies were identified in which exposure to DAAB is specifically mentioned. Because nomination of DAAB was based on its metabolism to benzene and aniline, the human cancer effects of these metabolites are summarized briefly in this section.

3.1 Benzene

Benzene is listed in the Report on Carcinogens as a *known to be human carcinogen* based on sufficient evidence of carcinogenicity in humans (NTP 2002b). The International Agency for Research on Cancer (IARC) also classified benzene as carcinogenic to humans (Group 1) based on sufficient evidence in humans (IARC 1982a, 1987a). The RoC and IARC based their conclusion on case reports, case series, case-control studies, and cohort studies that reported an association between exposure to benzene and leukemia (primarily acute myeloid leukemia), with the strongest evidence coming from the cohort studies.

The International Programme on Chemical Safety (IPCS) reviewed health effects of benzene in 1993. This report included a review of human epidemiological studies of exposure to benzene and cancer, including studies reviewed by IARC (1987a) and studies published since the 1987 IARC review. Increased risk of leukemia was identified in studies of benzene-exposed shoemakers, chemical and rubber workers, and workers in oil refineries and a study of benzene workers in various locations, with standardized mortality ratios (SMRs) ranging from 1.6 to 5.7. The IPCS task force concluded that the most consistent evidence was found for an association between benzene exposure and myeloid leukemia, although other types of leukemia, lymphoma, and multiple myeloma also were reported. Increased risks of other types of cancer have been reported in some studies, but the findings are not consistent. Some studies reported an exposure-response relationship between benzene exposure and leukemia, with very high SMR values (between 50 and 60 in two studies) for the groups with highest exposure.

Since the IARC review, Savitz and Andrews (1997) reviewed 18 community-based and 16 industry-based studies that provided data on benzene exposure and lymphatic and hematopoietic cancer. Their purpose was to look at the relationship between benzene exposure and types of leukemia other than acute myeloid leukemia (which is accepted to be associated with benzene exposure) and other types of hematopoietic cancers. Most studies found an association between benzene exposure and total lymphatic and hematopoietic cancer, total leukemia, and specific histologic types of leukemia, including chronic lymphocytic leukemia, as well as acute myeloid leukemia. Little evidence was found for an association between benzene exposure and multiple myeloma or non-Hodgkin's lymphoma. The authors concluded that the evidence supported a general association of benzene exposure with leukemia, rather than a specific association with acute myeloid leukemia.

3.2 Aniline

IARC classified aniline as not classifiable as to its carcinogenicity (Group 3), based on limited evidence in animals and inadequate evidence in humans (IARC 1982b, 1987b). IARC noted that although an excess of bladder cancer had been observed in aniline-dye workers, studies of workers exposed to aniline but not to other known bladder carcinogens had not reported an increased risk of cancer. However, these studies had methodological problems due to incomplete follow-up of workers who left the industry and to the absence of estimates of expected numbers of bladder cancer cases (IARC 1982b, 1987b).

4 Studies of Cancer in Experimental Animals

Kirby (1947, 1948) conducted carcinogenicity studies in mice using a chemical identified as *p*-diazoaminobenzene and DAAB. The chemical was purified by solution in ether or benzene, poured through a tower of alumina, concentrated, and precipitated by addition of petroleum ether. The purified chemical was described as a light tan powder with a melting point of 98°C. There is some question regarding the identity of this compound, because DAAB does not have para bonds and is golden-yellow to orange in color (see Section 1.2). Nevertheless, *p*-diazoaminobenzene is listed as a synonym for DAAB in some sources (see Section 1), and the melting point is correct. Furthermore, Kirby (1947) noted that DAAB is readily rearranged by chemical means to yield *p*-aminoazobenzene. This fact appears to establish the study chemical as DAAB, because chemical rearrangement of DAAB to *p*-aminoazobenzene is part of an industrial process for making phenylenediamine (Kirk-Othmer 1996). Although experimental details of these studies were lacking and the number of animals used was small, the data from these studies are presented in Section 4.1. No other carcinogenicity studies were identified.

Because DAAB is metabolized to benzene and aniline, the experimental carcinogenicity data for these chemicals reported by the National Cancer Institute (NCI 1978), the National Toxicology Program (NTP 1986), and the Chemical Industry Institute of Toxicology (CIIT 1982) are summarized in Section 4.2. Section 4.3 presents recent data on the carcinogenicity of benzene administered to genetically altered mice.

4.1 DAAB

Kirby (1947) investigated the effects of DAAB exposure by the oral, subcutaneous (s.c.) injection, and dermal routes. No control groups were reported for any of the studies.

For oral exposure, 6 male and 6 female mice (strain not reported) were fed a diet of rat cake powder containing 50 mg of DAAB per 100 g of diet; 10 additional mice were fed cake powder with 100 mg of DAAB per 100 g of diet; and 7 male and 3 female mice were fed a special restricted diet containing 100 mg of DAAB per 100 g of diet. Two male and all the female mice in the group given DAAB at 50 mg/100 g of cake powder died within 39 days as a result of liver and kidney toxicity. The remaining 4 male mice survived 122 to 125 days. Three mice in the group given DAAB at 100 mg/100 g of cake powder survived 240 to 291 days, and the other 7 mice died or were sacrificed on day 29. In the third group, 4 males and all 3 females died within 64 days. Two of the remaining males died by day 95, and one survived 331 days. No stomach lesions or tumors were reported in any of the three exposure groups.

In the injection experiments, 10 male and 10 female mice were given an initial s.c. injection in the right flank of 0.25 mL of arachis oil containing 2 mg of DAAB. About one month after the first injection, the survivors (1 male died) were injected with 4 mg of DAAB in the left flank. After 190 days, the surviving mice (8 males and 10 females) received a third injection of 10 mg of DAAB in the right flank; 4 males and 7 females died within a week of the last injection. Two mice of each sex survived 323 to 329 days. No tumors were reported in any of the mice.

The dermal studies consisted of painting mice (number not reported) in the interscapular region with a 0.5% solution of DAAB in acetone; after unspecified periods, the concentration was increased to 1%, then 2%, and finally 5%. Eight mice survived to be painted with the 5% solution, two of which developed squamous carcinoma. These mice survived for 346 to 601 days.

Kirby (1948) duplicated the dermal exposure study described above using a 5% solution of DAAB and added another experiment where DAAB was dissolved in a 0.5% solution of croton oil in acetone. Applications were made to the nape of the shaved neck, three times per week for up to 545 days. Croton oil had no effect on the outcome. Of 17 mice that survived more than 400 days, 5 developed squamous papilloma and 5 others developed squamous carcinoma at the application site. Two mice developed pulmonary adenoma, and one mouse developed pulmonary adenocarcinoma.

4.2 Benzene and aniline

The NTP (1986) conducted a two-year carcinogenicity assay of benzene in F344/N rats and $B6C3F_1$ mice. The NCI (1978) investigated the carcinogenicity of aniline hydrochloride in F344/N rats and $B6C3F_1$ mice, and CIIT (1982) investigated the carcinogenicity of aniline hydrochloride in CD-F rats. Table 4-1 shows the lowest doses associated with increased incidences of tumors of various types observed in these studies. In most cases, the lowest dose tested (25 mg/kg body weight [b.w.] in mice, 50 mg/kg b.w. in rats) induced increased incidences of at least one type of tumor. Exposure-response data from these studies are summarized in Tables 4-2a and 4-2b (benzene) and Table 4-3 (aniline). The data in Tables 4-1, 4-2a, 4-2b, and 4-3 all were derived from the NCI (1978), NTP (1986), and CIIT (1982) reports.

In the benzene studies, survival was significantly decreased in high-dose male rats, mid- and high-dose female rats, and in high-dose male and female mice; therefore, tumor incidence data in the exposed groups were adjusted for intercurrent mortality. Benzene induced Zymbal gland, oral cavity, and skin tumors in rats and Zymbal gland, lung, Harderian gland, ovarian, and preputial gland tumors and malignant lymphoma in mice. Aniline induced splenic sarcomas in rats but was not carcinogenic in mice.

Table 4-1. Lowest doses of benzene or aniline causing cancer in rats and mice

Chemical and strain	Exposure route[a]	Exposure duration (wk)	Dose level[b] (mg/kg b.w.)	Tumor types
Benzene				
Rat, F344/N				
Male	gavage	103	50[c]	oral cavity and skin squamous-cell papilloma or carcinoma
			100	Zymbal gland carcinoma
Female	gavage	103	25[c]	Zymbal gland carcinoma
			50	oral cavity squamous-cell papilloma or carcinoma
Mouse, $B6C3F_1$				
Male	gavage	103	25[c]	Harderian gland adenoma
			50	Zymbal gland and preputial gland carcinoma, alveolar/bronchiolar adenoma or carcinoma, malignant lymphoma
Female	gavage	103	25[c]	malignant lymphoma
			50	alveolar/bronchiolar adenoma or carcinoma, ovarian granulosa-cell tumor, mammary gland carcinoma
			100	Harderian gland and Zymbal gland carcinoma
Aniline				
Rat, CD-F				
Male	feed	104	100	splenic sarcoma
Rat, F344/N				
Male	feed	104	130[c,d]	splenic hemangiosarcoma
			260[d]	splenic and body-cavity sarcoma
Female	feed	104	240[d,e]	splenic and body-cavity sarcoma

Sources: NCI 1978, NTP 1986, CIIT 1982.

[a]Gavage exposure was for 5 days per week.

[b]The lowest dose at which tumor incidence exceeded control values.

[c]The lowest dose tested.

[d]The average daily dose (mg/kg per day) is approximate, assuming an average daily feed consumption of 11 g (females) and 15 g (males), an average body weight of 270 g (females) and 350 g (males), and an average concentration of aniline in the feed of 3,000 or 6,000 ppm.

[e]Based on the total incidence (7/50) of fibrosarcoma or sarcoma (spleen and body cavity combined), compared with historical controls. Results were not significantly different compared to concurrent controls.

Table 4-2a. Tumor incidence in F344 rats exposed to benzene by gavage

Sex	Dose (mg/kg b.w.)	Tumor incidence[a] (%)						
		Zymbal gland carcinoma	Oral cavity (squamous cell)			Skin (squamous cell)		
			Papilloma	Carcinoma	Combined	Papilloma	Carcinoma	All
M	0	2/32 (7)	1/50 (2)	0/50 (0)	1/50 (2)	0/50 (0)	0/50 (0)	1/50 (3)
	50	6/46 (15)	6/50 (18)	3/50 (7)	9/50 (24)*	2/50 (7)	5/50 (15)*	7/50 (22)*
	100	10/42 (29)*	11/50 (37)***	5/50 (15)*	16/50 (49)***	1/50 (4)	3/50 (10)	5/50 (17)
	200	17/42 (56)***	13/50 (48)***	7/50 (33)***	19/50 (69)***	5/50 (27)**	8/50 (30)***	11/50 (45)***
F	0	0/45 (0)	1/50 (2)	0/50 (0)	1/50 (2)	1/50 (NR)	0/50 (0)	1/50 (NR)
	25	5/40 (13)*	4/50 (11)	1/50 (3)	5/50 (13)	0/50 (0)	0/50 (0)	0/50 (0)
	50	5/44 (14)*	8/50 (21)**	4/50 (10)*	12/50 (29)***	1/50 (NR)	0/50 (0)	1/50 (NR)
	100	14/46 (42)***	5/50 (16)*	5/50 (15)**	9/50 (28)***	0/50 (0)	1/50 (NR)	1/50 (NR)

Source: NTP 1986.

*$P < 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared with controls (life-table tests).

[a]Number of animals with tumors/number of animals at risk. Percentages are based on the Kaplan-Meier estimated incidences after adjusting for intercurrent mortality. NR = not reported.

Table 4-2b. Tumor incidence and dose-response data in B6C3F$_1$ mice exposed to benzene by gavage

Sex	Dose (mg/kg b.w.)	Tumor incidence (%)[a]								
		Zymbal gland carcinoma	Malignant lymphoma	Lung (alveolar/bronchiolar)			Harderian gland combined	Preputial gland carcinoma[b]	Ovary granulosa cell[c]	Mammary gland carcinoma[d]
				Adenoma	Carcinoma	Combined				
M	0	0/43 (0)	4/49 (12)	6/49 (19)	5/49 (16)	10/49 (30)	1/49 (4)	0/21 (0)	NAP	NAP
	25	1/34 (3)	9/48 (31)	6/48 (21)	11/48 (36)	16/48 (49)	10/46 (36)**	5/28 (22)		
	50	4/40 (29)*	9/50 (42)*	8/50 (29)	12/50 (42)*	19/50 (60)**	13/49 (52)***	19/29 (82)***		
	100	21/39 (88)***	15/49 (69)***	12/49 (42)**	14/49 (59)***	21/49 (71)***	14/48 (61)***	31/35 (96)***		
F	0	0/43 (0)	15/49 (42)	4/49 (13)	0/49 (0)	4/49 (13)	5/48 (17)	NAP	1/47 (3)	0/49 (0)
	25	0/32 (0)	24/45 (68)*	2/42 (7)	3/42 (13)	5/42 (19)	6/44 (24)		1/44 (4)	2/45 (7)
	50	1/37 (5)	24/50 (63)*	5/50 (16)	6/50 (23)**	10/50 (33)*	10/50 (27)		6/49 (20)*	5/50 (16)*
	100	3/31 (19)*	20/49 (54)*	9/49 (44)*	6/49 (27)**	13/49 (57)***	10/47 (41)*		7/48 (29)**	10/49 (33)***

Source: NTP 1986.

*$P < 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared with controls (life-table tests).

[a]Number of animals with tumors/number of animals at risk. Percentages are based on the Kaplan-Meier estimated incidences after adjusting for intercurrent mortality. NAP = not applicable.

[b]All types.

[c]Increased incidences of benign mixed tumors at the mid and high dose also were reported (0/47, 1/44, 12/49, 7/48).

[d]Increased incidence of carcinosarcoma at the high dose also were reported (0/49, 0/45, 1/50, 4/49).

Table 4-3. Tumor incidence in rats exposed to aniline hydrochloride in the diet

Strain	Sex	Conc. in diet or daily dose[a]	No. with tumors/no. examined (%)			Reference
			Fibrosarcoma or sarcoma NOS[b] (spleen)	Hemangio-sarcoma (spleen)	Combined sarcomas (multiple body cavity organs)[c]	
F344	male	0	0/25 (0)	0/25 (0)	0/25 (0)	NCI 1978
		3,000 ppm	7/50 (14)	19/50 (38)***	2/50 (4)	
		6,000 ppm	9/46 (20)*	20/46 (43)***	9/48 (19)*	
F344	female	0	0/23 (0)	0/23 (0)	0/24 (0)	NCI 1978
		3,000 ppm	0/50 (0)	1/50 (2)	1/50 (2)	
		6,000 ppm	3/50 (6)	2/50 (4)	4/50 (8)	
CD-F	male	0	0/123 (0)	0/123 (0)	NR	CIIT 1982
		10 mg/kg	0/129 (0)	0/129 (0)	NR	
		30 mg/kg	1/128 (1)[d]	0/128 (0)	NR	
		100 mg/kg	28/130 (22)[e,f]	6/130 (5)[f]	NR	
CD-F	female	0	0/129 (0)	0/129 (0)	NR	CIIT 1982
		10 mg/kg	0/129 (0)	0/129 (0)	NR	
		30 mg/kg	0/130 (0)	0/130 (0)	NR	
		100 mg/kg	0/130 (0)	1/130 (1)	NR	

*$P < 0.05$, ***$P \leq 0.001$ compared with controls.

[a]Concentrations in the diet in the CIIT study were varied throughout the study: low dose = 117–248 ppm, mid dose = 310–762 ppm, high dose = 1,129–2,360 ppm.

[b]NOS = not otherwise specified.

[c]NR = not reported.

[d]Neoplasm identified as a stromal sarcoma.

[e]Neoplasms included 3 fibrosarcomas, 21 stromal sarcomas, 1 capsular sarcoma, and 3 osteogenic sarcomas.

[f]*P*-values were not provided.

4.3 Benzene studies in genetically altered mice

4.3.1 Dermal studies in transgenic mice

This section reviews three relatively recent studies (Blanchard *et al.* 1998, Spalding *et al.* 1999, French and Saulnier 2000) that examined the carcinogenicity of benzene applied to the skin of transgenic mice. The Tg.AC transgenic mouse model was used in each of these studies. These mice carry a v-Ha-*ras* oncogene, which imparts the characteristics of genetically initiated skin reported to be similar to those of the mouse-skin tumorigenesis model. The untreated skin of these animals appears normal, and spontaneous skin tumors are rare. However, exposure to a carcinogen activates the transgene, resulting in squamous-cell papilloma.

Blanchard *et al.* (1998) investigated the effects of dermal application of 200 µL of neat benzene, three times per week for 20 weeks, on tumor incidences in male and female homozygous and hemizygous Tg.AC mice. Benzene induced skin tumors in all groups of benzene-treated mice; however, tumor incidence was higher in the homozygous mice than the hemizygous mice (Table 4-4). Statistical comparisons with the control groups were not provided.

Spalding *et al.* (1999) applied benzene in doses of 200 (7,000 mg/kg), 400, or 800 µL, twice per week for 20 weeks. Acetone was used as a negative control. The mid and high doses were applied as two or four 200 µL portions of neat benzene. The low-dose was applied as a 1:1 solution of benzene in acetone (i.e. 100 µL of benzene in 100 µL of acetone). Chemicals were applied in the morning and afternoon. Groups of 15 to 20 female homozygous Tg.AC mice were used. Benzene induced papillomas in a dose-dependent manner (Table 4-4).

French and Saulnier (2000) applied benzene to Tg.AC mice in doses of 450 or 800 µL per week for 14 to 26 weeks. The low dose was applied three times per week as 150 µL benzene in 50 µL acetone, and the high dose was applied twice a week as two 200-µL portions of neat benzene. The authors reported dose-dependent increases in papilloma and spindle-cell tumors but did not provide the data. The incidence of granulocytic leukemia was significantly increased ($P < 0.05$) at both the low dose (4/14, 29%) and the high dose (11/15, 73%). None of the 19 vehicle-control animals developed leukemia.

Table 4-4. Skin tumor incidence in Tg.AC mice dermally exposed to benzene

Genotype	Sex	Exposure level (µL/wk)	Tumor incidence[a] (%)	Tumor multiplicity (tumors/mouse ± SD)	Reference
Hemizygous	M	0	6/65 (9)	NR[b]	Blanchard *et al.* 1998
	M	600	3/10 (30)	16.0 ± 3.6	
	F	0	2/65 (3)	NR[c]	
	F	600	4/10 (40)	17.3 ± 2.8	
Homozygous	M	600	10/10 (100)	15.9 ± 4.7	Blanchard *et al.* 1998
	F	600	9/10 (90)	15.6 ± 5.0	
Homozygous	F	0	3/5 (60)[d]	1.4 ± 1.7	Spalding *et al.* 1999
	F	400	7/10 (70)	7.0 ± 10.3	
	F	800	8/10 (80)	10.6 ± 8.5*	
	F	1,600	10/10 (100)*	12.6 ± 8.3*	

*$P < 0.05$ compared with controls (the Blanchard *et al*. 1998 study did not provide statistical comparisons with controls).

[a]Number of animals with tumors/number of animals at risk.

[b]Not reported; 5 mice had 1 tumor, and 1 mouse had 4 tumors; however, 2 tumors were observed before exposure.

[c]Not reported; each mouse only had 1 tumor.

[d]Among all other vehicle-control groups, the incidence was 7/67 (10%). The high incidences in this group were wound induced.

4.3.2 Gavage and inhalation studies in p*53 heterozygous mice*

In recent years the C57BL/6 *Trp*53 heterozygous (N5) mouse (*p*53$^{\pm}$ mice) has been used as an alternative model for carcinogenicity testing. Three inhalation studies (Boley *et al.* 2000, 2002, Healy *et al.* 2001) and two gavage studies (French *et al.* 2001, Hulla *et al.* 2001) of benzene exposure in these mice were reviewed.

Nearly 90% (24 of 27) of benzene-induced thymic lymphomas exhibited loss of the functional *p*53 allele locus, compared with 67% (4 of 6) in spontaneous tumors (Boley *et al.* 2000). Five different patterns of loss of heterozygosity (LOH) were observed in benzene-induced tumors, and six LOH patterns were observed in spontaneous tumors, with only one pattern common to both. Furthermore, 83% of these tumors retained two copies of the disrupted *p*53 locus, indicating that benzene exposure induced a high frequency of LOH on chromosome 11 in these mice which likely was mediated by aberrant chromosomal recombinations. Other inhalation studies with these mice indicated that benzene exposure (100 ppm [for 30 hours per week] or 200 ppm [for 15 hours per week] for up to six months) induced micronuclei in red blood cells and reticulocytes (Healy *et al.* 2001) and altered the mRNA expression of *p*53 target genes involved in cell-cycle control and apoptosis (Boley *et al.* 2002).

Exposure to benzene by gavage at a dose of 100 to 200 mg/kg b.w. per day for 26 weeks caused *p*53$^{\pm}$ mice to develop tumors with a high frequency of LOH (French *et al.* 2001, Hulla *et al.* 2001) (see Table 4-5). French *et al.* (2001) reported that benzene induced sarcomas around the head and neck or thoracic cavity and some thymic lymphomas. Hulla *et al.* (2001) also reported a high incidence of subcutaneous sarcomas, which were not observed in the NTP bioassay (see Section 4.2).

Table 4-5. Loss of heterozygosity in tumors induced in heterozygous *p*53$^{\pm}$ mice exposed to benzene by gavage

Group	Daily dose (mg/kg b.w.)	Tumor[a] incidence (%)	LOH (%)	Reference
Control	0	0/10 (0)	0/10 (0)	French *et al.* 2001
Exposed	200	20/39 (51)**	13/19 (68)***	
Exposed	100	19/39 (49)[b]	13/16 (81)[b]	Hulla *et al.* 2001

**$P < 0.01$, ***$P < 0.001$ compared with controls.

[a]Primarily subcutaneous sarcomas. French *et al.* (2001) reported 3 thymic lymphomas and 1 acinar carcinoma of the pancreas.

[b]Statistical analysis not reported.

4.4 Summary

In carcinogenicity studies conducted with DAAB in the late 1940s, dermal exposure resulted in skin and lung tumors in some mice. Furthermore, DAAB is metabolized to benzene and aniline, both of which are carcinogenic in laboratory animals. Oral exposure to benzene induced multiple tumors at multiple sites in rats and mice of both sexes. Rats

exposed to aniline in the diet developed sarcomas of the spleen and other body organs. In addition, transgenic (Tg.AC) mice developed skin tumors and leukemia following dermal exposure to benzene, and *p*53 heterozygous mice developed thymic lymphomas and various sarcomas following inhalation or gavage exposure to benzene. Therefore, DAAB is expected to be carcinogenic in animals, based on its metabolism to benzene and aniline.

5 Genotoxicity

Limited information is available on the genotoxicity of DAAB (see Section 5.3). However, there is a great deal of genotoxicity information available on benzene and some information on aniline, the principal metabolites of DAAB. This information is summarized in Sections 5.1 (benzene) and 5.2 (aniline).

5.1 Benzene

The genotoxicity of benzene has been investigated in a large number of *in vitro* and *in vivo* tests, and several recent reviews have been published (ATSDR 1997, Eastmond 2000, Whysner 2000). The following sections present a brief overview of genotoxic effects reported for benzene in *in vitro* and *in vivo* studies.

5.1.1 In vitro *assays*

The genotoxicity of benzene has been studied extensively in a variety of *in vitro* assays in prokaryotic and eukaryotic systems. *In vitro* studies have shown both positive and negative results with metabolic activation and generally negative results without metabolic activation for gene mutation in *Salmonella typhimurium* (at concentrations of 3 to 1,000 ppm) and *Escherichia coli* (10 μL). Positive results have been reported for DNA phosphorylation in rat liver epithelial cells, RNA synthesis inhibition in rat liver mitoplasts and in rabbit and cat bone marrow mitoplasts (1 mM), and DNA adduct formation in human cells (12.5 to 50 μg/mL) have been reported. In addition, benzene has been shown to cause sister chromatid exchange (SCE) in Chinese hamster ovary cells and intrachromosomal recombination in human lymphoblastoid cell culture (ATSDR 1997).

5.1.2 In vivo *assays*

Benzene has been shown to be genotoxic in *in vivo* studies in animals and humans (ATSDR 1997). Studies have shown that benzene is weakly effective in inducing point mutations and that it binds inefficiently to bone marrow (Eastmond 2000). In addition, benzene has been shown to cause chromosomal aberrations in spleen lymphocytes, bone marrow, and spermatogonial cells in mice (at exposure levels of 36 to 880 mg/kg b.w.) and, in several studies, to increase micronucleus formation in mouse bone marrow and peripheral blood erythrocytes (at 10 to 600 mg/kg b.w.). The comet assay (single-cell gel electrophoresis) has shown DNA lesions in multiple organs in mice and rats (at 2,000 mg/kg b.w.). Ress *et al*. (2002) reported significant increases in micronuclei in mouse bone marrow at all exposure levels of benzene tested (10, 20, and 40 mg/kg b.w.) (see Section 5.3.3 below).

In vivo studies in humans occupationally exposed to benzene (at concentrations of 0.1 to 68 mg/m^3 in air) have shown chromosomal aberrations in the bone marrow and lymphocytes to be associated with exposure to benzene (ATSDR 1997). The lengths of exposure in these studies ranged from 6 months to greater than 30 years. Chromosome breakage is the most common genetic damage caused by benzene, but chromosomal deletions and alterations also have been noted (Eastmond 2000). One study investigated

specific chromosomal effects, aneusomy of chromosomes 7 and 8 in lymphocytes, in workers exposed to benzene at a median concentration of 31 ppm (99 mg/m^3) benzene. The results showed higher incidence of aneusomy on both chromosomes in the exposed workers than in controls (Zhang *et al.* 1999).

5.1.3 Summary of benzene genotoxicity

Numerous studies in animals and humans have shown that benzene is effective in inducing chromosomal aberrations, chromosomal loss, DNA strand breakage, and micronuclei. However, benzene has been shown to be only weakly effective in inducing point mutations. Studies have shown that the genotoxicity of benzene in humans and in human cultured cells is the same as that found in animals and their cultured cells (Whysner 2000). The pattern of benzene genotoxicity is consistent with the patterns for other leukemia-inducing chemicals. Studies currently are under way investigating the mechanism of benzene's genotoxicity, in particular, the role of oxidative stress and non-DNA targets.

5.2 Aniline

Genotoxicity data are less extensive for aniline than for benzene. IARC (1987b) reviewed the genotoxicity of aniline but did not present an overall conclusion, because *in vitro* tests gave both positive and negative results, and no *in vivo* test results were available at the time of the review. No more recent reviews of the genotoxicity of aniline are available. More recent individual studies are summarized below.

5.2.1 In vitro *assays*

Aniline has not shown genotoxic effects in *S. typhimurium*, either with or without metabolic activation, in *E. coli*, or in *Saccharomyces cerevisiae*. Aniline did not induce DNA damage in primary rat hepatocyte cultures or in Chinese hamster lung fibroblasts. However, aniline induced SCE and chromosomal aberrations in several mammalian cell assays (IARC 1987b). Additional studies have shown results consistent with those reported by IARC: negative results in *S. typhimurium* (at exposure levels of 100 to 6,666 μg/plate) (Haworth *et al.* 1983) and positive results for SCE in rat liver epithelial cells (at concentrations of 100 to 1,000 μM) (Cunningham and Ringrose 1983) and human lymphocytes (at aniline hydrochloride concentrations of 50 to 1,000 μM) (Wilmer *et al.* 1984).

5.2.2 In vivo *assays*

In vivo assays of aniline have shown mixed results, with positive results for micronucleus formation in mouse bone marrow (only at the highest doses tested, 380 and 1,000 mg/kg b.w.) (George *et al.* 1990, Ashby *et al.* 1991, Westmoreland and Gatehouse 1991), DNA lesions in a number of organs in both rats and mice (at 100 mg/kg b.w.) (Sekihashi *et al.* 2002), and SCE in mice, but negative results for micronucleus formation in mice and DNA lesions in the liver and kidney of rats (IARC 1987b). A study in *Drosophila melanogaster* gave positive results for meiotic nondisjunction (at aniline hydrochloride concentrations of 5% to 15%) and negative results for translocation and sex-linked recessive lethal mutation (at aniline hydrochloride concentrations of 3% to 15%) (Muñoz

and Barnett 1998). Ress *et al.* (2002) reported a weak positive response for micronucleus formation in mice given aniline at doses of 23 and 470 mg/kg b.w. (see Section 5.3.3).

5.2.3 Summary of aniline genotoxicity

Aniline has not been shown to cause point mutations in *in vitro* tests, but it has been shown to cause SCE in *in vitro* tests. Mixed results have been seen for *in vivo* tests, with both positive and negative results for micronucleus formation and DNA lesions in rats and mice.

5.3 DAAB

5.3.1 Prokaryotic systems

DAAB (0.1 to 100 μg/plate) was tested in S. *typhimurium* strains TA98, TA100, and TA1537 both with and without rat or hamster liver S9 enzymes for metabolic activation. The results were positive for mutagenicity in strains TA98, TA100, and TA1537 with rat or hamster S9 activation. The results were negative in all strains without metabolic activation and in strain TA1535 both with and without metabolic activation (Zeiger *et al.* 1987). [The fact that DAAB was mutagenic in three of four *S. typhimurium* strains tested, whereas benzene and aniline generally are not mutagenic in these assays (see Sections 5.1 and 5.2, above), indicates that the structure of the parent molecule or free radicals generated in the course of its metabolism may account for additional mechanism(s) of genotoxicity.]

5.3.2 Plants

In a report of the U.S. Environmental Protection Agency Gene-Tox Program, Grant (1982) summarized results for induction of chromosomal aberrations in the common onion (*Allium cepa*) for 148 chemicals. DAAB induced chromosomal aberrations at a concentration of 1,250 ppm.

5.3.3 Micronucleus assay

Ress *et al.* (2002) tested DAAB, benzene, aniline, and a mixture of benzene and aniline for induction of micronuclei in polychromatic erythrocytes (PCEs) in the bone marrow of male B6C3F_1 mice (Table 5-1 shows the dose levels and results). The basis for the doses chosen was that DAAB is metabolized essentially into 40% benzene, 47% aniline, and 13% nitrogen. Micronuclei were induced by DAAB, benzene, and the mixture of benzene and aniline. This study provided the first evidence of DAAB-induced chromosomal damage. It is important to note, however, that benzene exposure in humans has been linked to clastogenic events, as indicated by the evidence of increased structural and numerical chromosomal damage in lymphocytes (Zhang *et al.* 1999, Giver *et al.* 2001).

In the comparative study of DAAB, benzene, aniline, and a benzene-aniline mixture (Ress *et al.* 2002), significant increases in the frequency of micronucleated PCEs were reported for all groups of mice exposed to DAAB, benzene, or the mixture; however, DAAB was the strongest inducer of micronuclei at the higher doses. In the mice exposed to aniline, significant increases were noted in the 23-mg/kg (0.25-mmol/kg) and 470-mg/kg (5.05-mmol/kg) dose groups; the authors considered these results to represent a

weak positive response. No differences in micronucleus formation were observed between the mice administered DAAB at 25 mg/kg and those given a 1:1 molar mixure of benzene and aniline equivalent to 25 mg/kg of DAAB. However, in mice given DAAB at 50 or 100 mg/kg, the frequency of micronucleated PCEs was 1.4 to more than 2 times that observed in the groups receiving benzene or the benzene-aniline mixture (Table 5-1). [It is noted that for all exposure groups, the standard error was large relative to the mean.] The authors concluded that DAAB was a potent inducer of micronuclei in mice and that its activity likely was related to that of benzene (a primary metabolite of DAAB).

Table 5-1. Micronuclei induction in mice (5 per exposure group)

Chemical	Dose (mg/kg)	MN-PCE (±SEM) per 1000[a]	% PCE
DAAB	0	0.70 ± 0.25	57.0
	25	2.10 ± 0.75*	61.2
	50	5.00 ± 1.75**	60.4
	100	9.00 ± 2.45**	63.4
		$P < 0.001$[b]	
Benzene	0	0.70 ± 0.25	57.0
	10	2.40 ± 0.40	
	20	2.50 ± 1.65**	55.2
	40	6.30 ± 2.10**	63.9
		$P < 0.001$[b]	
Aniline	0	0.70 ± 0.25	57.0
	12	1.20 ± 0.90	
	23	2.60 ± 0.95*	68.9
	47	1.40 ± 1.05	59.7
	120	2.10 ± 0.75	64.6
	470	3.30 ± 2.95**	63.6
		$P = 0.001$[b]	
mixture (Benzene/Aniline)	0	0.70 ± 0.25	57.0
	10/12	2.00 ± 1.60	
	20/23	3.00 ± 1.25**	62.4
	40/47	3.80 ± 1.90**	65.5
		$P < 0.001$[b]	
Cyclophosphamide (positive control)	20	17.9 ± 2.58	57.3

Sources: NTP (2002a) and Ress *et al.* (2002).

*$P < 0.005$, **$P \leq 0.001$

[a]Significance of pairwise comparison of treated group to vehicle (corn oil) control. A single control group was used for all test chemicals.

[b]One-tailed Cochran Armitage trend test.

5.4 Summary

Few genotoxicity studies of DAAB are available. These studies indicate that DAAB causes gene mutation in *S. typhimurium* with metabolic activation, chromosomal aberrations in the common onion, and micronucleus formation in the bone marrow of mice. Benzene, a major metabolite of DAAB, causes DNA adduct formation in human cells, inhibits RNA synthesis in *in vitro* tests, and induces micronuclei, chromosomal aberrations, and DNA lesions in mice and rats, but generally has not caused point mutations in *in vitro* tests. In addition, studies of workers occupationally exposed to benzene have reported increases in chromosomal aberrations and SCE. Aniline, another major metabolite of DAAB, induces SCE, micronuclei, and DNA lesions in mice and rats, but does not cause point mutations or DNA damage *in vitro*. Recent toxicity studies indicate that DAAB has toxic properties similar to those of its two main metabolites. However, DAAB may be a more potent genotoxicant than either benzene or aniline. DAAB is mutagenic in bacteria and induces micronucleus formation to a greater extent at doses ≥ 50 mg/kg b.w. than do benzene or a mixture of benzene and aniline at molar equivalent doses.

6 Other Relevant Data

The NTP sponsored a number of studies to characterize the toxicity and fate of DAAB in laboratory animals (NTP 2002a). As discussed above, DAAB was selected for study based on its potential for worker exposure, its presence as an impurity in foods, drugs, and cosmetics, the fact that it caused gene mutation in *Salmonella typhimurium*, and the lack of adequate toxicological data. It also was selected because its chemical structure suggested that it would be metabolized to benzene and aniline. Benzene is a known human carcinogen (NTP 2002b, IARC 1982a), and aniline was a positive carcinogen in chronic studies with rats (NCI 1978, IARC 1987b, CIIT 1982).

The metabolism and disposition of DAAB were characterized following oral, dermal, or intravenous administration to male and female F344/N rats and male B6C3F_1 mice as well as in human liver slices. As a follow-up to observations made in the course of metabolism studies, electron spin resonance studies were conducted to assess the possible formation of the phenyl radical in the course of DAAB metabolism. Toxicity studies were limited to16-day dermal studies using male and female F344/N rats and B6C3F_1 mice. Significant findings from each of these studies are described below.

6.1 Absorption, distribution, metabolism, and excretion studies

6.1.1 Disposition studies

Studies of the fate of ^{14}C-labeled DAAB (randomly labeled on the phenyl rings) in rats demonstrated that the radioactivity was readily and nearly completely absorbed from the gastrointestinal tract but was absorbed from skin to a lesser extent (≤ 7%). Application of a much higher dose of dermally applied radiolabeled DAAB did not result in a proportionally higher mass of labeled material absorbed. The absolute amount absorbed was similar for the 2 mg/kg and 20 mg/kg doses. Following absorption via either route, DAAB was rapidly metabolized, and the radioactive metabolites were excreted primarily in urine (NTP 2002a, Mathews and De Costa 1999). Approximately 80% of the radioactivity of an i.v. dose was excreted in urine by male rats within the first 24 hours after administration (Table 6-1). [Comparison of excretion in urine following oral vs. i.v. administration indicates that approximately 90% of the total radioactivity was absorbed from the gastrointestinal tract of rats.] Gastrointestinal absorption was similar in mice and rats, and urine was the primary route of excretion in both species; however, radiolabeled metabolites were excreted less rapidly by mice. Some of the differences in the relative importance of urinary vs. fecal routes of excretion for mice vs. rats also may be attributed to the fact that, for mice held in metabolic chambers, their feces tend to be contaminated with urine. In addition, total recovery of the labeled dose generally was lower in mice. Exhalation as volatile organics and carbon dioxide in the breath of male rats and mice accounted for less than 2% of the dose administered by any route.

Table 6-1. Excretion of ^{14}C-labeled DAAB in male rats and male mice

Route of administration	Dose	Percent of administered dose (mean ± SD)			
		Urine	Feces	Breath	Total
Male F344/N rats – 24 hours					
Intravenous	2 mg/kg	80.1 ± 3.3	5.2 ± 0.7	0.60 ± 0.08	85.9 ± 3.9
Gavage	20 mg/kg	75.5 ± 2.0	13.7 ± 1.6	1.30 ± 0.21	90.6 ± 3.0
Dermal	2 mg/cm^2	1.44 ± 0.38	0.09 ± 0.02	0.13 ± 0.03	1.66 ± 0.42
Dermal	20 mg/cm^2	0.16 ± 0.01	0.01 ± 0.00	0.05 ± 0.01	0.23 ± 0.01
Male F344/N rats – 72 hours (includes cage wash)					
Intravenous	2 mg/kg	87.0 ± 1.8	7.7 ± 0.4	0.67 ± 0.10	95.3 ± 1.6
Gavage	20 mg/kg	80.3 ± 0.8	15.7 ± 0.8	1.36 ± 0.22	97.5 ± 0.3
Dermal	2 mg/cm^2	4.67 ± 1.19	0.41 ± 0.08	0.36 ± 0.09	5.44 ± 1.44
Dermal	20 mg/cm^2	0.44 ± 0.03	0.05 ± 0.01	0.08 ± 0.01	0.57 ± 0.02
Male B6C3F$_1$ mice – 24 hours					
Intravenous	2 mg/kg	27.2 ± 10.5	11.4 ± 3.5	0.74 ± 0.31	39.7 ± 10.9
Gavage	20 mg/kg	44.3 ± 21.8	15.2 ± 6.7	0.87 ± 0.20	60.6 ± 16.9
Dermal	2 mg/cm^2	0.59 ± 0.25	0.42 ± 0.06	0.19 ± 0.14	1.20 ± 0.32
Dermal	20 mg/cm^2	0.08 ± 0.01	0.07 ± 0.06	0.04 ± 0.01	0.20 ± 0.05
Male B6C3F$_1$ mice – 72 hours (includes cage wash)					
Intravenous	2 mg/kg	43.4 ± 6.5	22.9 ± 5.6	1.02 ± 0.36	69.4 ± 5.2
Gavage	20 mg/kg	60.9 ± 15.0	20.0 ± 10.8	1.03 ± 0.27	82.2 ± 10.5
Dermal	2 mg/cm^2	1.92 ± 1.07	2.40 ± 0.84	0.48 ± 0.26	4.80 ± 0.26
Dermal	20 mg/cm^2	0.26 ± 0.01	0.36 ± 0.07	0.09 ± 0.01	0.71 ± 0.08

Source: NTP 2002a (Tables A1, A2, A4, A11, A12, and A13).

As evident from the excretion data, DAAB was not retained in tissues. DAAB-derived radioactivity was detected at low levels (< 1% total dose) in the adipose tissue, blood, kidney, liver, muscle, skin, and spleen in male and female rats 24 hours after oral administration of 20 mg/kg b.w. (Table 6-2) (Mathews and De Costa 1999, NTP 2002a). [The highest concentrations of DAAB-derived radioactivity were observed in kidney, but that likely was due to the fact that the primary route of excretion was in urine.]

Table 6-2. Tissue distribution of radioactivity in F344/N male and female rats

Tissue[b]	Male rats[a]		Female rats[a]	
	DAAB equivalents in tissue (ng/g)	Dose in total tissue (%)	DAAB equivalents in tissue (ng/g)	Dose in total tissue (%)
Adipose	438 ± 113[c]	0.155 ± 0.041	803 ± 443	0.273 ± 0.150
Blood	1,380 ± 80	0.361 ± 0.016	1,370 ± 300	0.346 ± 0.075
Kidney	1,910 ± 150	0.067 ± 0.006	2,430 ± 440	0.084 ± 0.016
Liver	912 ± 30	0.184 ± 0.002	1,090 ± 140	0.182 ± 0.025
Muscle	123 ± 11	0.297 ± 0.030	137 ± 17	0.319 ± 0.039
Skin	229 ± 11	0.196 ± 0.007	253 ± 43	0.210 ± 0.034
Spleen	671 ± 25	0.007 ± 0.001	976 ± 184	0.012 ± 0.002

Source: NTP 2002a (Table A9).

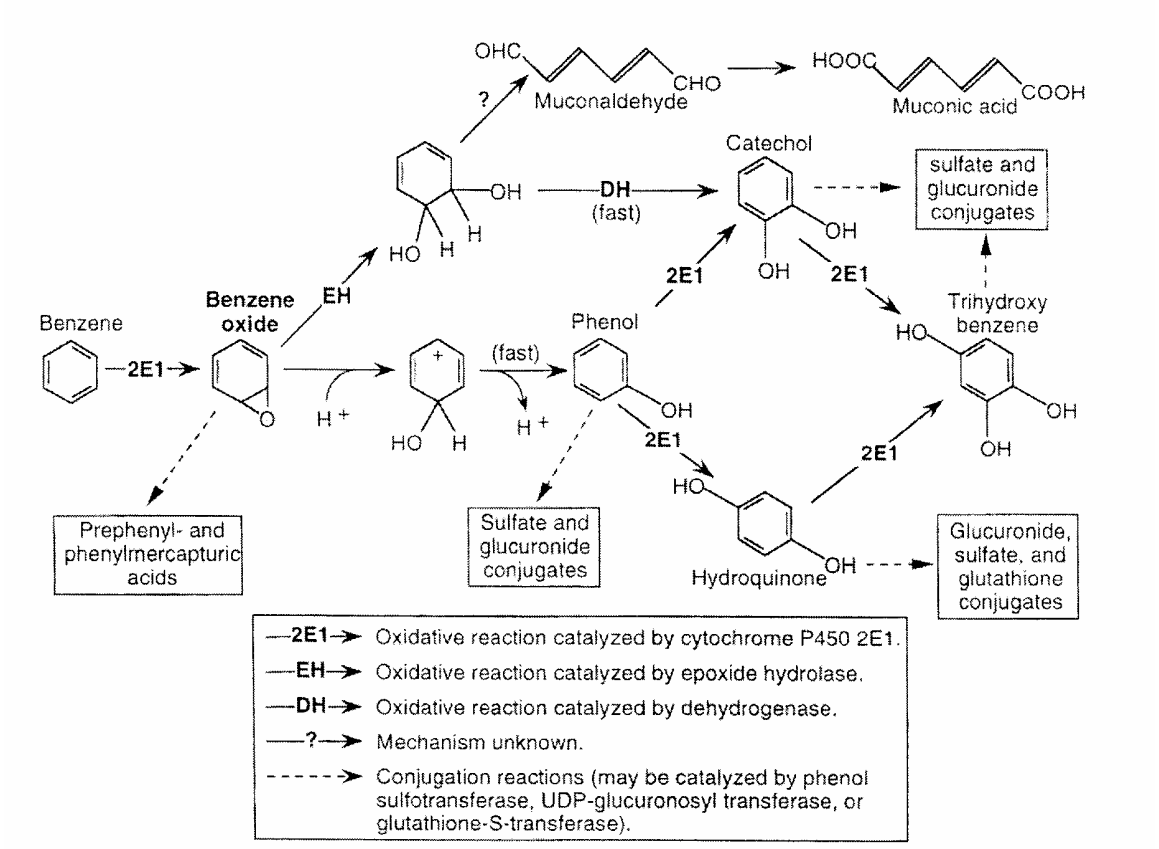
[a]Results obtained from three male and four female rats.

[b]Tissues were collected 24 hours after a single gavage of 20 mg/kg [^{14}C]DAAB.

[c]Mean ± SD.

6.1.2 Metabolism of benzene

Many of the urinary metabolites detected after administration of DAAB to rats and mice were known metabolites of benzene. The metabolism of benzene, reviewed recently by Lovern *et al.* (2001), is illustrated in Figure 6-1. Benzene initially is oxidized to benzene oxide, which may rearrange spontaneously to form phenol or may be converted enzymatically to a 1,2-dihydrodiol intermediate by epoxide hydrolase. Phenol may be oxidized by cytochrome P-450 2E1 to hydroquinone or catechol. Catechol also may be formed by dehydrogenation of the 1,2-dihydrodiol intermediate. The 1,2-dihydrodiol intermediate may give rise to muconic acid via the postulated intermediate muconaldehyde.



Source: Lovern *et al.* 2001.

Figure 6-1. Metabolism of benzene

The metabolism of benzene has been shown to be dose-dependent (Mathews *et al.* 1998). When benzene was administered at 100 mg/kg b.w., approximately 50% of the dose was exhaled unchanged, whereas at doses of 0.5 mg/kg or less, only about 2% was exhaled unchanged. In each case the remainder of the dose was excreted primarily in urine in the form of a number of metabolites. The major metabolites formed varied among rats, mice, and hamsters. Phenyl sulfate accounted for 64% to 73% of the dose excreted by rats. Phenyl sulfate (24% to 32%) and hydroquinone glucuronide (27% to 32%) were the major metabolites excreted by mice. Of the minor metabolites observed, mice formed more muconic acid than did rats (15% vs. 7%), and hamsters formed still more muconic acid (19% to 31%). The other major metabolite excreted by hamsters was hydroquinone glucuronide (24% to 29%). Minor metabolites observed in one or more species were prephenylmercapturic acid [the nonaromatic product of the oxirane ring opening of benzene oxide with thiol], phenylmercapturic acid, hydroquinone, phenol glucuronide, hydroquinone sulfate, and phenol. Trihydrobenzene and catechol sulfate were detected in hamsters only (Mathews *et al.* 1998).

6.1.3 Metabolism of aniline

When rats and mice were administered DAAB orally, the urine contained several metabolites also observed as metabolites of aniline. The urinary metabolites of aniline in F344 rats (sex not specified) and $B6C3F_1$ mice (sex not specified) were identified by HPLC 24 hours after intraperitoneal (i.p.) injection of ^{14}C-labeled aniline (McCarthy *et al.* 1985). The percentages of urinary metabolites for the lower dose administered to rats (50 mg/kg b.w.; 0.54 mmol/kg) are reported in Table 6-4, and those for the lower dose administered to mice (100 mg/kg b.w.; 1.07 mmol/kg) are reported in Table 6-5. Rats and mice also received doses of 250 mg/kg (2.68 mmol/kg) and 500 mg/kg (5.37 mmol/kg), respectively. A total of 8 metabolites were identified: aniline, *p*(4)-aminophenyl glucuronide, *p*(4)-aminophenyl sulfate, *o*(2)-aminophenyl sulfate, *p*(4)-acetamidophenol, *p*(4)-acetamidophenyl glucuronide, *p*(4)-acetamidophenyl sulfate, and *o*(2)-acetamidophenyl sulfate. HPLC chromatograms of urine from mice showed three unidentified peaks, representing 23.6% of the low dose and 25.7% of the high dose of radiolabeled aniline. Rat urine contained only one of the unknown metabolites, representing 0.5% of the total radioactivity.

Following oral administration of aniline to rats, sulfation was the major metabolic pathway for elimination, but the degree of sulfation varied with dose. *p*(4)-Acetamidophenyl sulfate accounted for > 70% of the urinary metabolites at a dose of 50 mg/kg, but only 30% at 250 mg/kg. Rats receiving the high dose excreted more *p*(4)-acetamidophenyl glucuronide and unconjugated metabolites (McCarthy *et al.* 1985).

6.1.4 Metabolism of DAAB

Benzene, aniline, and metabolites of benzene and aniline were detected in the blood of rats orally dosed with DAAB, and these metabolites were excreted in urine of dosed rats and mice (Mathews and De Costa 1999). The profile of DAAB metabolites observed in rats and mice was similar following dermal, oral, or intravenous administration. The metabolic conversion of DAAB to metabolites of benzene is consistent with the pathway illustrated in Figure 6-1.

6.1.4.1 Metabolites of DAAB in exhaled breath

Following oral administration of ^{14}C-labeled DAAB to rats and mice, benzene was the only radiolabeled product detected in exhaled breath, with 1.30% and 0.87% recovered 24 hours after gavage exposure in rats and mice, respectively (Mathews and De Costa 1999).

6.1.4.2 Common metabolites of DAAB, benzene, and aniline in blood

Within 15 minutes following oral administration, the predominant DAAB equivalents in blood of rats were known metabolites of benzene (i.e., phenol, hydroquinone glucuronide, muconic acid, prephenylmercapturic acid, phenol glucuronide, and phenyl sulfate) and aniline (i.e., 4-acetamidophenyl sulfate). Both benzene and aniline were detected at all time points, with peak concentrations at 1.0 and 0.5 hour, respectively. Of the benzene metabolites present, phenyl sulfate occurred at the highest concentration in both males and females. Muconic acid also was a major metabolite in both males and females, although in males, hydroquinone glucuronide was present at a higher

concentration than muconic acid. In both sexes, benzene was one of the four metabolites with the highest concentrations. Table 6-3 shows the metabolites observed at 6 hours after administration. No analysis was reported for metabolites in blood of mice.

Table 6-3. Metabolites of DAAB observed in blood of male and female rats following oral dosing

	Male rats[a,b]	Female rats[a,b]
Metabolites of benzene		
Benzene	51 ± 13	53 ± 13
Phenol	15 ± 15	2 ± 2
Hydroquinone glucuronide	89 ± 38	73 ± 15
Muconic acid	32 ± 12	102 ± 21
Prephenylmercapturic acid	22 ± 12	19 ± 3
Phenol glucuronide	14 ± 6	8 ± 4
Phenyl sulfate	210 ± 36	162 ± 40
Metabolites of aniline		
Aniline	27 ± 12	10 ± 7
4-Acetamidophenyl sulfate	366 ± 82	305 ± 13

Source: NTP 2002a (Table A10).

[a]Mean (± SD) blood concentration for rats in ng-Equivalents per g of blood 6 h after oral dosing.

[b]Metabolites of DAAB were identified in the blood of F344/N male and female rats after gavage administration of 20 mg/kg [^{14}C]DAAB.

Sabourin *et al.* (1989) profiled the metabolites of benzene in the blood of male F344 rats following oral exposure at doses of 1, 10, and 200 mg/kg b.w. The authors reported blood concentrations of two water-soluble benzene metabolites, which they described as representing a putative detoxification pathway (phenyl sulfate) and a putative toxification pathway (muconic acid). The highest concentration of each metabolite was present in blood collected at 0.5 hour after administration (the first time point), and both phenyl sulfate and muconic acid concentrations had decreased to baseline levels by 8 to 12 hours after administration. Area-under-the-curve analyses indicated that phenyl sulfate was the predominant metabolite, with smaller amounts of prephenylmercapturic acid and muconic acid.

The metabolites detected in the blood of rats exposed to 20 mg/kg of DAAB were qualitatively similar to those that resulted from oral exposure to benzene, with phenyl sulfate being the benzene-derived metabolite present in the highest concentration in blood of both male and female rats (Mathews and De Costa 1999). Muconic acid also was a major metabolite in both male and female rats exposed to DAAB, although in the blood of male rats, hydroquinone glucuronide was present at a higher concentration than was muconic acid. The blood of both male and female rats exposed to 20 mg/kg DAAB also contained benzene as one of the four metabolites with the highest concentration.

6.1.4.3 Common metabolites of DAAB, benzene, and aniline in urine

Urinary metabolites identified and quantified in the urine of rats and mice administered [^{14}C]benzene, [^{14}C]aniline, or [^{14}C]DAAB are listed in Tables 6-4 (rats) and 6-5 (mice). The pattern of metabolites excreted in urine indicated some evidence of species-specific differences in metabolism; however, DAAB metabolites common to both benzene and aniline were observed in the urine of both rats and mice. A quantitative comparison of these metabolites is difficult since recovery data were calculated in different ways, e.g., for rats Mathews *et al.* (1998) (benzene) and McCarthy *et al.* (1985) (aniline) reported results for metabolites as percentage of total urinary radioactivity recovered while Mathews and De Costa (1999) (DAAB) expressed results in terms of the percentage of the administered dose. The percentages for mice were based on total urinary radioactivity recovered for all three chemicals. The doses of chemicals also differed between mice and rats. In addition, the assay methods varied among the three studies; although HPLC was used in all three studies, the type of column and eluting reagents differed. It is not clear whether the differences in methodology contributed to differences in metabolite profiles among the three studies. [For these reasons, the data are best suited to qualitative comparisons.]

Mathews *et al.* (1998) administered benzene to rats at 0.02, 0.1, 0.5, 10 (results shown in Table 6-4), and 100 mg/kg b.w. and to mice at 0.1 (results shown in Table 6-5) and 100 mg/kg b.w. The profile of metabolites (rank order by amount recovered) was qualitatively similar across doses for rats; phenyl sulfate always represented the largest percentage of administered radioactivity, with prephenylmercapturic acid second or third in abundance. The results for mice varied more with the dose. At 0.1 mg/kg (see Table 6-5), hydroquinone glucuronide accounted for more radioactivity than did phenyl sulfate; however, at 100 mg/kg, the order was reversed, with 31.68% ± 0.46% phenyl sulfate and 26.49% ± 1.66% hydroquinone glucuronide.

McCarthy *et al.* (1985) administered aniline at doses of 50 mg/kg (shown in Table 6-4) and 250 mg/kg to rats and 100 mg/kg (shown in Table 6-5) and 500 mg/kg to mice. Percentages of urinary metabolites generally were similar for mice at both doses. The results for rats varied more with the dose; for example, comparing the results at the low and high doses, 4-acetamidophenyl sulfate decreased from 71.8% to 30.3%, and 4-acetamidophenyl glucuronide increased from 0.0% to 11.6%.

The results reported for DAAB by Mathews and De Costa (1999) were for a single dose level of 20 mg/kg in both rats and mice. Among the metabolites of DAAB, prephenylmercapturic acid, phenol glucuronide, and phenyl sulfate were observed only in the rat, and phenol was detected only in mouse urine. Rats excreted only a single metabolite of aniline, 4-acetamidophenyl sulfate, in urine, whereas mice excreted five aniline metabolites in urine (Mathews and De Costa 1999).

The metabolism studies showed that rats and mice metabolize DAAB almost exclusively to benzene, aniline, and their known metabolites. Based on the proposed mechanism of decomposition, DAAB is expected to yield approximately 40% benzene, 47% aniline, and 13% nitrogen. Within 24 hours after administration, the percentages of the oral dose of radiolabeled DAAB excreted in the urine as benzene metabolites were 30% in rats and

22% in mice; the percentages eliminated as aniline metabolites were 32% in rats and 35% in mice. In rats, the most prevalent urinary metabolite of both benzene and DAAB was phenyl sulfate, whereas in mice, it was hydroquinone glucuronide. Two metabolites of benzene, hydroquinone glucuronide and muconic acid, were common to both species. 4-Acetamidophenyl conjugates were the most common aniline-derived metabolites of both aniline and DAAB. In rats, the sulfate conjugate was the most common, whereas in mice, the glucuronide conjugate predominated.

Table 6-4. Urinary metabolites (0 to 24 hours) as percent of administered dose or of total urinary radioactivity in F344 rats administered benzene, aniline, or DAAB

Urinary metabolite	Metabolites of benzene[a]	Metabolites of aniline[b]	Metabolites of DAAB[c]
Phenyl sulfate	70.32 ± 0.49		15.0 ± 0.6
Prephenylmercapturic acid	11.24 ± 1.66		2.9 ± 0.4
Phenol	4.20 ± 0.16		
Hydroquinone glucuronide	3.77 ± 0.52		6.7 ± 0.3
Muconic acid	3.62 ± 1.28		2.4 ± 0.3
Phenol glucuronide	1.67 ± 0.20		2.3 ± 0.9
Phenylmercapturic acid	1.52 ± 0.23		
Hydroquinone	0.54 ± 0.07		
Hydroquinone sulfate	< 0.05		
4-Acetamidophenyl sulfate		71.8	32.3 ± 1.5
4-Aminophenyl sulfate		10.0	
2-Aminophenyl sulfate		9.4	
2-Acetamidophenyl sulfate		1.2	
4-Acetamidophenol		1.0	

[a]Mathews *et al.* (1998); 10.0 mg benzene/kg b.w. (0.13 mmol/kg) administered by gavage; mean ± SD (n = 4); percentage of urinary radioactivity appearing as each metabolite in the 0 to 24 hour pooled urine collection.

[b]McCarthy *et al.* (1985); 50 mg aniline/kg b.w. (0.54 mmol/kg) administered by i.p. injections; mean value (SD, and number of rats not reported); percentage of total radioactivity recovered in the 0 to 24 hour collection for each metabolite.

[c]Mathews and De Costa (1999); 20 mg DAAB/kg b.w. (0.10 mmol/kg) administered by gavage; mean ± SD (n = 4); percentage of the dose recovered in 0 to 24 hour urine collection for each metabolite.

Table 6-5. Urinary metabolites (0 to 24 hours) as percent of total urinary radioactivity in B6C3F$_1$ mice administered benzene, aniline, or DAAB

Urinary metabolite	Metabolites of benzene[a]	Metabolites of aniline[b]	Metabolites of DAAB[c]
Hydroquinone glucuronide	31.97 ± 1.31		13.0 ± 3.1
Phenyl sulfate	23.69 ± 0.61		
Muconic acid	15.32 ± 1.93		3.9 ± 0.9
Phenylmercapturic acid	3.71 ± 1.31		
Phenol glucuronide	3.08 ± 0.09		
Phenol	2.95 ± 0.29		4.7 ± 0.5
Hydroquinone sulfate	2.84 ± 0.34		
Prephenylmercapturic acid	2.03 ± 0.28		
Hydroquinone	1.40 ± 0.17		
4-Acetamidophenyl glucuronide		31.2	23.6 ± 2.3
2-Aminophenyl sulfate		16.5	1.5 ± 2.8
4-Acetamidophenol		9.0	0.8 ± 1.0
2-Acetamidophenyl sulfate		5.8	ND
4-Aminophenyl glucuronide		1.6	
4-Acetamidophenyl sulfate			4.5 ± 0.8
2-Acetamidophenol			5.0 ± 1.1

[a]Mathews *et al.* 1998; 0.1 mg benzene/kg b.w. (0.0013 mmol/kg) administered by gavage; mean ± SD (n = 4); percentage of urinary radioactivity appearing as each metabolite in the 0 to 24 hour pooled urine collection.

[b]McCarthy *et al.* 1985; 100 mg aniline/kg b.w. (1.07 mmol/kg) administered by i.p. injection; mean value (SD and number of mice not reported); percentage of total radioactivity recovered in the 0 to 24 hour collection for each metabolite.

[c]Mathews and De Costa 1999; 20 mg DAAB/kg b.w. (0.10 mmol/kg) administered by gavage; mean ± SD (n = 4); percentage of urinary radioactivity appearing as each metabolite in the 0 to 24 hour pooled urine collection

ND = not detected.

Sabourin *et al.* (1989) also profiled the metabolites of benzene in the urine of F344 rats following oral administration at 1, 10, and 200 mg/kg b.w. The profile of water-soluble benzene metabolites in 24-hour urine samples was similar to that found in blood. Phenyl sulfate constituted approximately 70% of the total metabolites, followed by either prephenylmercapturic acid or muconic acid, depending on the dose level.

6.1.4.4 Studies with human liver slices

A study of the metabolism of ^{14}C-labeled DAAB by human liver slices demonstrated that DAAB could be cleaved to yield metabolites of benzene and aniline (Mathews and De Costa 1999). After 5 hours of incubation, the medium still contained about 90% of the

radioactivity, but only 1% to 2% of that radioactivity represented metabolites of DAAB. Metabolites previously characterized in urine (4-acetamidophenyl sulfate, phenyl sulfate, aniline, and hydroquinone glucuronide) were confirmed in the media samples. In a similar study of benzene using liver slices and liver microsomal fractions from humans, rats, and mice, a similar spectrum of benzene metabolites was detected (Brodfuehrer *et al.* 1990).

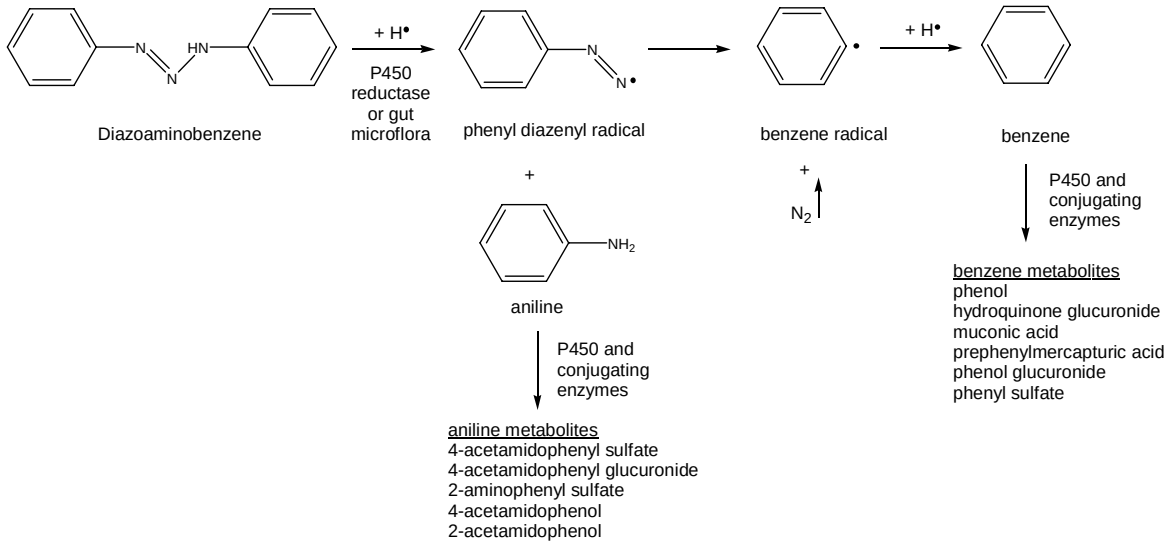
6.1.4.5 Proposed metabolic pathway for DAAB

As described above, the profile of DAAB metabolites observed in rats and mice was similar following dermal, oral, or intravenous administration. Metabolites detected in the blood and urine of rats or mice exposed to DAAB included those previously characterized for benzene (i.e., muconic acid, prephenylmercapturic acid, hydroquinone glucuronide, phenol glucuronide, phenyl sulfate, and phenol) and aniline (i.e., 2-aminophenyl sulfate, 4-acetamidophenyl glucuronide, 4-acetamidophenyl sulfate, 2-acetamidophenyl sulfate, 4-acetamidophenol, and 2-acetamidophenol).

Matthews and De Costa (1999) proposed that DAAB is cleaved reductively to form aniline and phenyl diazenyl radicals. The diazenyl radical fragments on further reaction ultimately yield nitrogen and benzene. The benzene and aniline are subsequently metabolized to form the metabolites detected in blood and urine of exposed animals (Figure 6-2). The metabolic conversion of DAAB to metabolites of benzene with benzene oxide as the initial metabolic step is consistent with the pathway illustrated in Figure 6-1. Metabolites of DAAB in blood of rats (Table 6-3), urine of rats (Table 6-4), and urine of mice (Table 6-5) included hydroquinone, muconic acid, and phenylmercapturic acid, which all share benzene oxide as a common intermediate in the pathway proposed by Lovern *et al.* (2001).

The involvement of cytochrome P-450 in the metabolism of DAAB or the further metabolism of benzene and aniline derived from DAAB was supported by data obtained from rats pretreated with 1-aminobenzotriazole (ABT) prior to oral administration of DAAB (Mathews and De Costa 1999). Urinary excretion of DAAB-derived radiolabel during the first 8 hours decreased from about 50% for non-ABT-treated rats to about 12% in ABT-treated rats. This change in metabolism was accompanied by an increase in the amount of unchanged benzene exhaled in the breath. Urinary excretion of the aniline metabolite 4-acetamidophenyl sulfate was delayed in rats pretreated with ABT; the majority of the metabolite was excreted in the 8-to-24-hour collection rather than in the first 8 hours following administration. The existence of transient free-radical metabolites of DAAB was confirmed in both *in vitro* and *in vivo* experiments using electron spin resonance (ESR) (see Section 6.1.4.6).

Because the micronucleus assays described in Section 5 were conducted with mouse bone marrow cells, metabolism of DAAB within the bone marrow could be a factor in the local effects of DAAB metabolites, including the phenyl radical. Although no studies on metabolism of DAAB by bone marrow were located, bone marrow cells do produce cytochrome P-450 (Heidel *et al.* 1998, Bernauer *et al.* 1999).



Source: Mathews and DeCosta 1999.

Figure 6-2. Proposed pathway for the metabolism of DAAB

6.1.4.6 Electron spin resonance studies

Because the metabolic pathway proposed for DAAB was thought to generate free radicals, a series of ESR studies were designed and conducted using the free radical trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (Kadiiska *et al.* 2000). *In vitro* studies using rat hepatic microsomes incubated with DAAB indicated that NADPH was required for the formation of the DMPO-phenyl adduct, indicative of free-radical formation. The phenyl radical also was formed in microsomes incubated with the mechanism-based P-450 inhibitor ABT and in the presence of carbon monoxide, indicating that interaction with the heme prosthetic group of P-450 is not required for initial cleavage of DAAB, although further metabolism of benzene and aniline do require P-450. Formation of the DMPO-phenyl adduct also was catalyzed by recombinant human NADPH P-450 reductase.

The possibility that metabolism of DAAB might be catalyzed by microbes in the gastrointestinal tract was investigated in an experiment that used anaerobic incubations of cecal contents isolated from rats (Kadiiska *et al.* 2000). Low levels of the DMPO-phenyl adduct were detected, suggesting that intestinal microbes could play a role in the metabolism of any DAAB that was not absorbed from the gastrointestinal tract before reaching the cecum.

Free-radical formation was demonstrated *in vivo* in studies in which intact bile duct-cannulated rats were administered DAAB via intragastric intubation and DMPO by i.p. injection. DMPO reacted with the phenyl radical, creating a more stable product that was collected in bile and characterized by ESR spectroscopy. The ESR spectrum was consistent with the formation of a DMPO-phenyl adduct, thus indicating that DAAB was absorbed into the systemic circulation and that the free radical was formed internally (Kadiiska *et al.* 2000).

ESR studies provide evidence that DAAB is reductively cleaved by hepatic enzymes to form phenyl radicals (Figure 6-2). *In vitro* detection of the phenyl radical required the full complement of microsomes or P-450 reductase, NADPH, DAAB, and DMPO (which was used to "trap" the phenyl radical). Addition of P-450 inhibitors, either carbon monoxide or ABT, did not alter the formation of the DMPO-phenyl adduct, indicating that the heme moiety of P-450 is not required for biotransformation. These results suggest that P-450 reductase, rather than the hemoprotein of P-450, catalyzes cleavage of the triazeno linkage, resulting in the formation of aniline and a phenyl diazenyl radical. The phenyl diazenyl radical fragments to form nitrogen and a phenyl radical, which in turn is reduced to benzene.

6.2 Short-term toxicity studies in rats and mice

The dermal route was selected for 16-day toxicity studies because it is the major route of exposure in occupational and nonoccupational circumstances (NTP 2002a). However, exposure to DAAB also can occur orally through ingestion of food and drugs. In the disposition study described above (NTP 2002a), the application site was protected from grooming, and dermal absorption accounted for a maximum of 7% of the dose when DAAB was applied at 2 mg/cm^2. In contrast, the application site in the 16-day toxicity studies was not protected, allowing for exposure to DAAB both dermally and orally through grooming by treated animals.

In these studies, groups of 5 male and 5 female F344/N rats and B6C3F$_1$ mice received dermal applications of 0, 12.5, 25, 50, 100, or 200 mg DAAB/kg b.w. in acetone, five days per week for 16 days. DAAB was not lethal to rats at any of the concentrations tested. In contrast, most male mice administered 50 mg/kg or more and 3 female mice administered 200 mg/kg died in the second week of the study. A decline in body weight gain was apparent in male and female rats and mice. Statistically significant dose-dependent decreases in absolute and relative thymus weights were observed in male and female rats and mice. Statistically significant increases in relative heart weights in male and female rats and mice also may have been DAAB-related. Relative spleen and liver weights were significantly increased in male and female rats, but not in mice, and relative kidney weights were significantly increased in male rats and male and female mice.

6.2.1 Hematologic effects of DAAB

Hematology data for male and female rats and mice administered DAAB in the NTP (2002a) study are summarized in Tables 6-6a,b (rats) and 6-7a,b (mice). In both sexes of both species, clinical pathology data indicated a DAAB-related methemoglobinemia. Heinz-body formation also was increased and was considered to be related to DAAB exposure. Further, there was a treatment-related decrease in erythroid mass, evidenced by a decrease in hematocrit, hemoglobin, and erythrocyte counts, suggesting a developing anemia. The erythron decrease was accompanied by an increased bone marrow response, as indicated by increased reticulocytes in male and female rats that received DAAB at doses of 50 mg/kg and higher and in all DAAB-exposed female mice. The frequency of nucleated erythrocytes was not increased in male mice, possibly because of poor survival at the three higher doses. In mice only, hemoglobin concentrations were increased in the higher-dose females. This latter response appears inappropriate, compared with other

estimates of red-cell mass, and may have been a spurious result related to the increased number of Heinz bodies. Associated with the anemia in mice was an increase in mean cell hemoglobin concentrations in both sexes, which would be consistent with intravascular hemolyses.

6.2.2 Dermal effects

Gross observations at necropsy revealed significant thickening of the skin at the site of application in all animals dermally exposed to DAAB. Microscopically, this corresponded to hyperplasia of the epidermis and hair follicles, which was evident in all dosed groups (Tables 6-8 to 6-11). Proliferation of hair follicles at the application site was a particularly prominent change of marked severity in the higher-dose groups. In some cases, this lesion was characterized by an extensive area of proliferation containing an increased density of hair follicles. This sometimes formed a raised, plaque-like lesion with a scalloped surface due to coalescence of dilated follicles containing multiple hair shafts. In other areas the interfollicular epidermis was thickened with variable cystic or hyaline-type degeneration in the stratum corneum. A slight infiltration of mixed inflammatory cells accompanied the hyperplastic change. Focal epidermal ulceration at the application site was present in some female mice in the higher-dose groups.

6.2.3 Nonneoplastic lesions

A number of internal nonneoplastic lesions were observed and considered to be related to chemical treatment (Tables 6-8 to 6-11). Lymphoid atrophy of the thymus (a depletion of cortical lymphocytes) was a common lesion; it increased from mild to moderate or marked severity with increasing dose in rats and mice of both sexes and was correlated with reduced thymus weight. A similar adverse effect of treatment on lymphoid tissue was indicated by a loss of mesenteric lymph tissue in rats and mice and mandibular lymph tissue in mice at the highest dose in both sexes. Presumably as a response to anemia, increased incidences of hematopoietic cell proliferation of generally mild severity occurred in the splenic red pulp of DAAB-exposed rats and mice and were correlated with increased spleen weight.

Table 6-6a. Hematology data for male rats dermally exposed to DAAB for 16 days (mean ± SE)

Parameter	Vehicle control	Dose (mg/kg b.w.)				
		12.5	25	50	100	200
n[a]	3	1[c]	1[c]	4	2	0
Hematocrit (%)	44.6 ± 1.4	42.3	43.6	41.3 ± 0.7*	41.5 ± 0.6	nd
Hemoglobin (g/dL)	15.4 ± 0.5	14.5	14.6	14.0 ± 0.2*	14.1 ± 0.1	nd
Erythrocytes (10^6/μL)	7.73 ± 0.23	7.51	7.77	7.26 ± 0.11	7.18 ± 0.11	nd
Reticulocytes (10^6/μL)	0.29 ± 0.04	0.35	0.40	0.42 ± 0.02*	0.55 ± 0.03**	nd
Nucleated erythrocytes/ 100 leukocytes	1.67 ± 0.67	1.00	1.00	3.75 ± 1.38	1.00 ± 0.00	nd
Mean cell volume (fL)	58.0 ± 0.0	56.0	56.0	57.3 ± 0.3	58.0 ± 0.0	nd
Mean cell hemoglobin (pg)	20.0 ± 0.0	19.3	18.8	19.4 ± 0.2*	19.7 ± 0.2	nd
Mean cell hemoglobin concentration (g/dL)	34.6 ± 0.1	34.3	33.5	34.0 ± 0.3	34.0 ± 0.3	nd
Methemoglobin (% hemoglobin)	0.85 ± 0.05[b]	1.40	2.10	3.28 ± 0.09	4.00 ± 0.90	nd
Heinz bodies (10^3/μL)	0	0	0	2 ± 2	8 ± 8	nd

Source: NTP 2002a (Table B7).

*$P \leq 0.05$, **$P \leq 0.01$, compared with the vehicle control group by Dunn's or Shirley's test, using unrounded data.

[a]Five blood samples were collected for analysis, but some were lost because of specimen clotting.

[b]n = 2.

[c]No standard error calculated.

nd = No data due to 100% mortality.

Table 6-6b. Hematology data for female rats dermally exposed to DAAB for 16 days (mean ± SE)

Parameter	Vehicle control	Dose (mg/kg b.w.)				
		12.5	25	50	100	200
n[a]	4	5	5	4	5	5
Hematocrit (%)	45.3 ± 1.7	44.4 ± 1.7	42.8 ± 1.1	40.7 ± 1.2*	39.7 ± 1.1**	39.2 ± 0.6**
Hemoglobin (g/dL)	15.6 ± 0.6	15.2 ± 0.5	14.5 ± 0.4	13.8 ± 0.3**	13.5 ± 0.3**	13.5 ± 0.2**
Erythrocytes (10^6/μL)	7.58 ± 0.32	7.69 ± 0.32	7.51 ± 0.20	7.14 ± 0.22	6.74 ± 0.14*	6.63 ± 0.11*
Reticulocytes (10^6/μL)	0.15 ± 0.02	0.27 ± 0.06	0.21 ± 0.02	0.27 ± 0.03*	0.33 ± 0.03**	0.32 ± 0.06**
Nucleated erythrocytes/100 leukocytes	0.25 ± 0.25	0.80 ± 0.37	0.80 ± 0.20	1.25 ± 0.48	3.00 ± 1.41*	6.40 ± 2.54*
Mean cell volume (fL)	60.0 ± 0.4	57.8 ± 0.4	56.8 ± 0.2**	57.3 ± 0.5*	58.8 ± 0.6	59.2 ± 0.7
Mean cell hemoglobin (pg)	20.6 ± 0.3	19.8 ± 0.2	19.4 ± 0.1*	19.4 ± 0.3*	20.0 ± 0.2	20.3 ± 0.2
Mean cell hemoglobin concentration (g/dL)	34.4 ± 0.3	34.4 ± 0.3	34.0 ± 0.2	34.0 ± 0.5	34.0 ± 0.2	34.4 ± 0.3
Methemoglobin (% hemoglobin)	0.65 ± 0.13	1.44 ± 0.11*	2.26 ± 0.16**	3.55 ± 0.36*	4.56 ± 0.28**	5.08 ± 0.69**
Heinz bodies (10^3/μL)	0	0	0	2 ± 2	3 ± 2	12 ± 4**

Source: NTP 2002a (Table B7).

*$P \leq 0.05$, **$P \leq 0.01$, compared with the vehicle control group by Dunn's or Shirley's test, using unrounded data.

[a]Five blood samples were collected for analysis, but some were lost because of specimen clotting.

Table 6-7a. Hematology data for male mice dermally exposed to DAAB for 16 days (mean ± SE)

Parameter	Vehicle control	Dose (mg/kg b.w.)				
		12.5	25	50	100	200
n[a]	5	4	5	1[b]	0	0
Hematocrit (%)	50.0 ± 1.6	47.0 ± 1.3	46.2 ± 0.6*	41.8	nd	nd
Hemoglobin (g/dL)	17.0 ± 0.6	16.3 ± 0.3	16.5 ± 0.3	16.7	nd	nd
Erythrocytes (10^6/μL)	10.58 ± 0.35	9.97 ± 0.26	9.80 ± 0.13	9.08	nd	nd
Reticulocytes (10^6/μL)	0.16 ± 0.03	0.13 ± 0.02	0.25 ± 0.03	0.22	nd	nd
Nucleated erythrocytes/100 leukocytes	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	nd	nd
Mean cell volume (fL)	47.2 ± 0.2	47.3 ± 0.3	47.0 ± 0.0	46.0	nd	nd
Mean cell hemoglobin (pg)	16.1 ± 0.1	16.4 ± 0.3	16.8 ± 0.2*	18.4	nd	nd
Mean cell hemoglobin concentration (g/dL)	34.0 ± 0.2	34.7 ± 0.7	35.7 ± 0.4*	40.0	nd	nd
Methemoglobin (% hemoglobin)	0.66 ± 0.05	2.45 ± 0.12*	5.92 ± 0.24**	10.00	nd	nd
Heinz bodies (10^3/μL)	11 ± 3	27 ± 10	20 ± 3	9	nd	nd

Source: NTP 2002a (Table B11).

*$P \leq 0.05$, compared with the vehicle control group by Shirley's test using unrounded data.

**$P \leq 0.01$, compared with the vehicle control group by Dunn's or Shirley's test using unrounded data.

[a]Five blood samples were collected for analysis, but some were lost because of specimen clotting.

[b]No standard error calculated.

nd = No data due to 100% mortality.

Table 6-7b. Hematology data for female mice dermally exposed to DAAB for 16 days (mean ± SE)

Parameter	Vehicle control	Dose (mg/kg b.w.)				
		12.5	25	50	100	200
n[a]	4	4	5	5	5	2
Hematocrit (%)	46.5 ± 1.5	44.0 ± 0.3	43.2 ± 0.5	39.6 ± 0.8**	42.9 ± 2.5	40.9 ± 0.3
Hemoglobin (g/dL)	16.5 ± 0.4	15.5 ± 0.1	15.5 ± 0.1	16.4 ± 0.3	18.5 ± 1.1	17.5 ± 0.6
Erythrocytes (10^6/μL)	9.65 ± 0.32	9.10 ± 0.09	8.77 ± 0.08	8.15 ± 0.13**	8.94 ± 0.52	8.35 ± 0.22
Reticulocytes (10^6/μL)	0.13 ± 0.02	0.20 ± 0.02*	0.35 ± 0.05**	0.41 ± 0.07**	0.41 ± 0.07**	0.39 ± 0.05*
Nucleated erythrocytes/100 leukocytes	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	48.5 ± 0.3	48.5 ± 0.3	49.4 ± 0.2	48.8 ± 0.5	48.0 ± 0.3	49.0 ± 2.0
Mean cell hemoglobin (pg)	17.1 ± 0.3	17.0 ± 0.1	17.7 ± 0.1	20.2 ± 0.3**	20.7 ± 0.1**	20.9 ± 0.1**
Mean cell hemoglobin concentration (g/dL)	35.5 ± 0.5	35.2 ± 0.3	36.0 ± 0.1	41.6 ± 0.7*	43.1 ± 0.2**	42.7 ± 1.6*
Methemoglobin (% hemoglobin)	1.05 ± 0.59	1.55 ± 0.13	4.68 ± 0.19**	10.92 ± 0.74**	15.46 ± 0.47**	19.00 ± 3.50**
Heinz bodies (10^3/μL)	5 ± 5	14 ± 3	23 ± 7	20 ± 2	39 ± 15	17 ± 17

Source: NTP 2002a (Table B11).

*$P \leq 0.05$, compared with the vehicle control group by Shirley's test using unrounded data.

**$P \leq 0.01$, compared with the vehicle control group by Dunn's or Shirley's test using unrounded data.

[a]Five blood samples were collected for analysis, but some were lost because of specimen clotting.

Table 6-8. Incidences of selected nonneoplastic lesions in male rats dermally exposed to DAAB for 16 days

	Dermal dose (mg/kg)					
Tissue[a]	Vehicle	12.5	25	50	100	200
Skin, application site						
Hyperplasia	0[b]	5** (1.6)[c]	5** (2.0)	5** (2.0)	5** (2.0)	5** (2.2)
Inflammation	0	5** (1.6)	5** (1.8)	3** (1.3)	5** (1.4)	5** (1.0)
Hair follicle hyperplasia	0	4* (1.3)	5** (2.8)	5** (3.2)	5** (3.6)	5** (4.0)
Epidermis, degeneration	0	0	5** (2.8)	5** (3.0)	5** (2.2)	5** (2.8)
Thymus						
Atrophy	0	5** (1.0)	5** (2.6)	5** (2.4)	5** (3.0)	5** (3.0)
Lymph node, mesenteric						
Atrophy	0	2[d] (1.5)	2 (2.0)	2 (2.0)	3 (1.0)	5** (2.2)
Spleen						
Hematopoietic cell proliferation	5 (1.2)	5 (1.0)	5 (1.2)	5 (1.6)	5 (2.0)	5 (2.0)

Source: 2002a (Table B8).

*$P \leq 0.05$, **$P \leq 0.01$, compared with the vehicle control group by the Fisher exact test.

[a]5 animals examined microscopically, unless otherwise noted.

[b]Number of rats with lesion.

[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

[d]4 animals examined microscopically.

Table 6-9. Incidences of selected nonneoplastic lesions in female rats dermally exposed to DAAB for 16 days

	Dermal dose (mg/kg)					
Tissue[a]	Vehicle	12.5	25	50	100	200
Skin, application site						
Hyperplasia	0[b]	5** (1.8)[c]	5** (2.0)	5** (2.2)	5** (2.0)	5** (2.0)
Inflammation	0	5** (1.8)	5** (1.0)	5** (1.0)	5** (1.0)	5** (1.0)
Hair follicle hyperplasia	0	5** (2.2)	5** (3.0)	5** (3.0)	5** (2.6)	5** (3.0)
Epidermis, degeneration	0	2 (2.0)	3 (2.3)	5** (3.2)	5** (3.0)	5** (2.4)
Thymus						
Atrophy	0	4* (1.0)	5** (1.2)	5** (2.6)	5** (2.8)	5** (3.0)
Lymph node, mesenteric						
Atrophy	0	0	0	0	0	4* (1.0)
Spleen						
Hematopoietic cell proliferation	0	4* (1.0)	5** (1.0)	5** (1.2)	5** (2.0)	5** (1.8)

Source: NTP 2002a (Table B8).

*$P \leq 0.05$, **$P \leq 0.01$, compared with from the vehicle control group by the Fisher exact test.

[a]5 animals examined microscopically.

[b]Number of rats with lesion.

[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Table 6-10. Incidences of selected nonneoplastic lesions in male mice dermally exposed to DAAB for 16 days

	Dermal dose (mg/kg)					
Tissue[a]	Vehicle	12.5	25	50	100	200
Skin, application site						
Epidermis, hyperplasia	0[b]	5** (2.0)[c]	5** (2.0)	5** (1.8)	5** (1.4)	5** (1.0)
Hair follicle, hyperplasia	0	5** (1.2)	5** (1.6)	5** (1.0)	5** (1.0)	2 (2.0)
Inflammation	0	5** (1.6)	5** (1.8)	5** (1.2)	5** (1.4)	5** (1.4)
Thymus						
Atrophy	0	0	0	5** (3.8)	5** (4.0)	5** (4.0)
Lymph node, mandibular						
Atrophy	0	0	0	3 (2.0)	4**[d] (2.8)	5** (2.8)
Lymph node, mesenteric						
Atrophy	0[d]	0	0[d]	2 (2.0)	3 (2.3)	4*[d] (2.5)
Spleen						
Hematopoietic cell proliferation	0	5** (2.0)	5** (2.0)	2 (1.5)	1 (2.0)	0
Lymphoid follicle, atrophy	0	0	0	1 (2.0)	2 (2.5)	5** (2.4)
Heart						
Atrium, thrombosis	0	0	0	2 (2.0)	5** (3.2)	5** (3.0)
Kidney						
Bilateral, cortex, renal tubule, necrosis	0	0	0	0	5** (2.0)	5** (3.0)
Liver						
Necrosis, focal	0	0	0	0	3 (1.3)	5** (2.6)

Source: NTP 2002a (Table B12).

*$P \leq 0.05$, **$P \leq 0.01$, compared with the vehicle group by the Fisher exact test.

[a]5 animals examined microscopically, unless otherwise noted.

[b]Number of rats with lesion.

[c]Average severity of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

[d]4 animals examined microscopically.

Table 6-11. Incidences of selected nonneoplastic lesions in female mice dermally exposed to DAAB for 16 days

	Dermal dose (mg/kg)					
Tissue[a]	Vehicle	12.5	25	50	100	200
Skin, application site						
Epidermis, hyperplasia	0[b]	5** (1.2)[c]	5** (3.0)	5** (2.6)	5** (2.4)	5** (2.0)
Hair follicle, hyperplasia	0	5** (1.6)	5** (3.2)	5** (3.0)	5** (3.0)	5** (3.0)
Inflammation	0	5** (2.0)	5** (1.2)	5** (2.4)	5** (2.2)	5** (2.2)
Ulcer	0	0	1 (1.0)	2 (1.5)	4* (1.3).	2 (1.0)
Thymus						
Atrophy	0[d]	0	0	4* (3.3)	5** (3.6)	3*[d] (4.0)
Lymph node, mandibular						
Atrophy	0	0	0	0	0[d]	3*[d] (2.0)
Lymph node, mesenteric						
Atrophy	0	0	0	0	1[e] (2.0)	1[d] (3.0)
Spleen						
Hematopoietic cell prolif.	0	5** (2.0)	5** (2.0)	5** (1.4)	2 (2.5)	2 (1.5)
Lymphoid follicle, atrophy	0	0	0	0	3** (2.7)	2 (2.5)
Heart						
Atrium, thrombosis	0	0	0	0	1 (1.0)	2 (2.5)
Kidney						
Bilateral, cortex, renal tubule, necrosis	0	0	0	0	4* (2.5)	1 (3.0)
Bilateral, cortex, renal tubule, dilation	0	0	0	0	0	4* (1.5)
Liver						
Necrosis, focal	0	0	0	0	0	2 (2.0)

Source: NTP 2002a (Table B12).

*$P \leq 0.05$, **$P \leq 0.01$, compared with the vehicle group by the Fisher exact test.

[a]5 animals examined microscopically, unless otherwise noted.

[b]Number of rats with lesion.

[c]Average severity of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

[d]4 animals examined microscopically.

[e]3 animals examined microscopically.

Several other microscopic findings in mice were considered related to DAAB exposure, many occurring in animals that died early. Atrial thrombosis of the heart was present, seen as a solid coagulum of proteinaceous material and embedded blood cells in the left auricle in all mice that died early. No myocardial changes were evident in either thrombotic hearts of early-death animals or in survivors with increased heart weights. Renal tubule necrosis was observed in early-death male mice and in female mice in the 100-mg/kg group that survived to study termination. Focal liver necrosis was observed in most early-death mice.

6.2.4 Summary of short-term toxicity studies

Results of these short-term toxicity studies indicate that DAAB is toxic to rats and mice of both sexes at the site of application and systemically. [Based on the small number of animals tested (n = 5), mice are more sensitive to the lethal effects of DAAB than rats, and male mice are more sensitive than females.] Systemic toxicity was most profound in the hematopoietic system of both species.

6.3 Potential mechanisms of toxicity and carcinogenicity of DAAB

Although animals were exposed to DAAB for only 16 days in short-term toxicity studies, the results suggest that DAAB has toxic effects characteristic of benzene and aniline; however, most studies with benzene and aniline have used longer exposures. The carcinogenicity of benzene and aniline are described briefly, below, together with possible mechanisms of toxicity and genotoxicity that may be relevant to the discussion of DAAB. As discussed in Section 4.1, Kirby (1947, 1948) reported on dermal exposure studies with DAAB in which mice developed a number of tumors, including squamous papillomas, squamous carcinomas, pulmonary adenomas, and a pulmonary adenocarcinoma.

Like DAAB, benzene and aniline are rapidly absorbed and metabolized and are excreted primarily in urine (McCarthy *et al.* 1985, Mathews *et al.* 1998). As observed in studies of DAAB, benzene was detected unchanged in the breath of rats and mice following administration of benzene by gavage (Mathews *et al.* 1998). The metabolism and excretion of benzene also are dose dependent (Mathews *et al.* 1998). The pathway of benzene metabolism that leads to the formation of muconic acid and hydroquinone is favored at low doses (0.1 mg/kg), whereas at higher doses, the predominant pathways lead to less toxic metabolites. [The dose of DAAB that would be equivalent on a molar basis to a benzene dose of 0.1 mg/kg would be 0.25 mg/kg, which is substantially lower than the dose levels reported above for studies of dermal exposure to DAAB (see Tables 6-6 to 6-10).] The dose- and species-dependent variations in benzene metabolism observed with DAAB previously were reported for benzene (Medinsky *et al.* 1989, Sabourin *et al.* 1989, Mathews and De Costa 1999).

[Analysis of DAAB toxicity is complicated by the fact that it is metabolized to two toxic molecules and also may induce symptoms of toxicity unique to the parent molecule. Analysis also is complicated by the fact that it is difficult to determine the exact internal dose animals received. That is, whereas it is likely that animals exposed dermally received much of the internal dose as a result of grooming, it is impossible to determine

the exact dose received or whether all animals receiving a similar dermally applied dose groomed an equal amount. In any case, conclusions still can be drawn from these short-term studies. As observed in previous studies of benzene, the erythrocyte and lymphoid systems were major targets of DAAB toxicity (IARC 1982a, ATSDR 1997 and references therein). Induction of lymphoid atrophy of the thymus and other lymphoid tissues characteristic of benzene toxicity was obvious in the 16-day study of DAAB toxicity. Symptoms of aniline exposure seen in the study of DAAB include methemoglobin formation, accompanying anemia, increased spleen weights, and regenerative hematopoiesis (CIIT 1982, Short *et al.* 1983, Khan *et al.* 1997).]

[In addition to toxicity induced by benzene and aniline, there is some evidence of toxicity unique to the parent molecule. DAAB apparently was more toxic at the application site than has been reported for equimolar doses of benzene or aniline. Further, though it is difficult to estimate the dose received internally, it appears that even when administered dermally, DAAB may be more toxic than equimolar doses of either benzene or aniline (IARC 1982a, ATSDR 1997 and references therein). The mechanism that accounts for the greater acute toxicity of DAAB has not been determined, but it may be attributable to properties of the parent molecule or to free radicals formed in its metabolism. DAAB induced a greater number of micronuclei than did a combination of equimolar doses of benzene and aniline (see Section 5.3.3, Table 5-1, and Section 6.3.3).]

6.3.1 Carcinogenicity of benzene and aniline

The carcinogenicity of DAAB has not been determined. However, the carcinogenicity of its two metabolites, benzene and aniline, has been evaluated, as reviewed in Section 4.2.

Benzene is a multi-site, multi-species carcinogen that has been extensively reviewed (IARC 1982a, WHO 1993, ATSDR 1997). Chronic inhalation exposure to benzene in rodents has been associated with lymphoma, leukemia, and neoplasia of the Zymbal gland, liver, mammary gland, and nasal cavity. Administration of benzene via oral gavage is associated with neoplasia of the Zymbal gland, oronasal cavity, mammary gland, liver, forestomach, skin, Harderian gland, preputial gland, ovary, and hematopoietic and lymphoreticular systems (NTP 1986). Benzene is listed in the 1st Report on Carcinogens (1980) as *a "known human carcinogen."* Benzene also is listed by IARC (1987a) as Group 1, based on sufficient evidence of carcinogenicity in humans based on numerous case reports and series that "suggested a relationship between exposure to benzene and the occurrence of various types of leukemia."

Aniline-induced carcinogenesis in spleens of rats has been observed in two separate chronic bioassays (NCI 1978, CIIT 1982). In the NCI study, the carcinogenic response in the spleen was specific to the rat and was not observed in $B6C3F_1$ mice exposed concurrently. IARC (1987a) listed aniline as not classifiable as to its carcinogenicity to humans (Group 3) based on inadequate evidence of carcinogenicity in humans and limited evidence of carcinogenicity in experimental animals. The NTP Report on the Metabolism, Toxicity, and Predicted Carcinogenicity of Diazoaminobenzene (2002) concluded that a daily dose of DAAB of 50 mg/kg b.w. given for two years to rats or mice would be equivalent to approximately 25 mg/kg each of benzene and aniline. As

noted in Table 4-1, the cancer effect level for benzene has been reported to be carcinogenic in rats and mice at doses of 25 mg/kg or lower.

6.3.2 Mechanisms of toxicity for benzene and aniline

The toxic effects of DAAB may be related to the toxicity of its metabolites, benzene and aniline. Numerous reports of short-term toxicity induced by benzene describe symptoms of myelotoxicity similar to those observed in the NTP study of DAAB (IARC 1982a, ATSDR 1997 and references therein). Further, as observed in the NTP study of DAAB, rats are more resistant to the toxicity of benzene than are mice. Also, as observed in the 16-week studies of DAAB, aniline is well known for its ability to induce methemoglobinemia (EPA 2000).

Ross (2000) reviewed the literature to address the question of which benzene metabolites are responsible for benzene toxicity and concluded that three different pathways may be responsible. These three pathways involve benzene oxide, the primary oxidation product of benzene; ring-opened reactive metabolites, such as *trans*,*trans*-muconaldehyde; and reactive polyphenols and quinones, such as hydroquinone. Although, as described in Section 6.1.4.2, the metabolite profiles in the blood of rats following oral exposure to DAAB and benzene were qualitatively similar, the DAAB-exposed rats had relatively greater amounts of hydroquinone glucuronide, a major metabolite in one of the putative pathways for benzene toxicity.

Lovern *et al.* (2001) summarized several potential metabolic pathways for benzene toxicity. The ultimate hematotoxin may be 1,4-benzoquinone formed from hydroquinone. 1,4-Benzoquinone is a genotoxin that is a direct alkylating agent, forms DNA adducts, and induces DNA strand breaks. Benzene oxide, the first metabolic product of benzene, also is capable of binding to protein. In addition, the postulated intermediate between 1,2-dihydrodiol and muconic acid, muconaldehyde, can induce DNA-protein crosslinks. Another metabolite of benzene, catechol (or 1,2-dihydroxybenzene), can be oxidized to 1,2-catechol quinone, which can form depurinating DNA adducts (Cavalieri *et al.* 2002). Golding and Watson (1999) concluded that the significance of DNA adduct formation in benzene-induced human leukemogenesis was unknown and suggested that other reactions of benzene metabolites with enzymes such as topoisomerase II also could play a role in the toxicity of benzene. Goldstein and Witz (2000) also reviewed the evidence for pathways of toxicity of benzene and concluded that “the effect of benzene is likely to be exerted through the action of multiple metabolites on multiple endpoints through multiple biological pathways.”

IARC (1982b) concluded that *N*-hydroxylation of aniline is probably related to the methemoglobinemia caused by aniline in humans. In rats and mice administered aniline, the predominant urinary metabolites were, respectively, 4-acetamidophenyl sulfate and 4-acetamidophenyl glucuronide (McCarthy *et al.* 1985). Following exposure to DAAB, these were the primary aniline metabolites detected in urine of mice, and 4-acetamidophenyl sulfate was the only aniline metabolite observed in the urine of rats. Mice also excreted three additional metabolites associated with aniline (Table 6-5).

6.3.3 Genotoxicity of DAAB, benzene, and aniline

The vast majority of organic chemicals identified as human carcinogens induce mutations in *S. typhimurium* and micronuclei and chromosomal aberrations in rodent bone marrow (Shelby 1988, Shelby and Zeiger 1990). Ress *et al*. (2002) demonstrated that DAAB, benzene, and aniline induce micronuclei in mice (see Section 5.3.3). The authors also noted that DAAB induced more micronuclei in mice than did equimolar doses of benzene or a mixture of benzene and aniline, an effect that they suggested could be due to formation of the phenyl radical during metabolism of DAAB. Aniline was considered a weak inducer of micronuclei. Benzene induced micronuclei at doses ranging from 10 to 40 mg/kg b.w., similar to the lowest doses associated with carcinogenicity (25 to 50 mg/kg b.w., as reported in Section 4.2). Similarly, aniline induced micronuclei at 23 and 470 mg/kg b.w. and tumors at 30 to 200 mg/kg b.w. Therefore, as concluded by Ress *et al.* (2002), the genotoxicity of DAAB in mouse bone marrow and its similarity to that of benzene could support a prediction of carcinogenicity for DAAB.

6.4 Summary

DAAB was almost completely absorbed from the gastrointestinal tract but sparingly absorbed from skin. For each route of administration, the absorbed portion of the dose was rapidly metabolized and excreted primarily in urine. Within 24 hours, approximately 60% of an oral dose of radiolabeled DAAB was accounted for as metabolites of benzene or aniline in the urine of rats and mice, and total recoveries of metabolites were quantitatively similar to the predicted values of 40% benzene and benzene metabolites and 47% aniline and aniline metabolites. The studies described above demonstrated that in rats and mice, DAAB is quantitatively metabolized to benzene, a known human and animal carcinogen, and to the rat carcinogen, aniline. In addition, i*n vitro* studies in human liver samples also demonstrated that DAAB could be cleaved to yield metabolites of benzene and aniline. Symptoms observed in animals administered DAAB are similar to those that would be anticipated in animals exposed to a combination of benzene and aniline. Skin painting studies with DAAB in mice resulted in several types of tumors, including squamous papilloma, squamous carcinoma, pulmonary adenoma, and pulmonary adenocarcinoma. DAAB also was observed to induce micronuclei in mouse bone marrow at molar doses similar to micronucleus-inducing doses of benzene and aniline. DAAB metabolism also results in the formation of a reactive phenyl radical, which could account for an additional risk of toxicity and/or carcinogenicity. Evidence for the possible involvement of the phenyl radical in DAAB mutagenicity is seen in the fact that DAAB causes mutations in *S. typhimurium* with metabolic activation, whereas benzene and aniline do not.

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Appendix A: NTP TR 73 (2002). NTP Report on the Metabolism, Toxicity, and Predicted Carcinogenicity of Diazoaminobenzene (CAS No 136-35-6). pp 1 - B24.

National Toxicology Program
Toxicity Report Series
Number 73

NTP Report on the Metabolism, Toxicity, and Predicted Carcinogenicity of

Diazoaminobenzene

(CAS No. 136-35-6)

September 2002

U.S. Department of Health and Human Services
Public Health Service
National Institutes of Health

FOREWORD

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control and Prevention. In July 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. The NTP coordinates the relevant programs, staff, and resources from these Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

The NTP develops, evaluates, and disseminates scientific information about potentially toxic and hazardous chemicals. This knowledge is used for protecting the health of the American people and for the primary prevention of disease.

The studies described in this Toxicity Study Report were performed under the direction of the NIEHS and were conducted in compliance with NTP laboratory health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals.

These studies are designed and conducted to characterize and evaluate the toxicologic potential of selected chemicals in laboratory animals (usually two species, rats and mice). Chemicals selected for NTP toxicology studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. The interpretive conclusions presented in this Toxicity Study Report are based only on the results of these NTP studies. Extrapolation of these results to other species and quantitative risk analyses for humans require wider analyses beyond the purview of these studies. Selection *per se* is not an indicator of a chemical's toxic potential.

Details about ongoing and completed NTP studies are available at the NTP's World Wide Web site: http://ntp-server.niehs.nih.gov. Abstracts of all NTP Toxicity Study Reports and full versions of the most recent reports and other publications are available from the NIEHS' Environmental Health Perspectives (EHP) http://ehp.niehs.nih.gov (800-315-3010 or 919-541-3841). In addition, printed copies of these reports are available from EHP as supplies last. A listing of all the NTP Toxicity Study Reports printed since 1991 appears on the inside back cover.

National Toxicology Program
Toxicity Report Series
Number 73

NTP Report
on the Metabolism, Toxicity,
and Predicted Carcinogenicity of

Diazoaminobenzene

(CAS No. 136-35-6)

Nancy B. Ress, Ph.D., Study Scientist

September 2002

NIH Publication No. 02-4412

U.S. Department of Health and Human Services
Public Health Service
National Institutes of Health

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CONTENTS

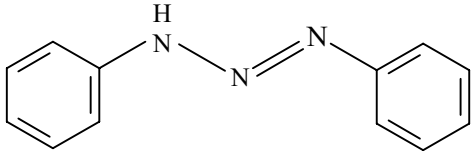
SUMMARY

Background: Diazoaminobenzene is used as a laboratory reagent and occurs as an impurity in cosmetics, food products, and pharmaceuticals. The structure of the chemical is a combination of benzene and aniline, both of which are known to cause cancer. We performed tests to determine if diazoaminobenzene might pose a similar hazard.

Methods: We exposed male and female rats and male mice to single doses of diazoaminobenzene (applied on the skin, injected into the blood, or inserted directly into the stomach through a tube) to determine if the chemical breaks down into benzene or aniline in the body. We also applied diazoaminobenzene to the skin of male and female rats and mice for 16 days to determine its pattern of toxicity.

Results: We found benzene, aniline, and their breakdown products (metabolites) in the blood of rats within 15 minutes after dosing with diazoaminobenzene. Benzene was detected in the breath of rats and mice, and all the metabolites in the urine were the same as those known to result from benzene and aniline in rats and mice. In the 16-day study, some toxic effects associated with aniline (methemoglobinemia) and with benzene (atrophy of the lymphoid tissue) occurred in rodents administered diazoaminobenzene.

Conclusions: Diazoaminobenzene is converted to the known carcinogens aniline and benzene and produces similar toxic effects as those two chemicals. Based on these results, we predict that diazoaminobenzene is also a carcinogen.



DIAZOAMINOBENZENE

CAS No. 136-35-6

Chemical Formula: $C_{12}H_{11}N_3$ Molecular Weight: 197.24

Synonyms: Anilinoazobenzene; benzeneazoanilide; benzeneazoaniline; DAAB; alpha-diazoamidobenzol; p-diazoaminobenzene; 1,3-diphenyltriazene; 1,3-diphenyl-1-triazene; DPT; N-(phenylazo)aniline
Trade names: Cellofor; Porofor DB

ABSTRACT

Diazoaminobenzene is used as an intermediate, complexing agent, and polymer additive. It is also an impurity in certain color additives used in cosmetics, food products, and pharmaceuticals. Diazoaminobenzene was selected for metabolism and toxicity studies based on the potential for worker exposure from its use in laboratories, positive *Salmonella typhimurium* gene mutation data, its presence as an impurity in foods and cosmetics, and the lack of adequate toxicity data. Several structural analogues and presumed metabolites of diazoaminobenzene are carcinogenic, providing evidence for the possible carcinogenicity of diazoaminobenzene. The chemical structure of diazoaminobenzene suggested that it would be metabolized into aniline and benzene; therefore, metabolism and disposition studies were performed in male and female F344/N rats and male B6C3F$_1$ mice administered a single oral, dermal, or intravenous dose of diazoaminobenzene. Electron spin resonance (ESR) studies were conducted to assess the possible formation of a phenyl radical from the reduction of diazoaminobenzene by components of the cytochrome P450 mixed-function oxidase (P450) system in microsomes or by gut microflora in anaerobic cecal incubations. Bile duct-cannulated male F344/N rats were administered diazoaminobenzene and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) for *in vivo* determination of the DMPO-phenyl radical. 16-Day toxicity studies were performed to identify target organs of diazoaminobenzene following dermal application to male and female F344/N rats and B6C3F$_1$ mice.

In the disposition and metabolism studies, oral doses of 20 mg/kg to male and female rats and male mice were readily absorbed and excreted mainly in the urine, with exhalation of volatile organics accounting for about 1% of the dose. The only volatile metabolite detected in the breath was benzene, and all the metabolites in the urine were those previously shown to result from the metabolism of benzene and aniline in rats and mice. While dermal doses to rats

and mice (2 and 20 mg/cm^2) were only slightly absorbed, benzene and aniline metabolites were nonetheless detected in the urine. High circulating levels of benzene, aniline, and their metabolites were detected in the blood of rats administered 20 mg/kg diazoaminobenzene as early as 15 minutes after exposure. At 24 hours after dosing, diazoaminobenzene was detected at low levels (<1%) in the adipose tissue, blood, kidney, liver, muscle, skin, and spleen. Metabolites of benzene and aniline were also formed in an *in vitro* study using human liver slices.

In the ESR spin-trapping experiments, the ESR spectrum of the DMPO-phenyl radical was detected when diazoaminobenzene was incubated with microsomes or P450 reductase, DMPO, and NADPH, or when incubated with cecal contents and DMPO. The DMPO-phenyl radical spectrum was not attenuated by the P450 inhibitor, 1-aminobenzotriazole, or carbon monoxide suggesting that P450s were not required. In *in vivo* experiments in which rats were administered diazoaminobenzene and DMPO, the DMPO-phenyl radical adduct was detected in bile within 1 hour after treatment.

In the 16-day toxicity studies, groups of five male and five female F344/N rats and B6C3F$_1$ mice received dermal applications of 0, 12.5, 25, 50, 100, or 200 mg diazoaminobenzene/kg body weight. Animals were evaluated for absolute and relative organ weights, for hematological effects, and for gross and microscopic lesions. No mortality occurred in rats. However, most male mice exposed to concentrations of 50 mg/kg or greater and female mice exposed to 200 mg/kg died. Body weights of male and female rats and female mice were less than those of the vehicle controls. Similar chemical-related toxicities were observed in both species. Clinical pathology data indicated a chemical-related methemoglobinemia and Heinz body formation in male and female rats and mice. Analysis of organ weights indicated possible chemical-related effects in the thymus, heart, spleen, kidney, and liver of rats and/or mice. Increases in the incidences of several skin lesions, including hyperplasia of the epidermis and hair follicles, and inflammation in rats and mice and ulceration in female mice were observed. Other nonneoplastic lesions that were considered to be related to diazoaminobenzene administration were atrophy of the thymus, mandibular and/or mesenteric lymph nodes, and white pulp of the spleen, as well as splenic hematopoietic cell proliferation in rats and mice. In mice, there were increased incidences of atrial thrombosis, and necrosis was observed in the renal tubules and liver.

Diazoaminobenzene was mutagenic in *S. typhimurium* strains TA98, TA100, and TA1537 with induced rat or hamster liver S9 enzymes; no activity was noted in strain TA1535, with or without S9. *In vivo*, two gavage administrations of either diazoaminobenzene or benzene induced highly significant increases in micronucleated polychromatic erythrocytes in bone marrow of male B6C3F$_1$ mice at all doses tested.

Diazoaminobenzene is metabolized to the known carcinogens benzene and aniline. Further evidence of this metabolism is that some toxic effects associated with aniline (methemoglobinemia) and benzene (atrophy of the lymphoid tissue) were identified. Based on these results, it is predicted that diazoaminobenzene is a carcinogen.

PEER REVIEW PANEL

The members of the Peer Review Panel who evaluated the draft report on the toxicity studies of diazoaminobenzene on October 18, 2001, are listed below. These reviewers serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determine if the design and conditions of these NTP studies are appropriate and ensure that this Toxicity Study Report presents the experimental results and conclusions fully and clearly. The comments of the reviewers were received and reviewed prior to the finalization of this document. Changes have been made such that the concerns of the reviewers have been addressed to the extent possible.

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SUMMARY OF PEER REVIEW COMMENTS

On October 18, 2001, the draft Technical Report on the toxicity studies of diazoaminobenzene received public review by the National Toxicology Program's Board of Scientific Counselors' Technical Reports Review Committee and associated Peer Review Panel. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. N.B. Ress, NIEHS, introduced the report on the metabolism, toxicity, and predicted carcinogenicity of diazoaminobenzene by describing the study design process and the results of metabolism and disposition studies and 16-day dermal toxicity studies. She also described results of a mouse bone marrow micronucleus study (not presented in the draft report) that showed that diazoaminobenzene, like benzene, is a potent inducer of micronuclei. The proposed conclusions to the report were:

> Diazoaminobenzene is metabolized to the known carcinogens benzene and aniline. Some toxic effects associated with aniline (Heinz body anemia, methemoglobinemia) and benzene (atrophy of the lymphoid tissue, hematopoietic cell proliferation) were identified. Based on these results, it is predicted that diazoaminobenzene is a carcinogen.

Dr. Thrall, a principal reviewer, agreed with the prediction of carcinogenicity based on the metabolism of diazoaminobenzene to benzene and aniline but questioned whether Heinz body anemia was truly an effect, as only one of 20 treated groups had a statistically significant increase in Heinz body formation. She asked for clarification of whether oral exposure occurred during the dermal study and suggested rearranging the conclusion statement to clarify that the prediction of carcinogenicity was based on metabolism. Dr. Ress replied that some oral exposure occurs in dermal studies as a result of the animals grooming themselves. In these studies the animals were housed individually to minimize such exposure.

Dr. Klaunig, the second principal reviewer, was unable to attend the meeting and his comments were read into the record by Dr. M.S. Wolfe, NIEHS. Dr. Klaunig agreed that the study results supported the premise that diazoaminobenzene may be carcinogenic.

Dr. Piegorsch, the third principal reviewer, agreed with the conclusions and asked if the results on the micronucleus studies would be included in the final version of the report. Dr. J.R. Bucher, NIEHS, indicated that the micronucleus data would be added with the understanding that these data were not used by the review panel in formulating the conclusion statement.

Dr. Hecht asked if phenyl hydrazine would also have been an expected metabolite of the compound and if any consideration had been given to possible interactive effects between the metabolites benzene and aniline. Dr. Ress replied that while phenyl hydrazine could be a metabolite, it was not observed in these studies. The possibility of interactive effects between the metabolites was being examined in further micronucleus tests.

Dr. Thrall moved that the conclusions be modified to eliminate mention of Heinz body anemia and hematopoietic cell proliferation. The revised conclusion was:

> Diazoaminobenzene is metabolized to the known carcinogens benzene and aniline. Further evidence of this metabolism is that some toxic effects associated with aniline (methemoglobinemia) and benzene (atrophy of the lymphoid tissue) were identified. Based on these results, it is predicted that diazoaminobenzene is a carcinogen.

Dr. Piegorsch seconded the motion, which was approved unanimously with five votes.

INTRODUCTION

Diazoaminobenzene is a golden-yellow crystal with a melting point of 98° C, a boiling point of 146° C, and a vapor density of 6.8 (Sax and Lewis, 1989). Diazoaminobenzene is soluble in ethyl alcohol, ethyl ether, benzene, pyridine, and hexane, and is insoluble in water (*Merck Index*, 1989; Lide, 1993). It decomposes when heated to temperatures above 130° C and explodes when heated to 150° C (*Merck Index*, 1989). The major decomposition products are benzene, *o*- and *p*-aminodiphenyl, diphenylamine, and azobenzene (Mortimore *et al.*, 1979).

Diazoaminobenzene is made by diazotizing aniline dissolved in hydrochloric acid with sodium nitrite and then adding a concentrated solution of sodium acetate (*Merck Index*, 1989). It also is formed through interaction of nitrous acid and an alcoholic solution of aniline (Lewis, 1993). Diazoaminobenzene is also prepared by the rapid reaction of aniline with isoamyl nitrite (Smith and Ho, 1990). Diazoaminobenzene also is formed as an intermediate during the preparation of iodobenzene from aniline (Smith and Ho, 1990).

Diazoaminobenzene has semiconducting properties (Shaaban *et al.*, 1993). It is used as a propellant for the molding of rubbers and plastics and as a coupler to promote adhesion of natural rubber to steel tire cords (*Kirk-Othmer*, 1982).

Occupational exposure to diazoaminobenzene occurs from its use as an intermediate during organic synthesis and in the manufacture of dyes and insecticides (Lewis, 1993). Other exposures to diazoaminobenzene may occur through its presence in cosmetics and food products. It has been identified as a contaminant in FD&C Red No. 33 and FD&C Yellow No. 5, which have been permitted for use in ingested and externally applied drugs and cosmetics (Bailey, 1985; Palmer and Mathews, 1986). Diazoaminobenzene has been identified in commercial products (unspecified) at concentrations up to 439 ppb with an average level of 99 ppb and in drugs (unspecified) at concentrations of 68 to 110 ppb (Palmer and Mathews, 1986).

Diazoaminobenzene is not listed in the National Occupational Exposure Survey (NOES) conducted by the National Institute for Occupational Safety and Health (NIOSH) (1990) from 1981 to 1983. No occupational exposure limits have been established by the American Conference of Governmental Industrial Hygienists, the NIOSH, or the Occupational Safety and Health Administration.

STUDY RATIONALE

Diazoaminobenzene was selected for toxicity and metabolism studies based on the potential for worker exposure from its use in laboratories, positive *Salmonella typhimurium* gene mutation data, its presence as an impurity in foods and cosmetics, and lack of adequate toxicological data. In addition, structural analogues of diazoaminobenzene are carcinogenic, providing evidence for the possible carcinogenicity of diazoaminobenzene as well (Table 1).

TABLE 1
Carcinogenic Compounds that are Structurally Similar to Diazoaminobenzene

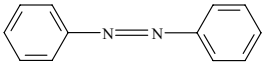
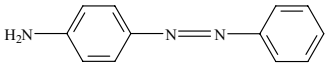
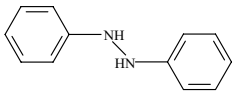
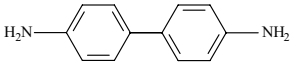
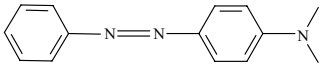
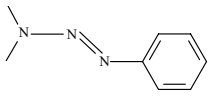
Structure	Classification	Mutagenicity Data
Azobenzene **CAS Number: 103-33-3** 	Carcinogenic in rats[a] IARC: 3[b]	*Salmonella* (+)[c] MN[d] Bone marrow (+)
***p*-Aminoazobenzene** **CAS Number: 60-09-3** 	Carcinogenic in neonate and male mice[e] IARC: 2B[b]	*Salmonella* (+)[f] MN[g] Bone marrow (+)
Hydrazobenzene **CAS Number: 122-66-7** 	Carcinogenic in rats and female mice[h] ROC: *Reasonably anticipated to be a human carcinogen*[i]	*Salmonella* (+)[j]
Benzidine **CAS Number: 92-87-5** 	IARC: 1[b] Carcinogenic in dogs, hamsters, mice, and rats[b] ROC: *Known to be a human carcinogen*[i]	*Salmonella* (+)[k] MN[g] Bone marrow (+)
***p*-Dimethylaminoazobenzene** **CAS Number: 60-11-7** 	IARC: 2B[b] Carcinogenic in mice, rats, and dogs[b] ROC: *Reasonably anticipated to be a human carcinogen*[i]	*Salmonella* (+)[l] MN[g] Bone marrow (+)

TABLE 1
Carcinogenic Compounds that are Structurally Similar to Diazoaminobenzene

Structure	Classification	Mutagenicity Data
Dimethylphenyltriazene **CAS Number: 7227-91-0** 	Carcinogenic in rats[m, n]	*Salmonella* (+)[o] MN[p] Peripheral blood (+)

[a] NCI, 1979
[b] IARC, 1987
[c] Haworth *et al.*, 1983
[d] George *et al.*, 1990
[e] Fujii, 1983
[f] Miyagoshi *et al.*, 1985
[g] Morita *et al.*, 1997
[h] NCI, 1978a
[i] NTP, 2001
[j] Dunkel *et al.*, 1984
[k] Reid *et al.*, 1984
[l] Dunkel *et al.*, 1985
[m] Kolar and Habs, 1984
[n] Frank *et al.*, 1992
[o] Malaveille *et al.*, 1976
[p] Heddle *et al.*, 1983

Based on its chemical structure, it was speculated that diazoaminobenzene would be metabolized into aniline and benzene. Therefore, metabolism and disposition studies were conducted by oral, dermal, and intravenous administration in male and female F344/N rats and B6C3F$_1$ mice. Electron spin resonance studies were conducted to assess the possible formation of the phenyl radical from the reduction of diazoaminobenzene by components of the P450 mixed-function oxidase system in microsomes or by gut microflora in cecal incubations. In addition, bile duct-cannulated male F344/N rats were administered diazoaminobenzene and DMPO for *in vivo* determination of the DMPO-phenyl radical. The 16-day toxicity studies were performed to identify target organs of diazoaminobenzene following dermal application to male and female F344/N rats and B6C3F$_1$ mice. Details of the disposition and 16-day toxicity studies are given in Appendixes A and B, respectively. The significant findings are described below.

NTP STUDIES

Absorption, Distribution, Metabolism, and Excretion Studies

The Materials and Methods and Results of the disposition and metabolism studies are presented in Appendix A. The disposition and metabolism studies on diazoaminobenzene were conducted using [^{14}C]-diazoaminobenzene to identify metabolites and their pathway of formation. The results of these studies showed that diazoaminobenzene is readily absorbed following oral and only slightly absorbed following dermal administration and is primarily excreted in the urine (Appendix A; Mathews and De Costa, 1999). When a single intravenous administration of 2 mg/kg diazoaminobenzene was given to male rats, urinary excretion accounted for most of the dose, with 80% being excreted within the first 24 hours (Table A1). Comparatively, in mice, only 27% of diazoaminobenzene was excreted in the urine after 24 hours, with only 57% being excreted in 72 hours (Table A11). In male and female rats orally administered 20 mg/kg diazoaminobenzene, 76% of the dose was excreted in the urine within 24 hours (Tables A2 and A3). In mice given a single oral dose of 20 mg/kg diazoaminobenzene, only 44% of the dose was excreted within 24 hours, and within 72 hours, 68% was excreted (Table A12). When given by intravenous injection, fecal elimination accounted for 8% of the dose in rats and 23% in mice, indicating bilary excretion. When administered orally, fecal elimination accounted for 16% of the dose in male rats and 20% of the dose in mice. It appears that mice excreted diazoaminobenzene to a greater extent in the feces; however, contamination of the feces with urine is a common problem in mouse metabolism studies and, as such, the excretion in urine and feces may be similar between species. Exhalation as volatile organics and CO_2 in the breath of rats and mice accounted for less than 2% of the dose by all routes (Tables A1, A2, A4, A11, A12, and A13). Seven percent or less of the applied dose was absorbed through the skin of rats and mice 72 hours after dermal exposure (Tables A5 and A14).

Benzene was the only radiolabeled product in the exhaled breath of rats and mice exposed orally to diazoaminobenzene (Tables A2 and A12). The profiles of the metabolites collected in the urine of rats treated intravenously or orally with diazoaminobenzene are presented in Tables A6 and A7. The metabolites detected in the urine from rats treated orally were benzene and aniline derivatives and constituted approximately 29% and 32%, respectively, of the diazoaminobenzene dose. Five of the urinary metabolites were common metabolites of benzene: hydroquinone glucuronide, muconic acid, prephenyl mercapturic acid (the nonaromatic product of the oxirane ring opening of benzene oxide with thiol), phenol glucuronide, and phenyl sulfate. The major urinary metabolite formed from the metabolism of aniline was 4-acetamidophenyl sulfate, which accounted for 32% of the dose. Although less than 7% of diazoaminobenzene was absorbed dermally, benzene and aniline metabolites were detected in the urine of a male F344/N rat administered a single dermal application of diazoaminobenzene (Table A8). In the urine of mice orally administered 20 mg/kg diazoaminobenzene, a different spectrum of benzene and aniline metabolites than that observed in rats was identified and similar metabolites occurred in different proportions than in rats (Table A15; Mathews and De Costa, 1999). Twenty-two percent of the initial dose was composed of the benzene metabolites

hydroquinone glucuronide, muconic acid, and phenol. Thirty-five percent of the diazoaminobenzene dose was composed of the aniline metabolites 4-acetamidophenyl glucuronide, 2-aminophenyl sulfate, 4-acetamidophenyl sulfate, 4-acetamidophenol, and 2-acetamidophenol.

Results from the metabolism studies show that diazoaminobenzene is metabolized to both benzene and aniline. Support of this pathway (Figure 1) was demonstrated in rats exposed to 1-aminobenzotriazole (ABT), a mechanism-based inhibitor of cytochrome P450, prior to oral administration of diazoaminobenzene (Table A2; Mathews and De Costa, 1999). Urinary excretion of radiolabeled product during the first 8 hours of dosing decreased from 49% for non ABT-treated rats to 12% in ABT-treated rats. An increase in the amount of unchanged benzene exhaled in the breath and a considerable decrease in the excretion of benzene metabolites in the urine were observed 24 hours after dosing (Table A7). Also, urinary excretion of the aniline metabolite 4-acetamidophenyl sulfate was delayed in rats pretreated with ABT, with the majority of the metabolite being excreted in the 8- to 24-hour collection.

Diazoaminobenzene was detected at low levels (<1%) in the adipose tissue, blood, kidney, liver, muscle, skin, and spleen in male and female rats 24 hours after oral administration of 20 mg/kg diazoaminobenzene (Table A9; Mathews and De Costa, 1999). The kidney accumulated more radioactivity than other organs and had a tissue/blood ratio greater than one for male and female rats.

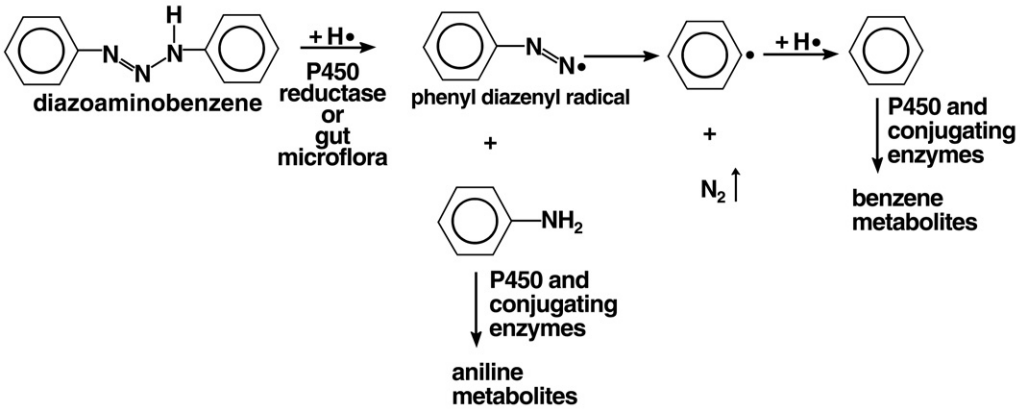


FIGURE 1
Proposed Pathway for the Metabolism of Diazoaminobenzene (Mathews and De Costa, 1999)

In rats, toxicokinetic studies demonstrated that diazoaminobenzene was rapidly eliminated from blood (Figures A1 and A2). The parent compound was present in smaller amounts than its metabolites (Table A10). The carcinogens benzene and aniline were detected at all time points, with peak concentrations at 1 hour and at 30 minutes, respectively. Within 15 minutes, the predominant circulating Equivalents were known metabolites of benzene and aniline and were detected at all time points during the study. The metabolites circulating in blood that were formed from the metabolism of benzene were hydroquinone glucuronide, muconic acid, prephenyl mercapturic acid, phenol glucuronide, phenyl sulfate, and phenol. The metabolite detected in the blood of rats that is formed from the metabolism of aniline was 4-acetamidophenyl sulfate.

An *in vitro* study (data not presented here) using liver slices from a human donor demonstrated that diazoaminobenzene is metabolized to benzene and aniline (Mathews and De Costa, 1999). The human slice incubations indicated that diazoaminobenzene was absorbed by the slices, but slowly metabolized. The distribution of radiolabel remained constant during the 5-hour incubation time with 87.6% ± 1.3% in the media and 6.36% ± 0.73% in the slices. The overall recovery of radioactivity from the incubations was greater than 94% and radiochemical purity did not decrease during a 5-hour incubation in control medium. Low rates of metabolism precluded accurate characterization of the metabolic profile, but metabolites previously characterized in urine (4-acetamidophenyl sulfate, phenyl sulfate, aniline, and hydroquinone glucuronide) were detected in the media samples. Because of the low rate of biotransformation and the likelihood that a major part of the reductive metabolism of diazoaminobenzene takes place in the intestinal tract, further *in vitro* metabolism studies with human liver slices were not pursued.

It was hypothesized that benzene and aniline were formed from diazoaminobenzene through cleavage of the triazene linkage by P450 reductase or gut microflora to produce a phenyl diazenyl radical and aniline. Loss of nitrogen from the phenyl diazenyl radical would create a phenyl radical, which can be detected with ESR spin trapping techniques. The phenyl radical abstracts a hydrogen atom from biological components, leaving benzene and a radical site on the biological component. This pathway was demonstrated *in vitro* and *in vivo* through a series of ESR spin trapping experiments. The phenyl radical was detected in rat hepatic microsomes treated with diazoaminobenzene, NADPH, which was required, and DMPO, which was used to "trap" the phenyl radical (Figure A3). The phenyl radical was also formed in microsomes incubated with the mechanism-based P450 inhibitor ABT and with carbon monoxide, indicating that interaction with the heme prosthetic group of P450 is not required (Figure A4). The formation of the DMPO-phenyl adduct was also catalyzed by recombinant human NADPH-P450 reductase (Figure A5). In anaerobic incubations using cecal contents isolated from rats, low levels of the DMPO-phenyl adduct were detected (Figure A6). In bile duct-cannulated rats administered diazoaminobenzene via intragastric intubation and DMPO by intraperitoneal injection, DMPO reacted with the phenyl radical, creating a more stable product that was collected in bile and characterized by ESR spectroscopy; the ESR spectrum obtained was consistent with the formation of a DMPO-phenyl adduct (Figures A7 and A8; Kadiiska *et al.*, 2000).

16-Day Toxicity Studies

The Materials and Methods and Results of the NTP 16-day studies involving dermal application of diazoaminobenzene to male and female F344/N rats and B6C3F$_1$ mice are presented in Appendix B. Diazoaminobenzene was not lethal to rats at any of the concentrations tested (Table B5). In contrast, in the second week of the study, most male mice administered 50 mg/kg or greater and three female mice administered 200 mg/kg died (Table B9). Body weight gains of all dosed groups of rats were significantly less than those of the vehicle controls (Table B5). Mice administered 50 mg/kg or greater lost weight during the study (Table B9). Final mean body weights and body weight gains of female mice administered 50 mg/kg or greater were significantly less than those of the vehicle controls.

Thymus weights were significantly decreased in all dosed groups of rats and female mice and in 25 mg/kg male mice (Tables B6 and B10). Spleen weights were increased in 100 and 200 mg/kg rats. Heart weights were significantly increased in 25 mg/kg male mice and in female mice administered 50 mg/kg or greater. Kidney weights were increased in female mice administered 50 mg/kg or greater. Relative liver weights were significantly increased in all dosed groups of male rats, female rats administered 25 mg/kg or greater, and 12.5 mg/kg mice. Other organ weight changes were likely associated with body weight changes.

Clinical pathology data indicated a chemical related methemoglobinemia and Heinz body formation (Tables B7 and B11). In rats and female mice, Heinz body formation was increased and considered to be chemically related. There was a treatment related decrease in erythroid mass evidenced by a decrease in hematocrit, hemoglobin, and erythrocyte counts suggesting a developing anemia. The erythron decrease was accompanied by an increased bone marrow response as indicated by increased reticulocytes in rats and mice and nucleated erythrocytes in rats. In mice only, the higher dose females had an increase in hemoglobin concentrations that would appear to be an inappropriate response compared to other estimates of red cell mass; this may have been a spurious result related to the increased number of Heinz bodies. Associated with the developing anemia was an increase in mean cell hemoglobin concentrations that would be consistent with the increased hemoglobin and possibly intravascular hemolyses related to Heinz body formation.

Gross observations at necropsy were limited to thickening of the skin at the site of application. Microscopically, this corresponded to hyperplasia of the epidermis and hair follicles which was evident in all dosed groups (Tables B8 and B12). Proliferation of hair follicles was a particularly prominent change of marked severity in the higher dose groups, characterized by an extensive area of the application site containing an increased density of hair follicles. This sometimes formed a raised, plaque-like lesion with a scalloped surface due to coalescense of dilated follicles

containing multiple hair shafts. In other areas the interfollicular epidermis was thickened with variable cystic or hyaline type degeneration in the stratum corneum. A slight mixed inflammatory cell infiltrate accompanied the hyperplastic change. Focal epidermal ulceration at the site of application was present in some female mice in the higher dose groups.

Various internal nonneoplastic lesions were observed and considered to be related to chemical treatment (Tables B8 and B12). Lymphoid atrophy of the thymus (a depletion of cortical lymphocytes) was a common lesion of mild to marked severity in treated rats and mice and corresponded to reduced thymus weights. A similar loss of lymphoid tissue was variably seen in the mesenteric and mandibular lymph nodes as well as in the white pulp of the spleen. Presumably, as a response to anemia, increased incidences of hematopoietic cell proliferation of generally mild severity occurred in the splenic red pulp of treated rats and mice and correlated with increased spleen weights.

Several other microscopic findings in mice were considered related to treatment, many occurring in early death animals. Atrial thrombosis of the heart was present and typically seen as a solid coagulum of proteinaceous material and embedded blood cells in the left auricle in all mice that died early. No myocardial changes were evident in either thrombotic hearts of early death animals or in survivors with increased heart weights. Renal tubule necrosis was found in early death male mice as well as in 100 mg/kg female mice that survived to study termination. Focal liver necrosis was found in most early death mice.

DISCUSSION

Diazoaminobenzene was nominated by the National Institute of Environmental Health Sciences to the National Toxicology Program for toxicity and metabolism studies based on the potential for widespread exposure to workers from its use in laboratories and to the public from its presence in food additives and cosmetics. Diazoaminobenzene was also shown to be mutagenic in *Salmonella typhimurium*, and several structural analogues were carcinogenic in rodents. It was also speculated that diazoaminobenzene would be metabolized to benzene and aniline, which are known human and/or rodent carcinogens. The purpose of these studies was to describe the metabolism and short-term toxicity of diazoaminobenzene.

The present studies show that rats and mice metabolize diazoaminobenzene almost exclusively to benzene, aniline, and their metabolites which have been previously identified (Table 2). Based on the proposed mechanism of decomposition, diazoaminobenzene is expected to yield approximately 40% benzene and 40% aniline, with the remaining percentage being nitrogen. Oral doses of diazoaminobenzene were well absorbed, rapidly metabolized, and excreted in the urine (Tables A2, A3, and A12). In rats, metabolites of benzene and aniline appeared rapidly in the blood following an oral dose of diazoaminobenzene, at levels that exceeded the parent compound (Table A10).

TABLE 2
Metabolites of Benzene and Aniline[a]

Aniline[b]	Benzene[c]
4-Aminophenyl glucuronide	Mucoaldehyde
4-Aminophenyl sulfate[M]	**Muconic acid**[R,M]
4-Acetamidophenyl glucuronide[M]	**Phenyl mercapturic acid**[R]
2-Aminophenyl sulfate	Prephenyl mercapturic acid
4-Acetamidophenyl sulfate[R,M]	Hydroquinone
2-Acetamidophenyl sulfate[M]	**Hydroquinone glucuronide**[R,M]
4-Acetamidophenol[M]	Hydroquinone sulfate
2-Acetamidophenol[M]	Benzoquinone
	Benzoquinone sulfate
	Benzoquinone glucuronide
	Phenol glucuronide[R]
	Phenyl sulfate[R]
	Phenol[M]
	Catechol
	Catechol sulfate
	Catechol glucuronide
	Trihydroxy benzene
	Trihydroxy benzene glucuronide
	Trihydroxy benzene sulfate

[a] Bold type indicates metabolites detected in the blood and urine of rats or mice exposed to diazoaminobenzene; R=rat; M=mouse.
[b] McCarthy *et al.*, 1985
[c] ATSDR, 1997; Mathews *et al.*, 1998

Within 24 hours, the percentage of the oral dose of radiolabeled diazoaminobenzene excreted in the urine of rats and mice was 30% or 22% for benzene metabolites and 32% or 35% for aniline metabolites, respectively (Tables A7 and A15). In rats, virtually all the metabolites formed following a dermal dose were common to those of benzene and aniline (Table A8).

Much like diazoaminobenzene, benzene and aniline are rapidly absorbed, metabolized, and excreted in the urine (Mathews *et al.*, 1998; McCarthy *et al.*, 1985). Similar to the diazoaminobenzene studies, benzene was detected unchanged in the breath of rats and mice gavaged with benzene (Mathews *et al.*, 1998). The concentration of benzene in the breath represents the biologically unavailable portion of the dose and at low doses of benzene (0.1 mg/kg), only 1% of benzene is present in the breath, while at high doses (100 mg/kg) the bioavailability of benzene decreases with up to 50% of benzene present in the breath. This effect was attributed to saturation of metabolic systems at the high dose (Mathews *et al.*, 1998). In the present studies, only a small amount of benzene was exhaled in the breath indicating that benzene was biologically available and that the metabolic systems were not saturated (Tables A1, A2, A4, A11, A12, and A13).

The excretion pattern of benzene-derived radioactivity is dose-dependent (Mathews *et al.*, 1998). The pathway of benzene metabolism that leads to the formation of muconic acid and hydroquinone is favored at low doses (0.1 mg/kg), while at higher doses the predominant pathways lead to less toxic metabolites. Medinsky *et al.* (1989) and Sabourin *et al.* (1989) showed that a greater proportion of the putative toxic metabolites, muconic acid and hydroquinone, are formed in mice than in rats. This same excretion pattern was observed in the present study in which muconic acid and hydroquinone accounted for 17% of the dose excreted in the urine of mice and only 9% of the dose in rats orally administered 20 mg/kg diazoaminobenzene (Tables A7 and A15; Mathews and De Costa, 1999). In rats and mice administered aniline, the predominant urinary metabolites formed were 4-acetamidophenyl sulfate and 4-acetamidophenyl glucuronide (McCarthy *et al.*, 1985). These metabolites also were the primary metabolites in rats and mice administered diazoaminobenzene; however, mice excreted four other metabolites associated with aniline (Tables 2, A7, and A15).

The pathway by which benzene and aniline are formed from diazoaminobenzene was described through a series of electron spin resonance studies performed *in vitro* and *in vivo* (Figures A3 to A8). These studies provide evidence that diazoaminobenzene is reductively cleaved to form a phenyl radical (Figure 1). *In vitro* formation of the phenyl radical required the full complement of microsomes or P450 reductase, NADPH, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and diazoaminobenzene (Figures A3 to A5). Addition of P450 inhibitors, carbon monoxide or 1-aminobenzotriazole, did not alter the formation of the DMPO-phenyl adduct, indicating that P450 is not required for biotransformation (Figures A3 and A4). The DMPO-phenyl radical also was observed in bile duct-cannulated rats administered DMPO and diazoaminobenzene (Figures A7 and A8). These results suggest that P450 reductase (Figure A5) as well as gut microflora (Figure A6) can catalyze the cleavage of the triazeno linkage resulting in the formation of aniline and a phenyl diazenyl radical that decomposes to form benzene and nitrogen gas. The resulting radical is free to react with other cellular components.

Diazoaminobenzene was metabolized to benzene and aniline in liver slices from a human donor (Mathews and De Costa, 1999). The yields of metabolites were relatively low given the amount of diazoaminobenzene available for metabolism, but were sufficient to establish the metabolic pathways involved. A similar study was performed with benzene using 10 human liver slices and livers from rats and mice (Brodfuehrer *et al.*, 1990). As with the diazoaminobenzene study, the metabolites formed from benzene were phenol, hydroquinone, phenyl sulfate, and phenyl glucuronide and were detected in each species tested. The metabolites concentrations in human liver slices in the benzene study ranged from nondetectable to values greater than those observed in rats and mice.

The 16-day diazoaminobenzene studies in rats and mice also gave evidence of toxicological effects that have been related to aniline or benzene. Dermal studies were performed with diazoaminobenzene because this is a likely

exposure scenario in both occupational and nonoccupational settings. As is customary, the dermal studies were performed without an occlusive patch. Because grooming can result in some ingestion of small quantities of the chemical, some of the toxicity at and away from the site of application in the present studies could have been associated with ingestion of diazoaminobenzene.

In the diazoaminobenzene studies, the erythrocyte and lymphoid systems were the major targets of toxicity. Because diazoaminobenzene is metabolized to benzene and aniline, the toxicity observed in the present studies was compared to that expected with its two major metabolites. The major toxic effect of benzene is bone marrow depression leading to aplastic anemia; the latter primarily occurs following longer exposure periods through inhalation and oral routes (ATSDR, 1997) and, as such, was not expected to be observed in the present study. This was confirmed through histological examination of bone marrow from treated rats and mice, which indicated that there were no treatment related changes. However, in the 16-day dermal toxicity studies of diazoaminobenzene, some toxicological effects possibly related to benzene were observed. Studies on benzene using various routes of administration and exposure durations have been reviewed and have reported increased liver and decreased thymus weights in rodents as well as central nervous system depression and death at high doses (ATSDR, 1997). Lymphoid depletion of the spleen was observed in F344/N rats exposed to benzene for 17 weeks by oral gavage, and splenic hematopoiesis was increased in mice exposed to benzene by oral gavage for 2 years (NTP, 1986). In the present studies, dose-related decreases in thymus weights in rats and mice, an increase in relative liver weights in rats and mice, atrophy of the lymphoid follicle of the spleen in mice, and hematopoietic cell proliferation in rats and mice were observed (Tables B6, B10, and B12). While these responses do not directly compare to those observed in the long-term benzene studies, there is concordance in the site of action.

Methemoglobinemia and splenotoxicity are major toxic events resulting from aniline exposure. In the diazoaminobenzene studies, methemoglobin formation, accompanying anemia, increased spleen weights, and splenic hematopoietic cell proliferation were indicative of erythrocyte toxicity (Appendix B). Aniline generates methemoglobin *in vivo*, presumably through bioactivation to aminophenols and N-hydroxylamines (CIIT, 1982; Short *et al.*, 1983; Firoze Khan *et al.*, 1997). In the present studies, methemoglobin and Heinz body formation were observed in rats and mice and were likely associated with the biotransformation of diazoaminobenzene to aniline and aniline metabolites. A direct effect of chemicals on mature red blood cells can cause oxidative red cell injury and formation of methemoglobin and Heinz bodies. This response often results in a regenerative hemolytic anemia characterized by the destruction of red blood cells and increased production of reticulocytes, which was observed in the present studies.

Aniline-induced carcinogenic responses in the spleen have been observed in two chronic bioassays (CIIT, 1982; NCI, 1978b). In these studies, the carcinogenic response in the spleen was specific to the rat and was not observed in $B6C3F_1$ mice exposed concurrently. The pathogenesis of aniline-induced splenotoxicity and carcinogenicity has been investigated (Goodman *et al.*, 1984; Weinberger *et al.*, 1985; Bus and Popp, 1987). Goodman *et al.* (1984) also postulated that aniline metabolites bind to methemoglobin and when methemoglobin is broken down in the red pulp of the spleen, the active metabolite is released and binds with splenic mesenchymal tissues, causing fibrosis and possibly leading to carcinogenicity. These conclusions are supported by McCarthy *et al.* (1985) in which high levels of aniline-derived radioactivity were associated with protein and RNA in the spleen of rats orally administered aniline for 7 days. A similar hypothesis has been proposed by Weinberger *et al.* (1985) and Bus and Popp (1987).

Benzene is a multi-site, multi-species carcinogen and has been extensively reviewed (IARC, 1982; WHO, 1993; ATSDR, 1997). Chronic inhalation exposure to benzene in rodents has been associated with various types of lymphomas and leukemia as well as neoplasms of the Zymbal's gland, liver, mammary gland, and oronasal cavity. Administration of benzene via oral gavage is associated with neoplasms of the Zymbal's gland, oronasal cavity, mammary gland, liver, forestomach, skin, harderian gland, preputial gland, ovary, and the hematopoietic and lymphoreticular systems (NTP, 1986).

Diazoaminobenzene was mutagenic in *Salmonella typhimurium* strains TA 98, TA100, and TA1537 with induced rat or hamster liver S9 enzymes (Table C1; Zeiger *et al.*, 1987). No additional genetic toxicity data have been published for diazoaminobenzene, but an abundance of literature exists for the two main metabolites, benzene and aniline. While benzene and aniline are not mutagenic in the *Salmonella* assay, both are active in other assays, particularly those that detect chromosomal damage (Haworth *et al.*, 1983; Zeiger and Haworth, 1985). Aniline and benzene each induced micronucleated erythrocytes in peripheral blood and bone marrow of mice exposed by gavage (Choy *et al.*, 1985; MacGregor *et al.*, 1990; Ashby *et al.*, 1991; Westmoreland and Gatehouse, 1991; Witt *et al.*, 2000). Benzene also induced micronuclei in spleen and lung cells of mice exposed via gavage and inhalation (Chen *et al.*, 1994; Ranaldi *et al.*, 1998). Both structural chromosome damage and aneuploidy events were implicated as mechanisms of micronucleus induction in these mouse studies (Chen *et al.*, 1994). Benzene induced chromosomal aberrations in mouse bone marrow cells and lymphocytes following inhalation exposure and chromosomal breakage in differentiating spermatogonial cells of CD-1 mice after oral exposure (Tice *et al.*, 1980; Rithidech *et al.*, 1987; Ciranni *et al.*, 1991). In humans, significant increases in numerical and structural chromosomal damage occurred in lymphocytes of benzene-exposed workers (Smith, 1996; Marcon *et al.*, 1999; Zhang *et al.*, 1999; Kašuba *et al.*, 2000). Because diazoaminobenzene is metabolized to benzene and aniline, it is reasonable to suspect it would possess similar genotoxic properties.

To further explore the relationship between diazoaminobenzene and benzene, a comparative toxicity study was performed to assess the formation of micronuclei in bone marrow following oral exposure to benzene or diazoaminobenzene. The acute mouse bone marrow micronucleus studies revealed chemical related increases in micronuclei in the bone marrow of male mice exposed to benzene or diazoaminobenzene (Table C2). Diazoaminobenzene induced a similar number of micronuclei as benzene, but at a 10-fold lower dose suggesting that aniline and possibly the phenyl radicals formed during the reductive metabolism of diazoaminobenzene induce micronuclei and may act in a synergistic manner with benzene. Further studies are being conducted with benzene, aniline, a mixture of benzene and aniline in proportions that are equivalent to that of the diazoaminobenzene molecule, or diazoaminobenzene. These studies will aid in the interpretation of the relative potencies of the single and combined metabolites in relation to diazoaminobenzene.

Based on these studies, it is predicted that diazoaminobenzene would be a carcinogen in the NTP 2-year rodent bioassays. This prediction is largely supported by the almost exclusive metabolism of diazoaminobenzene to the two known carcinogens, benzene and aniline. The dermal toxicity study also demonstrated that diazoaminobenzene possesses similar toxicological properties to those of benzene, and in particular, aniline; this information complements the metabolism information. However, these toxicity studies do not directly address whether diazoaminobenzene would be carcinogenic in rodents exposed by the dermal route in long-term bioassays. Due to the toxicity of diazoaminobenzene administered dermally, it is likely that the doses acceptable for use in a 2-year study would not be high enough to elicit a carcinogenic response. To address this issue, the Cancer Effect Level (CEL), the lowest dose for which the increase in tumor incidence was chemically related, was determined in rats and mice orally exposed to benzene and aniline (Table 3). Unfortunately, there were no 2-year dermal carcinogenicity studies with benzene or aniline for comparison. The CEL for benzene administered by oral gavage is 50 mg/kg for male F344/N rats and 25 mg/kg for female F344/N rats and male and female B6C3F$_1$ mice. It is important to note that in these studies the CEL is the lowest dose tested, so a chemical related increase in tumors at a lower dose is possible. For aniline, the CEL was 30 mg/kg for male CD-F rats and 200 mg/kg for female F344/N rats. Based on the studies assessing micronuclei formation in the bone marrow of mice treated with diazoaminobenzene by oral gavage, the maximum tolerated dose was 100 mg/kg. Assuming diazoaminobenzene could be given at daily doses as high as 50 mg/kg for 2 years to rats and mice, this would be approximately equivalent to daily doses of aniline and benzene at 25 mg/kg for each chemical. It would appear from the data in Table 3 that this would be sufficient to produce carcinogenic effects in both rats and mice administered diazoaminobenzene orally. It is also possible that benzene and aniline may act in a synergistic manner and, as such, diazoaminobenzene would be more potent than either benzene or aniline tested separately.

TABLE 3
Cancer Effect Levels (CEL)[a] in Rats and Mice Exposed to Benzene or Aniline

	Route	Exposure Duration	CEL	Tumor Types
Benzene[b, c, d]				
Rat, F344/N				
Male	gavage	103 weeks	50 mg/kg	Zymbal's gland carcinoma Oral cavity: squamous cell papilloma and/or carcinoma Skin: squamous cell papilloma and/or carcinoma
Female	gavage	103 weeks	25 mg/kg	Zymbal's gland carcinoma Oral cavity: squamous cell papilloma and/or carcinoma
Mice, $B6C3F_1$				
Male	gavage	103 weeks	25 mg/kg	Zymbal's gland carcinoma Malignant lymphoma Alveolar/bronchiolar adenoma and/or carcinoma Harderian gland adenoma Preputial gland squamous cell carcinoma
Female	gavage	103 weeks	25 mg/kg	Malignant lymphoma Alveolar/bronchiolar adenoma and/or carcinoma
Aniline				
Rat, CD-F				
Male[e]	feed	104 weeks	30 mg/kg	Splenic sarcomas
Rat, F344/N				
Female[f, g]	feed	103 weeks	200 mg/kg	Splenic sarcomas

[a] Cancer effect level (CEL) is the lowest dose at which tumor incidence is increased above control values.
[b] CEL was the lowest dose tested.
[c] NTP, 1986
[d] A greater spectrum of tumors was observed at higher doses.
[e] CIIT, 1982
[f] NCI, 1978b
[g] The average daily dose is approximate assuming average daily feed consumption of 11 g for female rats and an average body weight of 330 g for female rats; the concentrations of aniline in the feed were 0, 3,000, and 6,000 ppm. In a subsequent evaluation by Weinberger *et. al* (1985), the CEL in female rats was 100 mg/kg.

CONCLUSIONS

Diazoaminobenzene is metabolized to the known carcinogens benzene and aniline. Further evidence of this metabolism is that some toxic effects associated with aniline (methemoglobinemia) and benzene (atrophy of the lymphoid tissue) were identified. Based on these results, it is predicted that diazoaminobenzene is a carcinogen.

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APPENDIX A
ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION STUDIES OF DIAZOAMINOBENZENE IN F344/N RATS AND B6C3F$_1$ MICE

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION STUDIES OF DIAZOAMINOBENZENE IN F344/N RATS AND B6C3F$_1$ MICE

INTRODUCTION

Studies were conducted in adult male and female F344/N rats and male B6C3F$_1$ mice to determine the absorption, distribution, metabolism, and excretion of diazoaminobenzene following intravenous injection, gavage dosing, or dermal application. Also, a series of ESR spin trapping experiments was performed on diazoaminobenzene *in vitro* and *in vivo* to demonstrate the creation of a phenyl radical. These studies were conducted by Research Triangle Institute (Research Triangle Park, NC).

MATERIALS AND METHODS

[^{14}C]-Diazoaminobenzene (37.9 mCi/mmol; lot 960508), randomly labeled on the phenyl rings, was obtained from Wizard Laboratories, Inc. (West Sacramento, CA). The radiochemical purity was determined to be approximately 97% using a high-performance liquid chromatography (HPLC) Supelcosil LC-18-DB analytical column (Bellefonte, PA). An isocratic mobile phase of acetonitrile was used at a flow rate of 1.0 mL/minute. The column effluent was monitored by a Ramona 5-LS radioactivity detector with a solid scintillator-packed flow cell. Radioactivity eluting in each fraction was measured by liquid scintillation spectrometry (LSS). Nonradiolabeled diazoaminobenzene (lot A008385701) was obtained from ACROS Organics (Pittsburgh, PA); the chemical was identified as nonradiolabeled diazoaminobenzene by proton nuclear magnetic resonance spectrometry and by mass spectrometry.

5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) (lot 16023AN) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI).

Male and female F344/N rats and male B6C3F$_1$ mice were obtained from Charles Rivers Laboratories, Inc. (Raleigh, NC). Animals were quarantined at least 1 week; rats and mice were 10 to 11 weeks old when the studies began. Animals received certified Purina Rodent Chow No. 5002 and tap water *ad libitum*. Rats and mice were housed in polycarbonate cages; the day before dosing, animals were transferred to individual glass metabolism chambers that allowed for separate collection of urine, feces, and breath components.

Intravenous dose formulations for rats and mice contained 18.0 to 19.5 µCi [^{14}C]-diazoaminobenzene, an appropriate amount of nonradiolabeled diazoaminobenzene, and a sufficient amount of Emulphor EL-620 and water (1:9) for a dosing volume of 1 mL/kg. The doses were injected into a lateral tail vein using a Hamilton syringe fitted with a 27-gauge hypodermic needle.

The 20 mg/kg single intragastric gavage dose formulations for rats and mice contained [^{14}C]-diazoaminobenzene (10.0 to 24.1 µCi for rats; 5.7 to 7.1 µCi for mice), an appropriate amount of nonradiolabeled diazoaminobenzene, and a sufficient amount of Emulphor EL-620 and water (2:8) for a dosing volume of 5 mL/kg. Dose formulations for the electron spin resonance experiments in rats delivered 16 mg/kg diazoaminobenzene directly into the stomach and contained an appropriate amount of nonradiolabeled diazoaminobenzene and a sufficient amount of Emulphor EL-620 and water (2:8) for a dosing volume of 5 mL/kg. The dose was contained in a 2.5 mL Hamilton No. 1002 syringe fitted with a Teflon®-tipped plunger and a gavage ball-tipped needle (16 gauge for rats; 18 gauge for mice). The concentration of [^{14}C]-diazoaminobenzene in each dose formulation was measured in two weighed

aliquots taken before, one during, and two after dosing each series of animals. To measure residual diazoaminobenzene left on the dosing apparatus after dosing, the needle was wiped clean with a Kimwipe® that was placed into a scintillation vial containing 2 mL ethanol and analyzed by LSS after addition of fluor. The dose for each route was calculated as the difference between the weights of the filled and empty dosing apparatus, less the amount found in the wipe. 1-Aminobenzotriazole, used as a pretreatment in the gavage studies, was administered intraperitoneally. The 100 mg/kg dose of 1-aminobenzotriazole was prepared by dissolving an appropriate amount in deionized distilled water for a dosing volume of 4 mL/kg.

Dermal dose formulations for rats contained 22.2 to 31.2 µCi [^{14}C]-diazoaminobenzene, an appropriate amount of nonradiolabeled diazoaminobenzene, and acetone for a total volume of 50 to 80 µL per dose. Dermal dose formulations for mice contained 13.1 to 14.6 µCi [^{14}C]-diazoaminobenzene, an appropriate amount of nonradiolabeled diazoaminobenzene, and acetone for a total volume of 25 to 50 µL per dose. Approximately 24 hours before dermal doses were applied, animals were anesthetized with an intramuscular injection of ketamine:xylazine (7:1). The fur on the back of each animal was clipped and the dosing area was wiped with acetone, marked, and examined; animals with nicks in the clipped area were excluded from the study. The doses were applied to 2 cm^2 (rats) or 1 cm^2 (mice) areas of skin using a Wiretrol (Drummond Scientific Co., Broomall, PA). Prior to dosing, a tissue capsule constructed of wire mesh with a nonocclusive linen cloth glued over it with cyanoacrylate was attached to each animal to protect the dose site.

Determination of Excretion, Urinary Metabolites, and Tissue Distribution of [^{14}C]-Diazoaminobenzene in Rats and Mice

Groups of four male rats were administered single intravenous injections of 2 mg [^{14}C]-diazoaminobenzene per kilogram body weight, single gavage doses of 20 mg/kg, or single dermal applications of 2 or 20 mg/cm^2; additionally, a group of four female rats was administered a single gavage dose of 20 mg/kg. Groups of four male mice were administered single intravenous injections of 2 mg/kg, single gavage doses 20 mg/kg, or single dermal applications of 2 or 20 mg/cm^2. Urine and feces were collected separately into round-bottom flasks cooled with dry ice 8 (urine only), 24, 48, and 72 hours after dosing and were stored in the dark at –20° C until analysis.

At the end of the study, rats were anesthetized with an intramuscular injection of 60 mg/kg ketamine and 8.6 mg/kg xylazine and mice with an intraperitoneal injection of 180 mg/kg sodium pentobarbital. Blood was withdrawn by cardiac puncture with a syringe containing heparin. Rats were then sacrificed by an intracardiac injection of 300 mg/kg sodium pentobarbital and mice by cervical dislocation. For animals administered diazoaminobenzene dermally, the skin at the site of application was excised with the appliance attached. The appliance was removed from the skin with acetone to dissolve the adhesive. The nonocclusive linen cover was removed from the appliance and placed into a scintillation vial for analysis. The appliance was rinsed. The skin from the application site was rinsed with acetone and ethanol, washed with cotton gauzes soaked in soapy water, and swabbed with cotton gauzes soaked in water. Rinses were collected; the gauzes were placed into 20-mL scintillation vials containing 2 mL water. The skin from the site of application was digested in approximately 70 mL 2 N ethanolic sodium hydroxide.

For determinations of total radioactivity, aliquots of urine and the breath trap collections were added directly to vials containing scintillation cocktail (Ultima Gold™; Packard Instrument Company, Inc., Meriden, CT). Samples of feces and blood (0.1 to 0.3 g) were digested in 2 mL Soluene®-350 (Packard Instrument Company, Inc.). After digestion, samples requiring bleaching were decolorized with perchloric acid/hydrogen peroxide prior to addition of scintillation cocktail. For rats administered gavage doses of 20 mg/kg [^{14}C]-diazoaminobenzene, adipose tissue, blood, kidney, liver, muscle, skin, and spleen were analyzed for carbon-14 content.

Urinary Metabolites

Urinary metabolite profiles were obtained using a Zorbax ODS analytical column with a C_{18} precolumn (Newport, DE). Urinary metabolites were eluted using a linear gradient, changing from 10% to 90% methanol in 35 mM aqueous tetrabutylammonium hydrogen sulfate over a 35-minute period. The flow rate was 1 mL/min and

the column was maintained at 40° C. Volatile components in breath were analyzed using a Zorbax ODS analytical column with a C_{18} precolumn and an isocratic mobile phase consisting of 60% methanol in water. The flow rate was 1 mL/min. Column effluents were monitored by UV absorbance at 270 nm (Applied Biosystems 757, Foster City, CA) and a Ramona 5-LS flow through radioactivity detector equipped with a 600 µL solid scintillate flow cell.

The assignment of one metabolite as 4-acetamidophenyl sulfate and another as phenyl sulfate was made by treatment of urine with sulfatase followed by demonstration of coelution of the resulting analytes in the incubation solution with those of standards of 4-acetamidophenol and phenol, respectively. An aliquot of urine was incubated with sulfatase (prepared from *Aerobacter aerogenes*) in TRIZMA® buffer for 3 hours at 37° C.

Stability Study of Diazoaminobenzene in Blood

Blood was incubated with [^{14}C]-diazoaminobenzene (0.1 or 1 mM) at 37° C for 10 or 30 minutes prior to extraction. Aliquots of blood (150 µL) were then extracted with 1 mL of methanol or acetone, centrifuged, and the supernatent was removed. The pellet was extracted with an additional 500 µL of solvent, and the supernatants were combined, evaporated to dryness, reconstituted, and analyzed by HPLC using the same methods described for radiochemical purity confirmation. The supernatants were kept separate from the samples where the radioactivity was to be followed to determine extraction efficiencies.

Electron Spin Resonance Studies

In vitro Experiments

In vitro experiments were performed on rat cecal contents, liver microsomes, and purified human NADPH-P450-reductase (Panvera Corporation, Madison, WI) (Kadiiska *et al.*, 2000). Incubations of the cecal contents (approximately 100 mg/mL 100 mM phosphate buffer; pH 7.4) with diazoaminobenzene (25 mM final concentration) were performed in a glove bag saturated with nitrogen gas. The incubation mixture was extracted with nitrogen-sparged toluene to detect the proposed phenyl radical. The incubation of diazoaminobenzene (25 mM final concentration) with microsomes (1 mg protein/mL 100 mM phosphate buffer) and 1 mM NADPH was performed under ambient conditions. Each permutation of the control experiment excluded one component from the complete incubation system. The incubation of diazoaminobenzene (25 mM final concentration) with reductase (2.4 pmol/mL 100 mM phosphate buffer) and 1 mM NADPH was performed under ambient conditions. DMPO (200 mM) was used as the spin trap in all of these *in vitro* experiments. ESR spectra were recorded using a Varian E-109 spectrometer (Varian, Inc., Palo Alto, CA) equipped with a TM_{110} cavity operating at 9.33 GHz, a power of 20 mW, and a modulation frequency of 100 kHz.

In vivo Experiments

In vivo experiments were performed on male F344/N rats (Charles River Laboratories, Inc., Raleigh, NC) anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and then bile duct-cannulated. Diazoaminobenzene was administered intragastrically and DMPO intraperitoneally. Bile samples were collected from cannulated bile ducts at 20-minute intervals for 2 hours after administration of DMPO (1 g/kg) and diazoaminobenzene (16 mg/kg) in four rats. An aliquot (50 µL) of the iron chelating agent, 2,2-dipyridyl (30 mM) was added to four bile collections to inhibit the formation of iron radical adducts generated *ex vivo*. ESR spectra were recorded by procedures similar to those described for the *in vitro* experiments.

RESULTS

The disposition and metabolism studies on diazoaminobenzene were conducted using [^{14}C]-diazoaminobenzene to identify metabolites and their pathway of formation. The results of these studies showed that diazoaminobenzene is readily absorbed following oral and only slightly absorbed following dermal administration and is primarily excreted in the urine (Mathews and De Costa, 1999). When a single intravenous administration of 2 mg/kg

diazoaminobenzene was given to male rats, urinary excretion accounted for most of the dose, with 80% being excreted within the first 24 hours (Table A1). Comparatively, in mice, only 27% of diazoaminobenzene was excreted in the urine after 24 hours, with only 57% being excreted in 72 hours (Table A11). In male and female rats orally administered 20 mg/kg diazoaminobenzene, 76% of the dose was excreted in the urine within 24 hours (Tables A2 and A3). In mice given a single oral dose of 20 mg/kg diazoaminobenzene, only 44% of the dose was excreted within 24 hours, and within 72 hours, 68% was excreted (Table A12). When given by intravenous injection, fecal elimination accounted for 8% of the dose in rats and 23% in mice indicating bilary excretion. When administered orally, fecal elimination accounted for 16% of the dose in male rats and 20% of the dose in mice. It appears that mice excreted diazoaminobenzene to a greater extent in the feces; however, contamination of the feces with urine is a common problem in mouse metabolism studies and, as such, the excretion in urine and feces may be similar between species. Exhalation as volatile organics and CO_2 in the breath of rats and mice accounted for less than 2% of the dose by all routes (Tables A1, A2, A4, A11, A12, and A13). Seven percent or less of the applied dose was absorbed through the skin of rats and mice 72 hours after dermal exposure (Tables A5 and A14).

Benzene was the only radiolabeled product in the exhaled breath of rats and mice exposed orally to diazoaminobenzene (Tables A2 and A12). The profiles of the metabolites collected in the urine of rats treated intravenously or orally with diazoaminobenzene are presented in Tables A6 and A7. The metabolites detected in the urine from rats treated orally were benzene and aniline derivatives and constituted approximately 29% and 32%, respectively, of the diazoaminobenzene dose. Five of the urinary metabolites were common metabolites of benzene: hydroquinone glucuronide, muconic acid, prephenyl mercapturic acid (the nonaromatic product of the oxirane ring opening of benzene oxide with thiol), phenol glucuronide, and phenyl sulfate. The major urinary metabolite formed from the metabolism of aniline was 4-acetamidophenyl sulfate, which accounted for 32% of the dose. Although less than 7% of diazoaminobenzene was absorbed dermally, benzene and aniline metabolites were detected in the urine of a male F344/N rat administered a single dermal application of diazoaminobenzene (Table A8). In the urine of mice orally administered 20 mg/kg diazoaminobenzene, a different spectrum of benzene and aniline metabolites than that observed in rats was identified and similar metabolites occurred in different proportions than in rats (Table A15; Mathews and De Costa, 1999). Twenty-two percent of the initial dose was composed of the benzene metabolites hydroquinone glucuronide, muconic acid, and phenol. Thirty-five percent of the diazoaminobenzene dose was composed of the aniline metabolites 4-acetamidophenyl glucuronide, 2-aminophenyl sulfate, 4-acetamidophenyl sulfate, 4-acetamidophenol, and 2-acetamidophenol.

Results from the metabolism studies show that diazoaminobenzene is metabolized to both benzene and aniline. Support of this pathway was demonstrated in rats exposed to 1-aminobenzotriazole (ABT), a mechanism-based inhibitor of cytochrome P450, prior to oral administration of diazoaminobenzene (Table A2; Mathews and De Costa, 1999). Urinary excretion of radiolabeled product during the first 8 hours of dosing decreased from 49% for non ABT-treated rats to 12% in ABT-treated rats. An increase in the amount of unchanged benzene exhaled in the breath and a considerable decrease in the excretion of benzene metabolites in the urine were observed 24 hours after dosing (Table A7). Also, urinary excretion of the aniline metabolite 4-acetamidophenyl sulfate was delayed in rats pretreated with ABT, with the majority of the metabolite being excreted in the 8- to 24-hour collection.

Diazoaminobenzene was detected at low levels (<1%) in the adipose tissue, blood, kidney, liver, muscle, skin, and spleen in male and female rats 24 hours after oral administration of 20 mg/kg diazoaminobenzene (Table A9; Mathews and De Costa, 1999). The kidney accumulated more radioactivity than other organs and had a tissue/blood ratio greater than one for male and female rats.

In rats, toxicokinetic studies demonstrated that diazoaminobenzene was rapidly eliminated from blood (Figures A1 and A2). The parent compound was present in smaller amounts than its metabolites (Table A10). The carcinogens benzene and aniline were detected at all time points, with peak concentrations at 1 hour and at 30 minutes, respectively. Within 15 minutes, the predominant circulating Equivalents were known metabolites of benzene and aniline and were detected at all time points during the study. The metabolites circulating in blood that were formed from the metabolism of benzene were hydroquinone glucuronide, muconic acid, prephenyl mercapturic acid, phenyl glucuronide, phenyl sulfate, and phenol. The metabolite detected in the blood of rats that is formed from the metabolism of aniline was 4-acetamidophenyl sulfate.

An *in vitro* study (data not presented here) using liver slices from a human donor demonstrated that diazoaminobenzene is metabolized to benzene and aniline (Mathews and De Costa, 1999). The human slice incubations indicated that diazoaminobenzene was absorbed by the slices, but slowly metabolized. The distribution of radiolabel remained constant during the 5-hour incubation time with 87.6% ± 1.3% in the media and 6.36% ± 0.73% in the slices. The overall recovery of radioactivity from the incubations was greater than 94% and radiochemical purity did not decrease during a 5-hour incubation in control medium. Low rates of metabolism precluded accurate characterization of the metabolic profile, but metabolites previously characterized in urine (4-acetamidophenyl sulfate, phenyl sulfate, aniline, and hydroquinone glucuronide) were detected in the media samples. Because of the low rate of biotransformation and the likelihood that a major part of the reductive metabolism of diazoaminobenzene takes place in the intestinal tract, further *in vitro* metabolism studies were not pursued.

It was hypothesized that benzene and aniline were formed from diazoaminobenzene through cleavage of the triazene linkage by P450 reductase or gut microflora to produce a phenyl diazenyl radical and aniline. Loss of nitrogen from the phenyl diazenyl radical would create a phenyl radical, which can be detected with ESR spin trapping techniques. The phenyl radical abstracts a hydrogen atom from biological components, leaving benzene and a radical site on the biological component. This pathway was demonstrated *in vitro* and *in vivo* through a series of ESR spin trapping experiments. The phenyl radical was detected in rat hepatic microsomes treated with diazoaminobenzene, NADPH, which was required, and DMPO, which was used to "trap" the phenyl radical (Figure A3). The phenyl radical was also formed in microsomes incubated with the mechanism-based P450 inhibitor ABT and with carbon monoxide, indicating that interaction with the heme prosthetic group of P450 is not required (Figure A4). The formation of the DMPO-phenyl adduct was also catalyzed by recombinant human NADPH-P450 reductase (Figure A5). In anaerobic incubations using cecal contents isolated from rats, low levels of the DMPO-phenyl adduct were detected (Figure A6). In bile duct-cannulated rats administered diazoaminobenzene via intragastric intubation and DMPO by intraperitoneal injection, DMPO reacted with the phenyl radical, creating a more stable product that was collected in bile and characterized by ESR spectroscopy; the ESR spectrum obtained was consistent with the formation of a DMPO-phenyl adduct (Figures A7 and A8; Kadiiska *et al.*, 2000).

TABLE A1
Cumulative Excretion of Radioactivity by Male F344/N Rats after a Single Intravenous Injection of 2 mg/kg [^{14}C]-Diazoaminobenzene[a]

Time (hours after dosing)	Urine	Feces	Breath[b]	Total
8	48.2 ± 12.5	—[c]	0.45 ± 0.07	48.7 ± 12.5
24	80.1 ± 3.3	5.2 ± 0.7	0.60 ± 0.08	85.9 ± 3.9
48	85.5 ± 2.0	7.3 ± 0.3	0.65 ± 0.09	93.5 ± 1.9
72	87.0 ± 1.8	7.7 ± 0.4	0.67 ± 0.10	95.3 ± 1.6
Cage wash	87.4 ± 1.7			95.7 ± 1.6
Total	87.4 ± 1.7	7.7 ± 0.4	0.67 ± 0.10	95.7 ± 1.6

[a] Four rats were examined; data are presented as cumulative percentage of dose (mean ± standard deviation).
[b] A single radiolabeled product was present as volatile organic chemicals in the exhaled breath. Analysis by reverse phase HPLC determined that it coeluted with benzene.
[c] Feces were not collected at this time point.

TABLE A2
Cumulative Excretion of Radioactivity by Male F344/N Rats after a Single Gavage Dose of 20 mg/kg [^{14}C]-Diazoaminobenzene[a]

Time (hours after dosing)	Urine	Feces	Breath[b]	CO_2	Total
No Pretreatment					
8	48.9 ± 1.9	—[c]	1.15 ± 0.21	0.03 ± 0.00	50.1 ± 1.9
24	75.5 ± 2.0	13.7 ± 1.6	1.30 ± 0.21	0.04 ± 0.00	90.6 ± 3.0
48	79.5 ± 1.0	15.6 ± 0.8	1.35 ± 0.22	0.04 ± 0.00	96.5 ± 0.9
72	80.3 ± 0.8	15.7 ± 0.8	1.36 ± 0.22	0.05 ± 0.01	97.5 ± 0.3
Cage wash	80.6 ± 0.8				97.7 ± 0.4
Total	80.6 ± 0.8	15.7 ± 0.8	1.36 ± 0.22	0.05 ± 0.01	97.7 ± 0.4
Pretreatment with 1-Aminobenzotriazole[d]					
8	11.6 ± 6.3	—	6.57 ± 2.95	0.014 ± 0.01	18.2 ± 6.4
24	50.3 ± 7.5	11.8 ± 0.8	11.9 ± 3.9	0.024 ± 0.01	74.1 ± 4.1
48	60.2 ± 6.6	17.2 ± 3.1	—	—	89.3 ± 1.8
Cage wash	61.8 ± 6.2				90.9 ± 1.6
Total	61.8 ± 6.2	17.2 ± 3.1	11.9 ± 3.9	0.024 ± 0.01	90.9 ± 1.6

[a] Four rats were examined; data are presented as cumulative percentage of dose (mean ± standard deviation).
[b] A single radiolabeled product was present as volatile organic chemicals in the exhaled breath. Analysis by reverse phase HPLC determined that it coeluted with benzene.
[c] Not measured at this time point.
[d] Intraperitoneal injection of 100 mg/kg 1-aminobenzotriazole was 4 hours prior to the single gavage dose of [^{14}C]-diazoaminobenzene.

TABLE A3
Cumulative Excretion of Radioactivity by Female F344/N Rats after a Single Gavage Dose of 20 mg/kg [^{14}C]-Diazoaminobenzene[a]

Time (hours after dosing)	Urine	Feces	Total
8	35.9 ± 29.0	—[b]	35.9 ± 29.0
24	76.3 ± 8.78	2.21 ± 2.21	78.5 ± 8.96
Cage wash	85.3 ± 4.90		87.5 ± 6.0
Total	85.3 ± 4.90	2.21 ± 2.21	87.5 ± 6.0

[a] Four rats were examined; data are presented as cumulative percentage of dose (mean ± standard deviation).
[b] Feces were not collected at this time point.

TABLE A4
Cumulative Excretion of Radioactivity by Male F344/N Rats after a Single Dermal Application of [^{14}C]-Diazoaminobenzene[a]

Dose	Time (hours after dosing)	Urine	Feces	Breath[b]	Total
2 mg/cm^2					
	8	0.41 ± 0.17	—[c]	0.05 ± 0.01	0.45 ± 0.18
	24	1.44 ± 0.38	0.09 ± 0.02	0.13 ± 0.03	1.66 ± 0.42
	48	2.96 ± 0.76	0.25 ± 0.05	0.24 ± 0.05	3.44 ± 0.86
	72	4.67 ± 1.19	0.41 ± 0.08	0.36 ± 0.09	5.44 ± 1.44
	Cage wash	4.85 ± 1.16			5.62 ± 1.31
	Total	4.85 ± 1.16	0.41 ± 0.08	0.36 ± 0.09	5.62 ± 1.31
20 mg/cm^2					
	8	0.05 ± 0.01	—	0.03 ± 0.00	0.07 ± 0.01
	24	0.16 ± 0.01	0.01 ± 0.00	0.05 ± 0.01	0.23 ± 0.01
	48	0.30 ± 0.02	0.04 ± 0.01	0.07 ± 0.01	0.41 ± 0.01
	72	0.44 ± 0.03	0.05 ± 0.01	0.08 ± 0.01	0.57 ± 0.02
	Cage wash	0.46 ± 0.03			0.60 ± 0.03
	Total	0.46 ± 0.03	0.05 ± 0.01	0.08 ± 0.01	0.60 ± 0.03

[a] Three rats were examined in the 2 mg/cm^2 group and four rats were examined in the 20 mg/cm^2 group; data are presented as cumulative percentage of dose (mean ± standard deviation).
[b] A single radiolabeled product was present as volatile organic chemicals in the exhaled breath. Analysis by reverse phase HPLC determined that it coeluted with benzene.
[c] Feces were not collected at this time point.

TABLE A5
Disposition of [^{14}C]-Diazoaminobenzene in Male F344/N Rats 72 Hours after a Single Dermal Application[a]

Site	2 mg/cm^2	20 mg/cm^2
Absorbed Dose		
Urine	4.85 ± 1.16	0.46 ± 0.03
Feces	0.41 ± 0.08	0.05 ± 0.01
Volatile breath	0.36 ± 0.09	0.08 ± 0.01
Dose site	0.70 ± 0.24	0.17 ± 0.19
Total for absorbed dose	6.32 ± 1.46	0.77 ± 0.19
Unabsorbed Dose		
Appliance cover	1.07 ± 0.81	0.21 ± 0.33
Gauze	1.07 ± 0.90	0.15 ± 0.19
Skin wash	77.0 ± 6.2	89.3 ± 5.7
Total for unabsorbed dose	79.1 ± 6.7	89.6 ± 5.3
Total Recovery	85.5 ± 7.9	90.3 ± 5.0

[a] Three rats were examined in the 2 mg/cm^2 group and four rats were examined in the 20 mg/cm^2 group; data are presented as percentage of dose (mean ± standard deviation).

TABLE A6
Urinary Metabolites in Male F344/N Rats after a Single Intravenous Injection of 2 mg/kg [^{14}C]-Diazoaminobenzene[a]

	Rat 1		Rat 2		Rat 3		Rat 4		Mean ± Standard Deviation
	0 to 8	8 to 24	0 to 8	8 to 24	0 to 8	8 to 24	0 to 8	8 to 24	0 to 24
Hydroquinone glucuronide	13.8	2.9	7.4	5.2	10.8	3.3	6.8	5.6	14.0 ± 2.0
Muconic acid	1.1	0.3	0.8	0.5	1.0	0.4	0.6	0.8	1.4 ± 0.1
Prephenyl mercapturic acid	1.6	0.5	1.3	0.9	1.7	0.6	1.0	1.3	2.2 ± 0.1
Phenol glucuronide	1.7	0.7	1.5	0.9	1.6	0.7	1.2	1.4	2.4 ± 0.1
4-Acetamidophenyl sulfate	15.1	9.6	12.7	12.8	16.3	9.5	8.3	16.2	25.1 ± 0.6
Phenyl sulfate	11.9	3.9	9.3	6.4	13.0	5.0	7.8	9.7	16.8 ± 1.2
Total identified metabolites	45.2	17.9	33.0	26.7	44.4	19.5	25.7	35.0	61.9 ± 2.0
Total in urine	59.8	23.1	41.8	33.8	57.4	24.8	33.8	45.8	80.1 ± 3.3

[a] Data are presented as percentage of dose recovered during the collection period (hours after dosing).

TABLE A7
Urinary Metabolites in Male F344/N Rats after a Single Gavage Dose of 20 mg/kg [^{14}C]-Diazoaminobenzene[a]

	Rat 1		Rat 2		Rat 3		Rat 4		Mean ± Standard Deviation
	0 to 8	8 to 24	0 to 8	8 to 24	0 to 8	8 to 24	0 to 8	8 to 24	0 to 24
No Pretreatment									
Hydroquinone glucuronide	4.0	2.2	5.1	1.9	4.8	1.8	5.3	1.5	6.7 ± 0.3
Muconic acid	1.4	1.0	2.4	0.4	1.4	0.9	1.5	0.7	2.4 ± 0.3
Prephenyl mercapturic acid	2.4	1.0	1.6	0.9	2.0	0.8	2.0	0.9	2.9 ± 0.4
Phenol glucuronide	1.7	0.4	0.6	0.6	2.3	1.1	1.8	0.5	2.3 ± 0.9
4-Acetamidophenyl sulfate	19.0	13.7	20.2	11.1	20.3	13.8	20.7	10.2	32.3 ± 1.5
Phenyl sulfate	9.5	6.4	10.1	4.7	10.1	4.6	10.6	3.9	15.0 ± 0.6
Total identified metabolites	38.0	24.7	40.0	19.6	40.9	23.0	41.9	17.7	61.5 ± 2.2
Total in urine	46.2	30.3	50.1	25.7	48.8	28.3	50.5	22.2	75.5 ± 2.0
Pretreatment with 1-Aminobenzotriazole[b]									
Hydroquinone glucuronide	0.8	1.7	0.4	2.5	0.5	1.5	0.5	1.5	2.4 ± 0.4
Muconic acid	0.3	0.7	0.1	0.9	0.2	0.5	0.2	0.7	0.9 ± 0.1
Prephenyl mercapturic acid	0.4	0.9	0.1	0.9	0.2	0.6	0.3	0.8	1.1 ± 0.2
Phenol glucuronide	0.4	0.8	0.3	1.0	0.8	0.9	1.6	0.5	1.6 ± 0.4
4-Acetamidophenyl sulfate	3.2	21.0	1.0	23.8	2.5	20.5	3.4	25.3	25.2 ± 2.5
Phenyl sulfate	1.6	1.9	1.0	3.1	2.7	2.9	6.8	3.0	5.8 ± 2.8
Total identified metabolites	6.7	27.0	2.9	32.2	6.9	26.9	12.8	31.8	36.8 ± 5.2
Total in urine	11.2	34.8	4.7	43.1	10.4	35.5	20.0	41.6	50.3 ± 7.6

[a] Data are presented as percentage of dose recovered during the collection period (hours after dosing).
[b] Intraperitoneal injection of 1-aminobenzotriazole (100 mg/kg) was 4 hours prior to the single oral gavage dose of [^{14}C]-diazoaminobenzene.

TABLE A8
Urinary Metabolites in a Male F344/N Rat after a Single Dermal Application of 2 mg/cm^2 [^{14}C]-Diazoaminobenzene[a]

	Collection Period (hours after dosing)			
	0 to 8	8 to 24	24 to 48	48 to 72
Hydroquinone glucuronide	0.056	0.086	0.195	0.238
Muconic acid	0.028	0.047	0.047	0.053
Prephenyl mercapturic acid	0.028	0.054	0.073	0.078
Phenol glucuronide	0.015	0.022	0.039	0.042
4-Acetamidophenyl sulfate	0.207	0.435	0.607	0.721
Phenyl sulfate	0.092	0.179	0.261	0.280
Total identified metabolites	0.426	0.823	1.22	1.41
Total in urine	0.56	1.06	1.55	1.74

[a] Data are presented as percentage of dose recovered during the collection period.

TABLE A9
Tissue Distribution of Radioactivity in F344/N Rats 24 Hours after a Single Gavage Dose of 20 mg/kg [^{14}C]-Diazoaminobenzene[a]

	Tissue	Diazoaminobenzene Equivalents in Tissue (ng/g)	Tissue-to-Blood Ratio	Dose in Total Tissue (%)
Male				
	Adipose	438 ± 113	0.320 ± 0.098	0.155 ± 0.041
	Blood	1,380 ± 80	—[b]	0.361 ± 0.016
	Kidney	1,910 ± 150	1.38 ± 0.10	0.067 ± 0.006
	Liver	912 ± 30	0.662 ± 0.047	0.184 ± 0.002
	Muscle	123 ± 11	0.089 ± 0.010	0.297 ± 0.030
	Skin	229 ± 11	0.166 ± 0.006	0.196 ± 0.007
	Spleen	671 ± 25	0.485 ± 0.008	0.007 ± 0.001
Female				
	Adipose	803 ± 443	0.579 ± 0.256	0.273 ± 0.150
	Blood	1,370 ± 300	—	0.346 ± 0.075
	Kidney	2,430 ± 440	1.80 ± 0.15	0.084 ± 0.016
	Liver	1,090 ± 140	0.830 ± 0.195	0.182 ± 0.025
	Muscle	137 ± 17	0.104 ± 0.027	0.319 ± 0.039
	Skin	253 ± 43	0.191 ± 0.043	0.210 ± 0.034
	Spleen	976 ± 184	0.722 ± 0.076	0.012 ± 0.002

[a] Three male and four female rats were examined; data are presented as mean ± standard deviation.
[b] Unity

TABLE A10
Concentration of Diazoaminobenzene and Diazoaminobenzene Metabolites in Blood Extracts of F344/N Rats after a Single Gavage Dose of 20 mg/kg [^{14}C]-Diazoaminobenzene[a]

	Time (hours after dosing)					
	0.25	0.5	1	2	4	6
Male						
Diazoaminobenzene	144 ± 50	90 ± 61	66 ± 69	57 ± 95	26 ± 24	18 ± 14
Diazoaminobenzene metabolites						
Hydroquinone glucuronide	1,637 ± 445	2,181 ± 547	2,049 ± 498	839 ± 393	318 ± 102	89 ± 38
Muconic acid	123 ± 17	196 ± 48	247 ± 80	144 ± 74	110 ± 55	32 ± 12
Prephenyl mercapturic acid	162 ± 12	255 ± 43	244 ± 41	102 ± 52	49 ± 20	22 ± 12
Phenol glucuronide	45 ± 9	89 ± 19	69 ± 20	35 ± 24	18 ± 8	14 ± 6
4-Acetamidophenyl sulfate	645 ± 45	1,324 ± 155	1,727 ± 223	1,261 ± 39	536 ± 62	366 ± 82
Phenyl sulfate	950 ± 141	1,908 ± 294	2,008 ± 482	1,324 ± 233	336 ± 30	210 ± 36
Benzene	69 ± 31	135 ± 63	178 ± 120	124 ± 91	58 ± 21	51 ± 13
Phenol	97 ± 145	190 ± 259	200 ± 235	109 ± 121	18 ± 19	15 ± 15
Aniline	320 ± 114	382 ± 83	247 ± 95	102 ± 60	22 ± 18	27 ± 12
Female						
Diazoaminobenzene	305 ± 165	199 ± 201	92 ± 176	34 ± 176	7 ± 176	14 ± 175
Diazoaminobenzene metabolites						
Hydroquinone glucuronide	1,320 ± 409	2,865 ± 857	2,941 ± 1,143	893 ± 428	136 ± 53	73 ± 15
Muconic acid	315 ± 118	546 ± 49	692 ± 35	620 ± 53	146 ± 72	102 ± 21
Prephenyl mercapturic acid	266 ± 53	386 ± 66	411 ± 71	172 ± 34	43 ± 1	19 ± 3
Phenol glucuronide	69 ± 26	121 ± 21	101 ± 19	39 ± 6	17 ± 10	8 ± 4
4-Acetamidophenyl sulfate	531 ± 49	864 ± 88	1,210 ± 68	1,539 ± 139	649 ± 38	305 ± 13
Phenyl sulfate	1,179 ± 207	2,200 ± 381	2,494 ± 251	2,271 ± 519	523 ± 104	162 ± 40
Benzene	78 ± 69	111 ± 49	115 ± 25	103 ± 21	78 ± 15	53 ± 13
Phenol	25 ± 8	21 ± 6	26 ± 8	13 ± 9	7 ± 7	2 ± 2
Aniline	324 ± 242	633 ± 217	521 ± 332	139 ± 74	38 ± 11	10 ± 7

[a] Three male and four female rats were examined; data are presented as ng-Equivalents diazoaminobenzene per gram of blood.

TABLE A11
Cumulative Excretion of Radioactivity by Male B6C3F$_1$ Mice after a Single Intravenous Injection of 2 mg/kg [^{14}C]-Diazoaminobenzene[a]

Time (hours after dosing)	Urine	Feces	Breath[b]	Total
8	12.5 ± 17.0	—[c]	0.55 ± 0.29	13.1 ± 17.2
24	27.2 ± 10.5	11.4 ± 3.5	0.74 ± 0.31	39.7 ± 10.9
48	36.7 ± 8.5	17.2 ± 6.0	0.93 ± 0.34	54.8 ± 7.4
72	43.4 ± 6.5	22.9 ± 5.6	1.02 ± 0.36	69.4 ± 5.2
Cage wash	56.7 ± 7.0			80.6 ± 5.3
Total	56.7 ± 7.0	22.9 ± 5.6	1.02 ± 0.36	80.6 ± 5.3

[a] Four mice were examined; data are presented as cumulative percentage of dose (mean ± standard deviation).
[b] A single radiolabeled product was present as volatile organic chemicals in the exhaled breath. Analysis by reverse phase HPLC determined that it coeluted with benzene.
[c] Feces were not collected at this time point.

TABLE A12
Cumulative Excretion of Radioactivity by Male B6C3F$_1$ Mice after a Single Gavage Dose of 20 mg/kg [^{14}C]-Diazoaminobenzene[a]

Time (hours after dosing)	Urine	Feces	Breath[b]	CO_2	Total
8	4.54 ± 8.82	—[c]	0.73 ± 0.17	0.16 ± 0.03	5.43 ± 8.85
24	44.3 ± 21.8	15.2 ± 6.7	0.87 ± 0.20	0.21 ± 0.05	60.6 ± 16.9
48	54.6 ± 17.5	18.5 ± 9.6	0.98 ± 0.25	0.23 ± 0.06	74.3 ± 11.8
72	60.9 ± 15.0	20.0 ± 10.8	1.03 ± 0.27	0.24 ± 0.07	82.2 ± 10.5
Cage wash	68.3 ± 11.6				89.6 ± 4.40
Total	68.3 ± 11.6	20.0 ± 10.8	1.03 ± 0.27	0.24 ± 0.07	89.6 ± 4.40

[a] Four mice were examined; data are presented as cumulative percentage of dose (mean ± standard deviation).
[b] A single radiolabeled product was present as volatile organic chemicals in the exhaled breath. Analysis by reverse phase HPLC determined that it coeluted with benzene.
[c] Feces were not collected at this time point.

TABLE A13
Cumulative Excretion of Radioactivity by Male B6C3F$_1$ Mice after a Single Dermal Application of [^{14}C]-Diazoaminobenzene[a]

Dose	Time (hours after dosing)	Urine	Feces	Breath[b]	Total
2 mg/cm^2					
	8	0.00 ± 0.00	—[c]	0.10 ± 0.11	0.10 ± 0.11
	24	0.59 ± 0.25	0.42 ± 0.06	0.19 ± 0.14	1.20 ± 0.32
	48	1.02 ± 0.43	1.04 ± 0.31	0.30 ± 0.19	1.92 ± 1.07
	72	1.92 ± 1.07	2.40 ± 0.84	0.48 ± 0.26	4.80 ± 0.26
	Cage wash	3.87 ± 1.32			6.76 ± 1.31
	Total	3.87 ± 1.32	2.40 ± 0.84	0.48 ± 0.26	6.76 ± 1.31
20 mg/cm^2					
	8	0.01 ± 0.01	—	0.02 ± 0.01	0.02 ± 0.01
	24	0.08 ± 0.01	0.07 ± 0.06	0.04 ± 0.01	0.20 ± 0.05
	48	0.18 ± 0.01	0.17 ± 0.05	0.07 ± 0.01	0.42 ± 0.04
	72	0.26 ± 0.01	0.36 ± 0.07	0.09 ± 0.01	0.71 ± 0.08
	Cage wash	0.55 ± 0.05			1.00 ± 0.11
	Total	0.55 ± 0.05	0.36 ± 0.07	0.09 ± 0.01	1.00 ± 0.11

[a] Four mice were examined per dose group; data are presented as cumulative percentage of dose (mean ± standard deviation).
[b] A single radiolabeled product was present as volatile organic chemicals in the exhaled breath. Analysis by reverse phase HPLC determined that it coeluted with benzene.
[c] Feces were not collected at this time point.

TABLE A14
Disposition of [^{14}C]-Diazoaminobenzene in Male B6C3F$_1$ Mice 72 Hours after a Single Dermal Application[a]

Site	2 mg/cm^2	20 mg/cm^2
Absorbed Dose		
Urine	3.87 ± 1.32	0.55 ± 0.05
Feces	2.40 ± 0.84	0.36 ± 0.07
Volatile breath	0.48 ± 0.26	0.09 ± 0.01
Dose site	0.28 ± 0.05	0.19 ± 0.17
Total for absorbed dose	7.04 ± 1.30	1.19 ± 0.23
Unabsorbed Dose		
Appliance cover	1.01 ± 1.58	0.12 ± 0.05
Gauze	0.41 ± 0.35	0.18 ± 0.15
Skin wash	78.0 ± 3.69	63.0 ± 6.2
Total for unabsorbed dose	79.4 ± 2.2	63.3 ± 6.2
Total Recovery	86.4 ± 2.8	64.5 ± 6.3

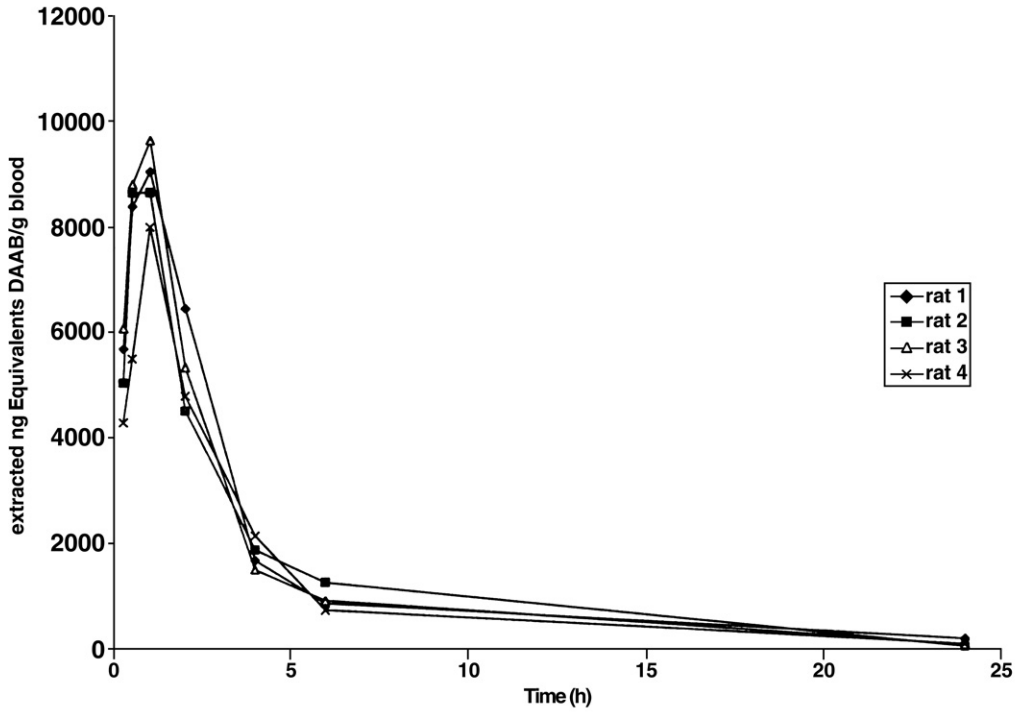
[a] Four mice were examined per dose group; data are presented as percentage of dose (mean ± standard deviation).

TABLE A15
Urinary Metabolites in Male B6C3F$_1$ Mice Administered 20 mg/kg of [^{14}C]-Diazoaminobenzene[a]

Metabolite	Percent of Urinary Radioactivity
Hydroquinone glucuronide	13.0 ± 3.1
Muconic acid	3.9 ± 0.9
Phenol	4.7 ± 0.5
4-acetamidophenyl glucuronide	23.6 ± 2.3
2-aminophenyl sulfate	1.5 ± 2.8
4-acetamidophenyl sulfate	4.5 ± 0.8
2-acetamidophenyl sulfate	ND[b]
4-acetamidophenol	0.8 ± 1.0
2-acetamidophenol	5.0 ± 1.1

[a] Urine collected 0 to 24 hours after oral administration to four mice; data are given as mean ± standard error.
[b] None detected. (Mathews and De Costa, 1999)

a. Total Radioactivity



b. Diazoaminobenzene

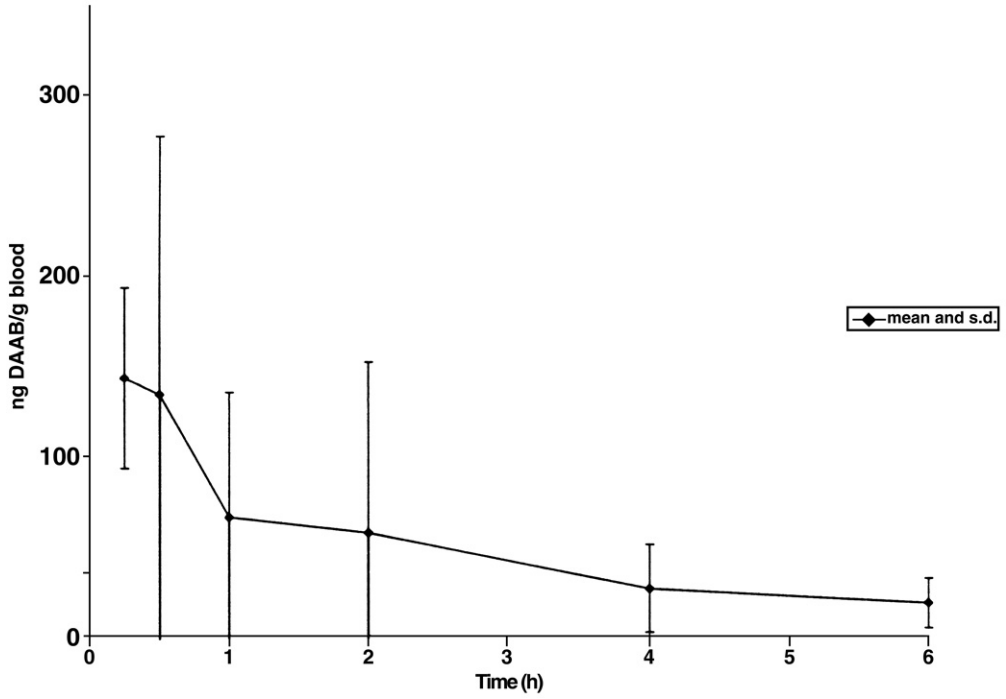
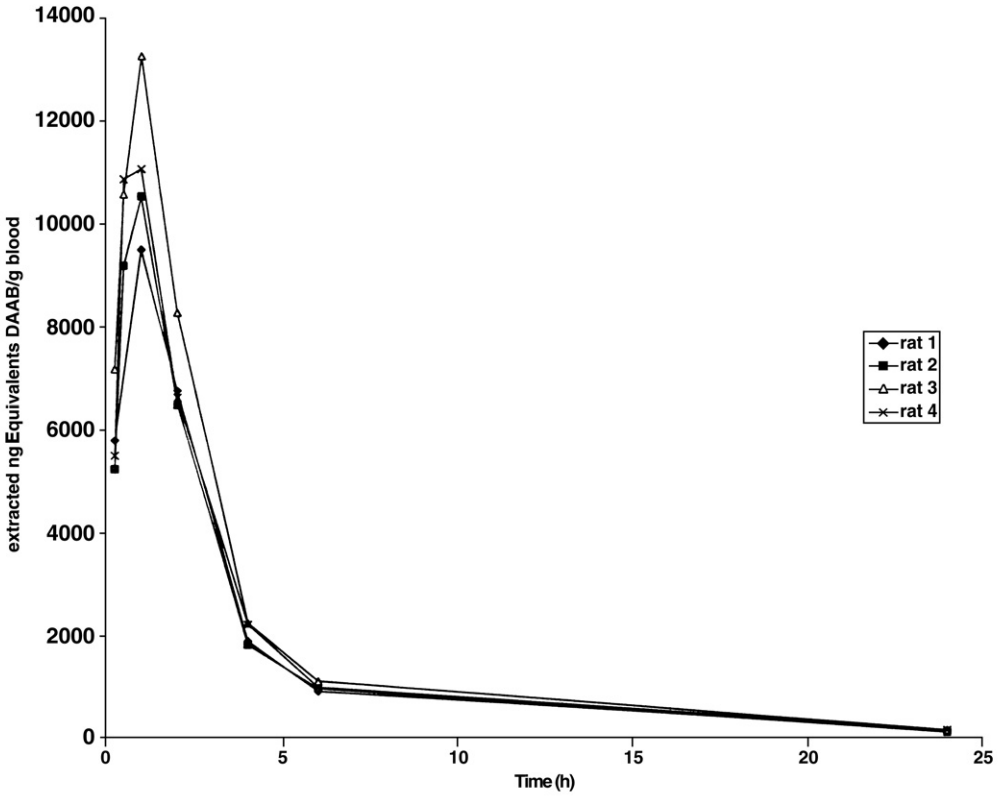


FIGURE A1
Concentration of (a) Total Diazoaminobenzene-Derived Compounds and (b) Diazoaminobenzene in the Blood of Male F344/N Rats after a Single Gavage Dose of 20 mg/kg [^{14}C]-Diazoaminobenzene

a. Total Radioactivity



b. Diazoaminobenzene

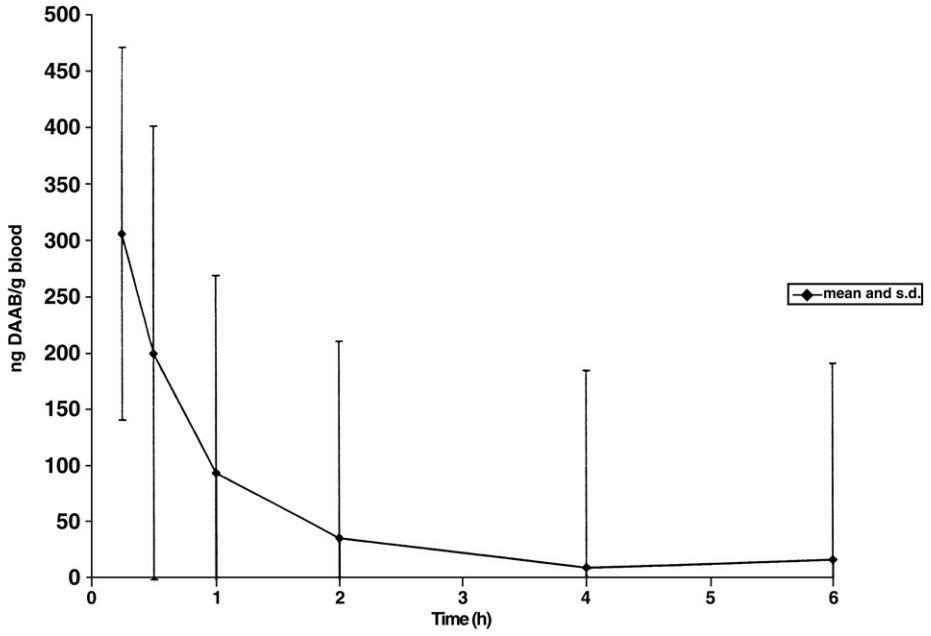
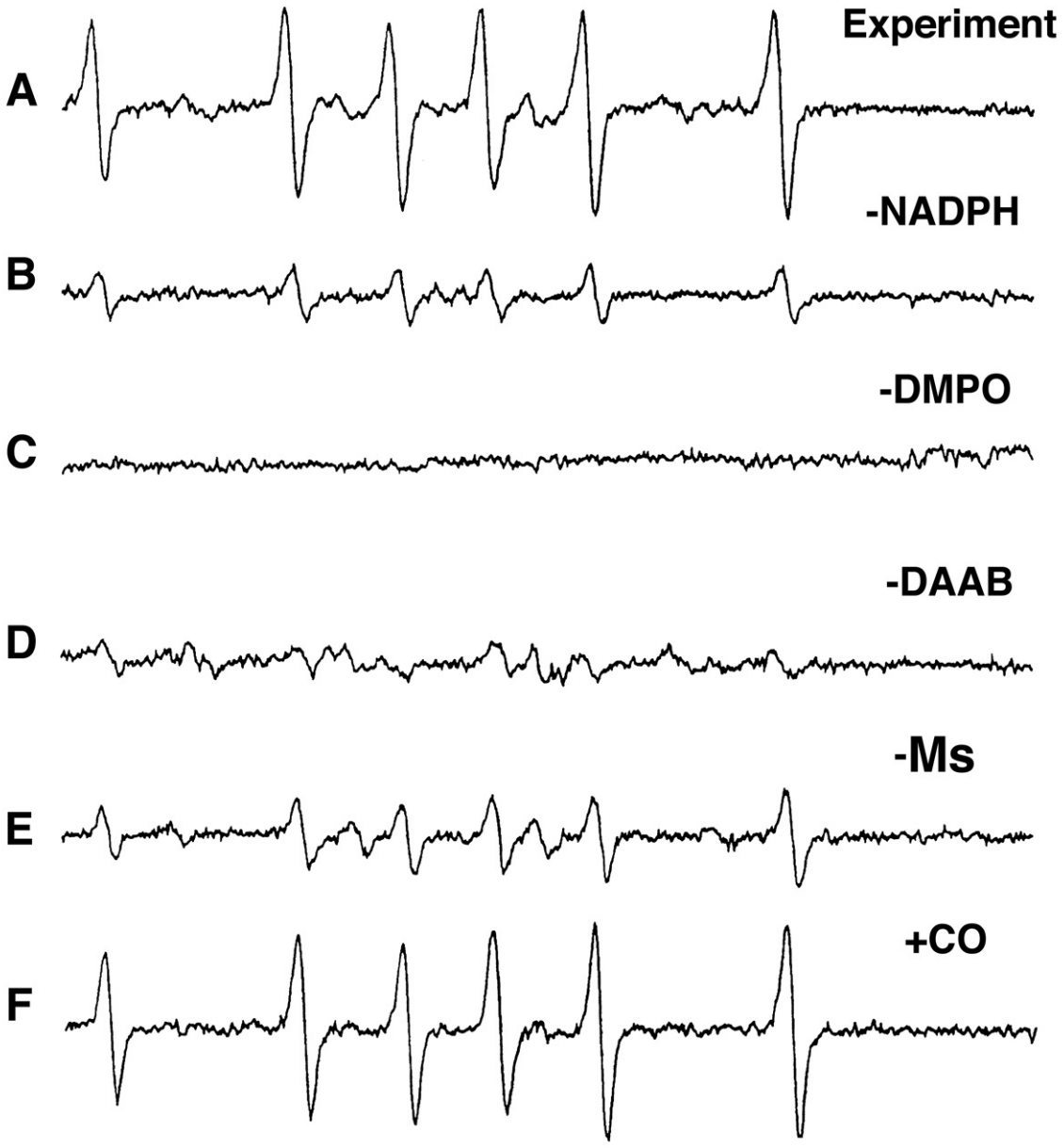
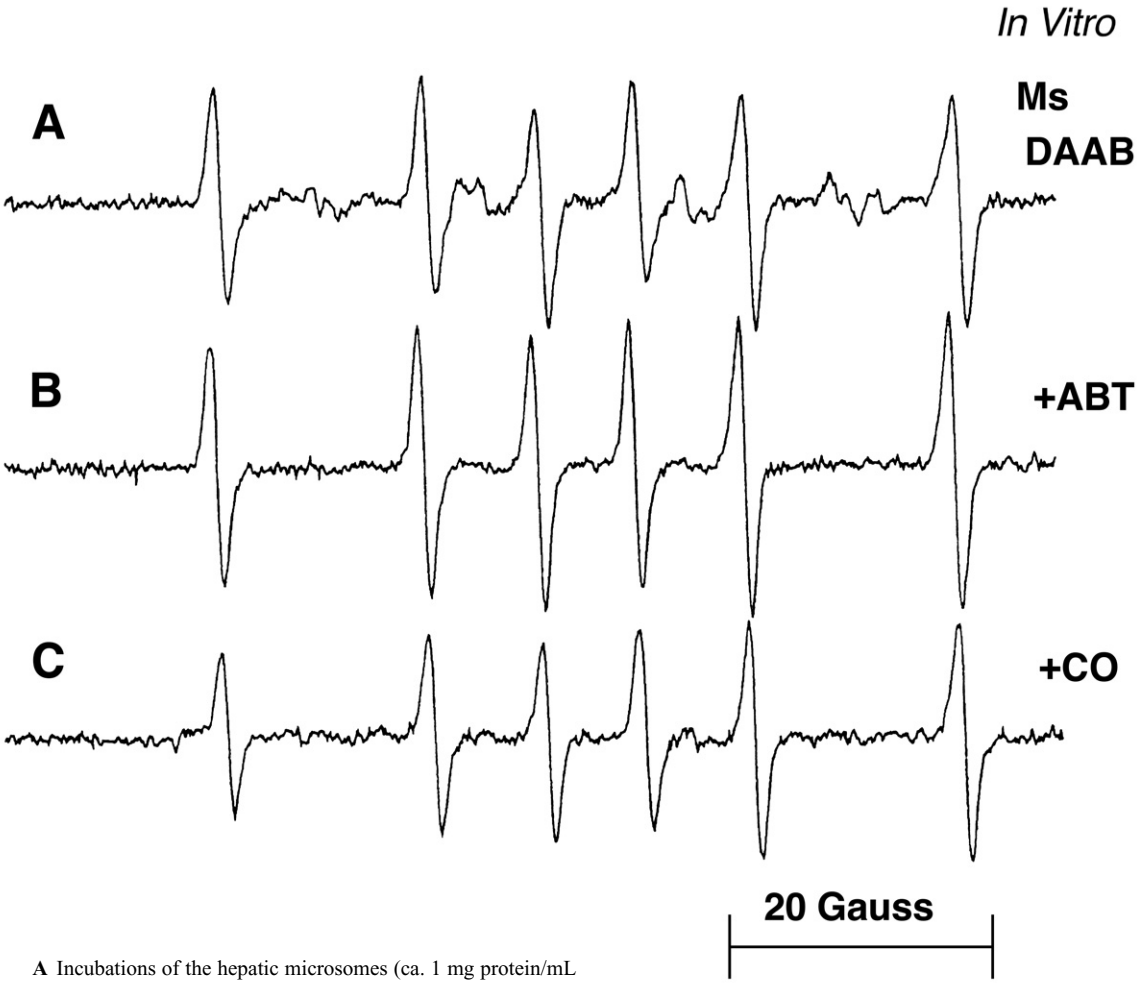


FIGURE A2
Concentration of (a) Total Diazoaminobenzene-Derived Compounds and (b) Diazoaminobenzene in the Blood of Female F344/N Rats after a Single Gavage Dose of 20 mg/kg [^{14}C]-Diazoaminobenzene



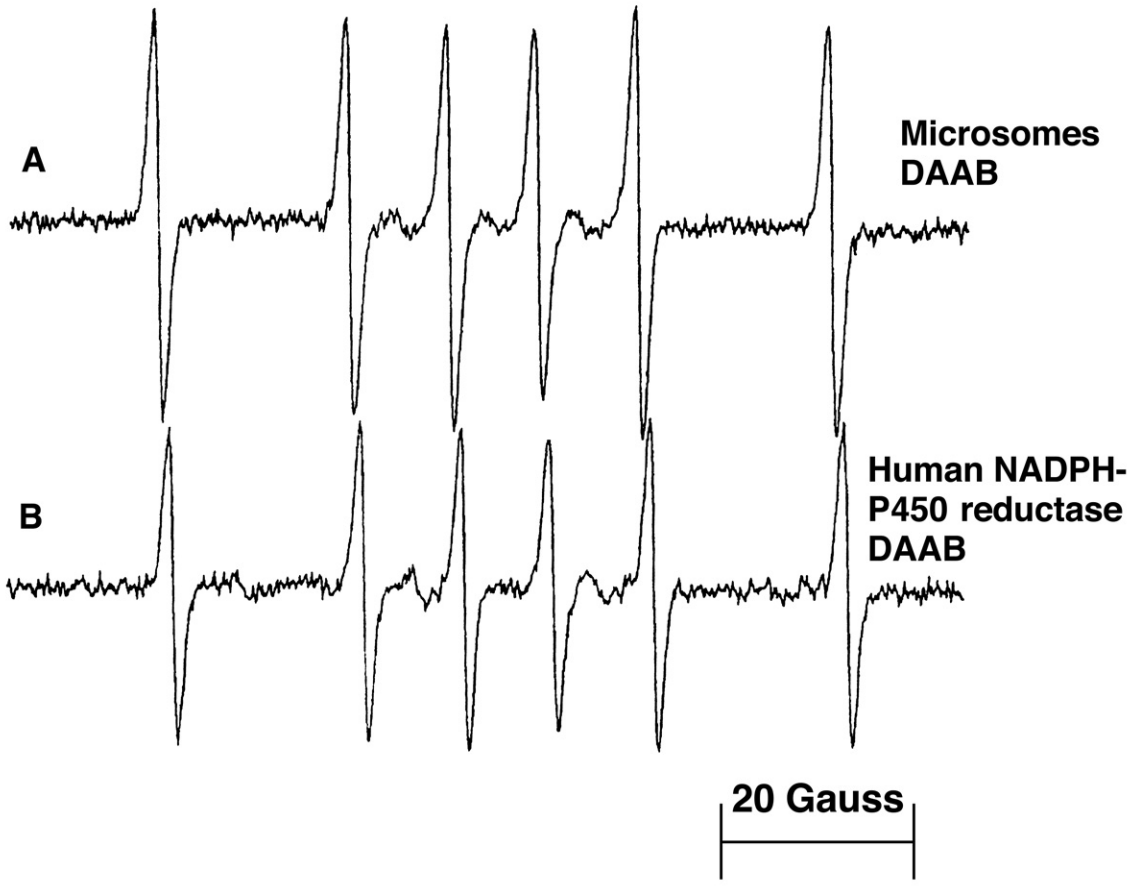
A Incubations of the hepatic microsomes (ca. 1 mg protein/mL 100 mM phosphate buffer) with diazoaminobenzene (25 mM final concentration), DMPO (200 mM), and NADPH (1 mM)
B All components of A except NADPH
C All components of A except DMPO
D All components of A except diazoaminobenzene
E All components of A except hepatic microsomes
F All components of A, microsomes were saturated with carbon monoxide prior to incubation.

FIGURE A3
Electron Spin Resonance Spectra of Phenyl Radical Adducts Detected in Rat Hepatic Microsomes



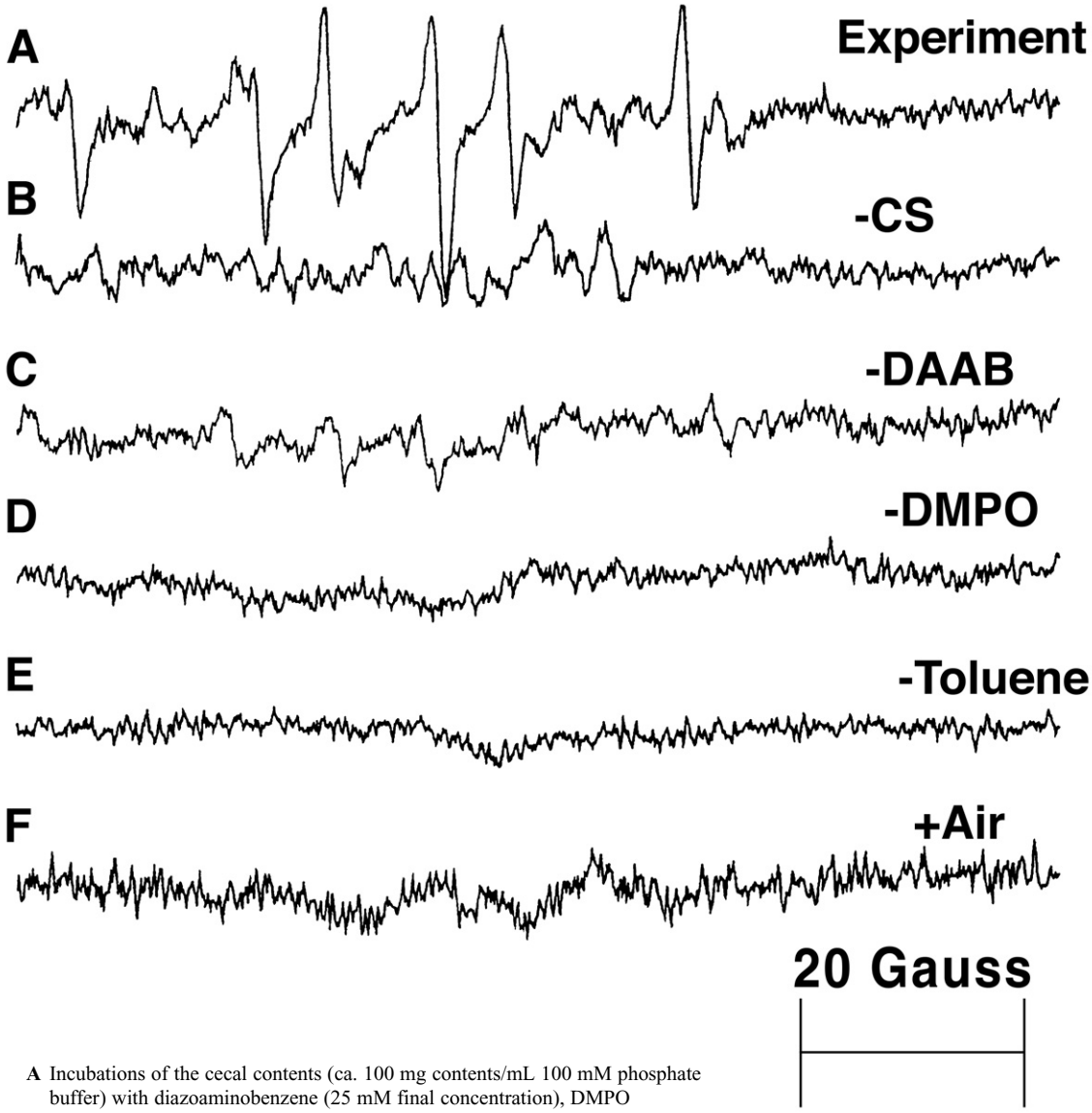
A Incubations of the hepatic microsomes (ca. 1 mg protein/mL 100 mM phosphate buffer) with diazoaminobenzene (25 mM final concentration), DMPO (200 mM), and NADPH (1 mM)
B All components of A, microsomes were preincubated for 15 minutes with NADPH and 1-aminobenzotriazole (ABT) prior to incubation with diazoaminobenzene.
C All components of A, microsomes were saturated with carbon monoxide prior to incubation.

FIGURE A4
Effect of Carbon Monoxide and 1-Aminobenzotriazole on the Electron Spin Resonance Spectrum of Phenyl Radical Adducts Detected in Rat Hepatic Microsomes



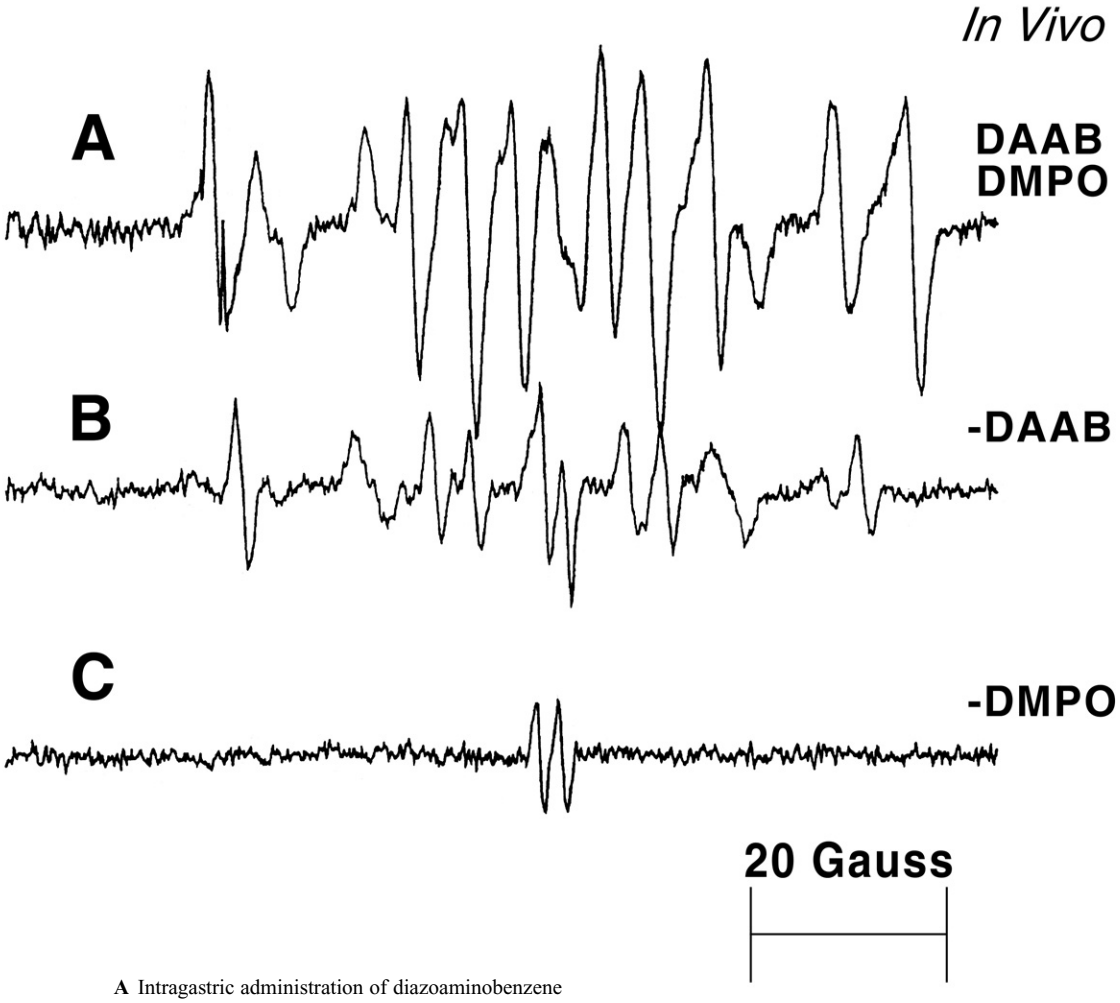
A Incubations of the hepatic microsomes (ca. 1mg protein/mL 100 mM phosphate buffer) with diazoaminobenzene (25 mM final concentration), DMPO (200 mM), and NADPH (1 mM)
B All components of **A** except human P450 reductase (2.4 pmol/mL 100 mM phosphate buffer) were substituted for hepatic microsomes.

FIGURE A5
Effect of Human NADPH-P450 Reductase on the Electron Spin Resonance Spectrum of Phenyl Radical Adducts Detected in Rat Hepatic Microsomes



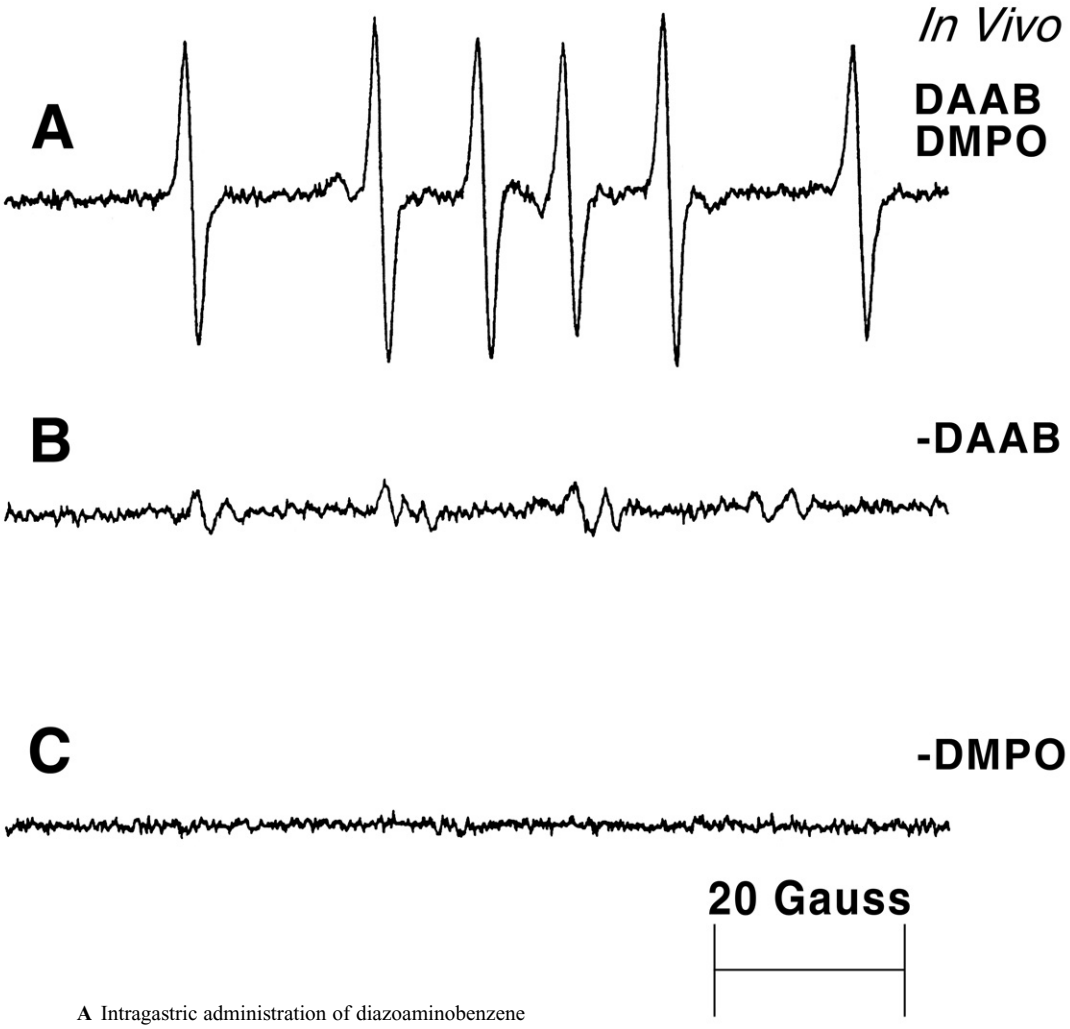
A Incubations of the cecal contents (ca. 100 mg contents/mL 100 mM phosphate buffer) with diazoaminobenzene (25 mM final concentration), DMPO (200 mM), under nitrogen gas and extraction of the incubation mixture with nitrogen-sparged toluene
B All components of A except cecal contents
C All components of A except diazoaminobenzene
D All components of A except DMPO
E All components of A but no toluene extraction
F All components of A but incubated in the presence of air

FIGURE A6
Electron Spin Resonance Spectra of Phenyl Radical Adducts Detected in Rat Cecal Contents



A Intragastric administration of diazoaminobenzene formulated in Emulphor EL-620 and water (2:8), DMPO (1 g/kg, ip)
B Intragastric administration of Emulphor EL-620 and water (2:8), DMPO (1 g/kg, ip)
C Intragastric administration of diazoaminobenzene formulated in Emulphor EL-620 and water (2:8)

FIGURE A7
Electron Spin Resonance Spectra of Phenyl Radical Adducts Detected in Bile of a Male F344/N Rat 21 to 40 Minutes after a Single Gavage Dose of 16 mg/kg Diazoaminobenzene



A Intragastric administration of diazoaminobenzene formulated in Emulphor EL-620 and water (2:8), DMPO (1 g/kg, ip)
B Intragastric administration of Emulphor EL-620 and water (2:8), DMPO (1 g/kg, ip)
C Intragastric administration of diazoaminobenzene formulated in Emulphor EL-620 and water (2:8)

FIGURE A8
Electron Spin Resonance Spectra of Phenyl Radical Adducts Detected in Bile of a Male F344/N Rat 41 to 60 Minutes after a Single Gavage Dose of 16 mg/kg Diazoaminobenzene

APPENDIX B
16-DAY TOXICITY STUDIES
IN F344/N RATS AND B6C3F$_1$ MICE

16-DAY TOXICITY STUDIES IN F344/N RATS AND B6C3F$_1$ MICE

INTRODUCTION

Studies were conducted in male and female F344/N rats and B6C3F$_1$ mice to obtain toxicity information on diazoaminobenzene when administered dermally for 16 days. These studies were conducted by BioReliance (Rockville, MD).

MATERIALS AND METHODS

Procurement and Characterization of Diazoaminobenzene

Diazoaminobenzene was obtained from the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO), in one lot (MRI 051997KH). Identity and purity analyses were conducted by the analytical chemistry laboratory and the study laboratory. Reports on analyses performed in support of the diazoaminobenzene studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a light brown, crystalline powder, was identified as diazoaminobenzene by the analytical chemistry laboratory using infrared and nuclear magnetic resonance spectroscopy. The spectra were consistent with the literature spectra (Smith and Ho, 1990; Shaaban *et al*., 1993). Identity was confirmed by the study laboratory using infrared spectrophotometry. The spectrum was consistent with the literature spectrum (*Aldrich,* 1981) of diazoaminobenzene. The infrared and nuclear magnetic resonance spectra are presented in Figures B1 and B2.

The purity of lot MRI 051997KH was determined by the analytical chemistry laboratory and the study laboratory using high-performance liquid chromatography (HPLC) systems A and B, respectively (Table B1). HPLC system A indicated a major peak and two impurities with a combined area of approximately 1.9% relative to the major peak area. No impurities were detected by HPLC system B. The overall purity was determined to be greater than 98%.

Stability studies of the bulk chemical were not performed. Information provided by the manufacturer indicated that diazoaminobenzene was stable as a bulk chemical when stored protected from heat, direct sunlight, and oxidizing agents. To ensure stability, the bulk chemical was stored frozen, under a nitrogen headspace, and in a dry, dark, and well-ventilated area protected from physical damage.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared once by mixing diazoaminobenzene and acetone to give the required concentrations (Table B2). The dose formulations were sonicated, placed in vials, sealed under a nitrogen headspace, and stored refrigerated at 2° to 8° C.

Stability studies of 3 and 100 mg/mL dose formulations were performed by the analytical chemistry laboratory using HPLC system C (Table B1). Stability was confirmed for at least 35 days for samples stored frozen or at room temperature in the dark. Stability was confirmed for at least 3 hours for samples stored under animal room conditions (room temperature, open to air and light).

Analyses of the dose formulations of diazoaminobenzene were conducted by the study laboratory at the beginning of the studies using HPLC system D (Tables B1 and B3). All five dose formulations for rats or mice were within 10% of the target concentrations. In addition, animal room samples collected at the end of the studies were analyzed. All five animal room samples for rats and three of five for mice were within 10% of the target concentrations.

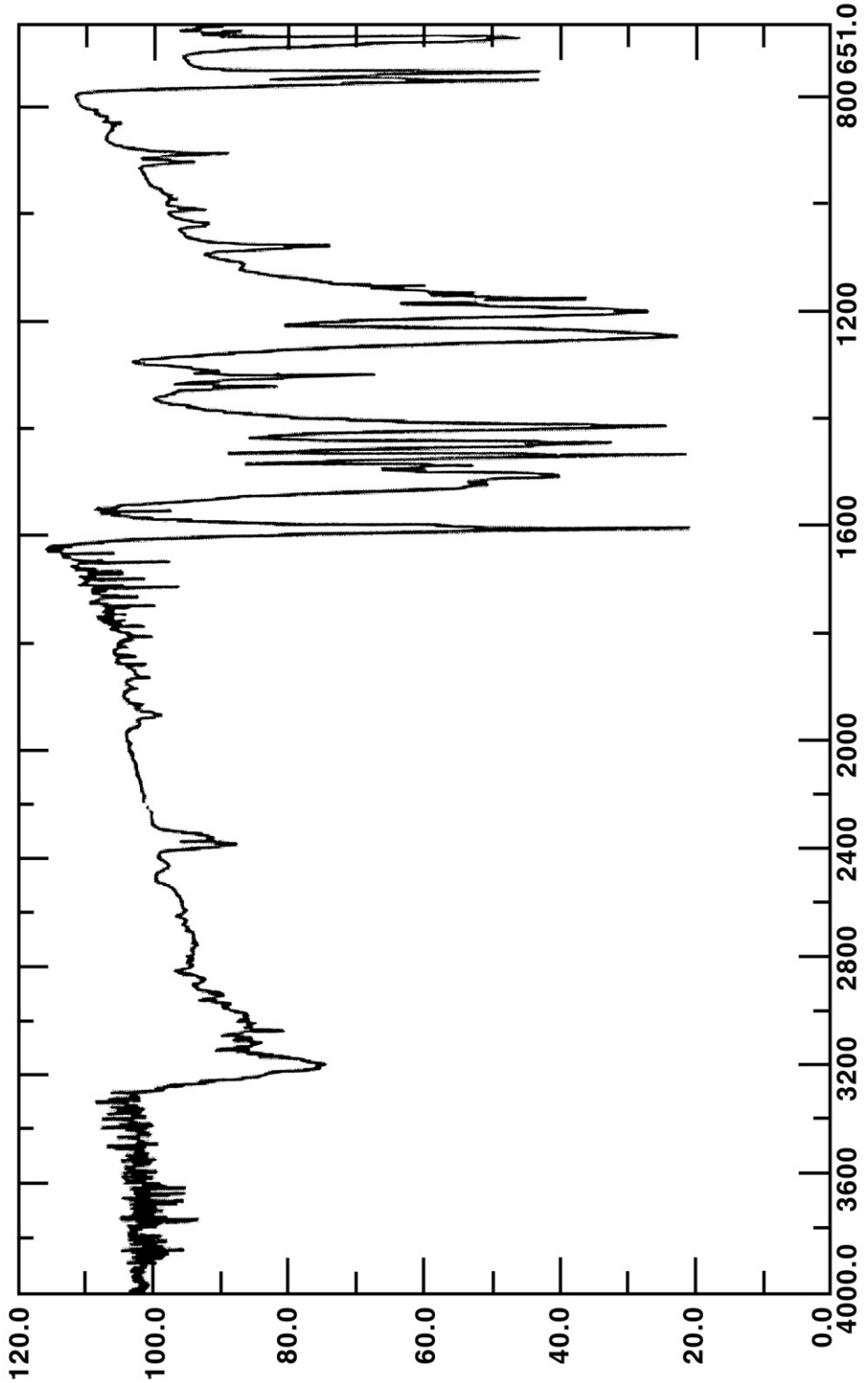


FIGURE B1
Infrared Absorption Spectrum of Diazoaminobenzene

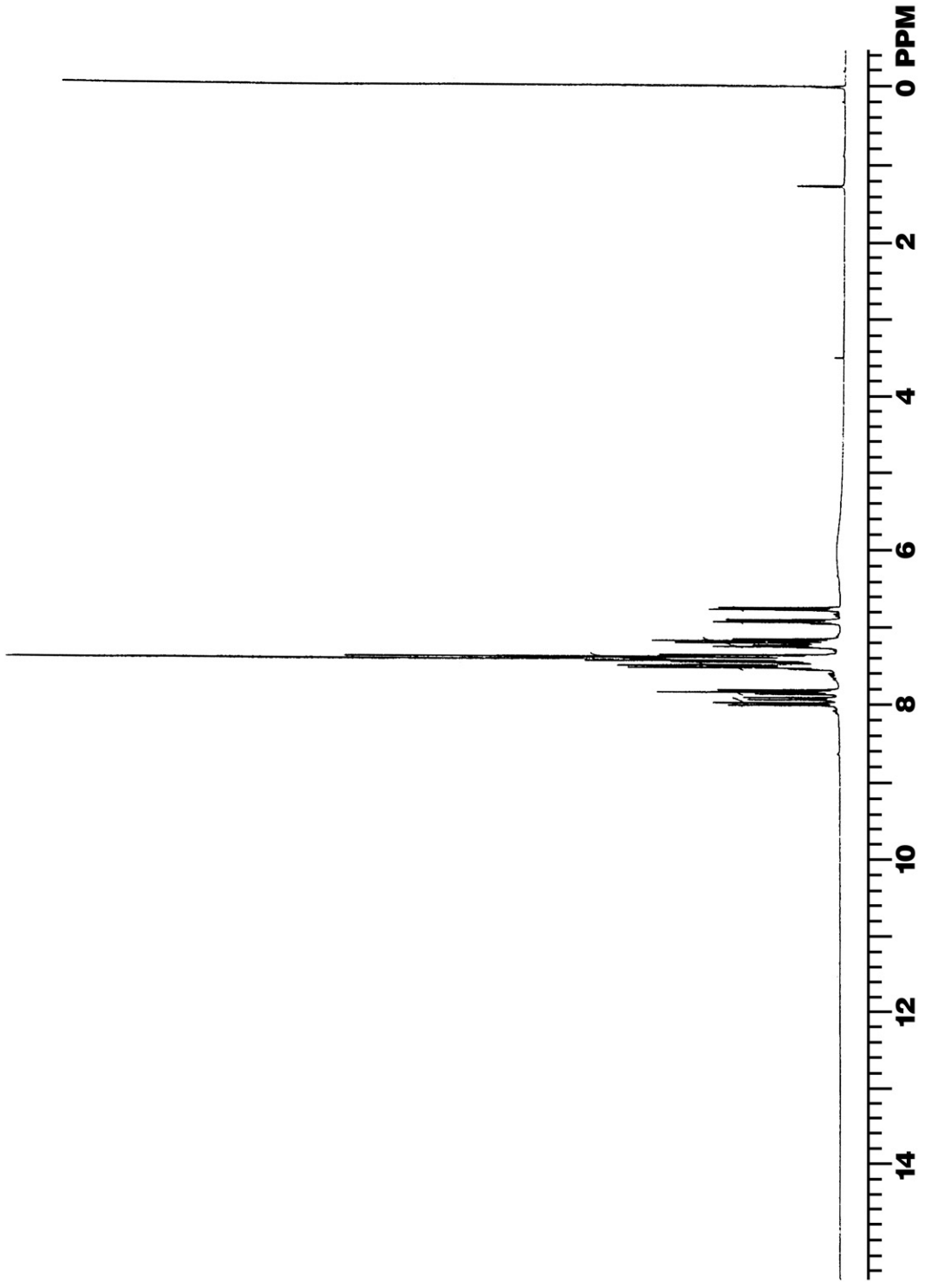


FIGURE B2
Nuclear Magnetic Resonance Spectrum of Diazoaminobenzene

TABLE B1
High-Performance Liquid Chromatography Systems Used in the 16-Day Dermal Studies of Diazoaminobenzene[a]

Detection System	Column	Solvent System
System A		
Ultraviolet (365 nm) light	Hypersil ODS, 25 cm × 3.2 mm, 5 µm (Thermo Hypersil-Keystone, Cheshire, UK)	Aqueous ammonium acetate (1.5% weight/volume):acetonitrile (40:60); flow rate 0.75 mL/minute
System B		
Ultraviolet (254 nm) light	Alltech Hypersil C18, 250 mm × 4.6 mm, 5 µm (Thermo Hypersil-Keystone)	Aqueous ammonium acetate (1.5% weight/volume):acetonitrile (40:60); flow rate 1.5 mL/minute
System C		
Ultraviolet (365 nm) light	C18 ODS, 250 mm × 4.6 mm, 5 µm (Burdick & Jackson, Muskegon, MI)	Water with 0.1% triethylamine:acetonitrile (30:70); flow rate 1.0 mL/minute
System D		
Ultraviolet (365 nm) light	C18 ODS, 250 mm × 4.6 mm, 5 µm (Burdick & Jackson)	Water with 0.1% triethylamine:acetonitrile (30:70); flow rate 2.2 mL/minute

[a] High-performance liquid chromatographs were manufactured by Waters Corp. (Millford, MA) (system A), Hewlett-Packard (Palo Alto, CA) (systems B and D), and Spectra-Physics (Mountain View, CA) (system C).

TABLE B2
Preparation and Storage of Dose Formulations in the 16-Day Dermal Studies of Diazoaminobenzene

Preparation
Doses formulations were prepared by mixing diazoaminobenzene with acetone and sonicating. The doses were mixed once.

Chemical Lot Number
MRI 051997KH

Maximum Storage Time
16 days

Storage Conditions
Stored in vials sealed under a nitrogen headspace in and refrigerated at 2° to 8° C

Study Laboratory
BioReliance (Rockville, MD)

TABLE B3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 16-Day Dermal Studies of Diazoaminobenzene

Date Prepared	Date Analyzed	Target Concentration (mg/mL)	Determined Concentration[a] (mg/mL)	Difference from Target (%)
Rats				
December 22, 1997	December 22, 1997	25	23.5	–6
		50	50.8	+2
		100	105	+5
		200	209	+5
		400	417	+4
	January 16, 1998[b]	25	25.2	+1
		50	50.3	+1
		100	91.4	–9
		200	179	–10
		400	377	–6
Mice				
December 22, 1997	December 22, 1997	6.25	6.33	+1
		12.5	12.6	+1
		25	23.5	–6
		50	50.8	+2
		100	105	+5
	January 16, 1998[b]	6.25	7.21	+15
		12.5	14.0	+12
		25	24.8	–1
		50	53.7	+7
		100	96.8	–3

[a] Results of duplicate analyses. Dosing volume = 0.5 mL/kg (rats) or 2.0 mL/kg (mice)
[b] Animal room samples

Study Design

Male and female F344/N rats and B6C3F$_1$ mice were obtained from Taconic Farms (Germantown, NY). On receipt, the rats and mice were 3 to 4 weeks old. Animals were quarantined for 13 or 14 days and were 6 weeks old on the first day of the study. Groups of five male and five female rats and mice received dermal application of diazoaminobenzene at concentrations of 0, 12.5, 25, 50, 100, or 200 mg diazoaminobenzene/kg body weight in acetone, 5 days per week for 16 days. Feed and water were available *ad libitum*. Rats and mice were housed individually. Clinical findings were recorded on dosing days. The animals were weighed initially, on day 8, and at the end of the studies. Details of the study design and animal maintenance are summarized in Table B4.

Blood was collected from the retroorbital sinus of all animals surviving to the end of studies for hematology analyses. Rats and mice were anesthetized with carbon dioxide during a 3- to 5-hour collection period. Methemoglobin concentration was measured within 30 minutes using an IL 682 CO-Oximeter (Instrumentation Laboratory, Inc., Lexington, MA). Erythrocyte, leukocyte, and platelet counts; hematocrit values; hemoglobin concentration; mean cell volume; mean cell hemoglobin; and mean cell hemoglobin concentration were determined using a Serono-Baker System 9010 hematology analyzer (Serono-Baker Diagnostics, Allentown, PA). Differential leukocyte smears were air dried, fixed in absolute methanol, stained with Wright's stain, and evaluated microscopically. Reticulocyte smears were stained with methylene blue. Heinz body smears were stained with crystal violet stain, counterstained with Wright's stain, and allowed to air dry before being evaluated microscopically. The parameters measured are listed in Table B4.

Necropsies were performed on all animals. The heart, right kidney, liver, lung, spleen, right testis, and thymus were weighed. Histopathologic examinations were performed on all vehicle control rats and mice, 200 mg/kg rats, 25 mg/kg and greater male mice, and 100 and 200 mg/kg female mice. Additionally, all gross lesions and selected tissues of rats and mice in other dose groups were examined. Table B4 lists the tissues and organs examined.

TABLE B4
Experimental Design and Materials and Methods in the 16-Day Dermal Studies of Diazoaminobenzene

Study Laboratory
BioReliance (Rockville, MD)

Strain and Species
Rats: F344/N
Mice: $B6C3F_1$

Animal Source
Taconic Farms (Germantown, NY)

Time Held Before Studies
Rats: 13 days
Mice: 14 days

Average Age When Studies Began
6 weeks

Date of First Dose
Rats: December 29, 1997
Mice: December 30, 1997

Duration of Dosing
5 days per week for 16 days

Date of Last Dose
Rats: January 13, 1998
Mice: January 14, 1998

Necropsy Dates
Rats: January 14, 1998
Mice: January 15, 1998

Average Age at Necropsy
8 weeks

Size of Study Groups
5 males and 5 females

Method of Distribution
Animals were distributed randomly into groups of approximately equal initial mean body weights.

Animals per Cage
1

Method of Animal Identification
Tail tattoo

Diet
Irradiated NTP-2000 open formula pelleted diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*

Water
Tap water (Washington Suburban Sanitary Commission Potomac Plant) via automatic watering system (Edstrom Industries, Inc., Waterford, WI), available *ad libitum*

Cages
Polycarbonate (Lab Products, Inc., Seaford, DE), changed weekly

TABLE B4
Experimental Design and Materials and Methods in the 16-Day Dermal Studies of Diazoaminobenzene

Bedding
Heat-treated Sani-Chip® (P.J. Murphy Forest Products, Montville, NJ), changed once weekly

Cage Filters
Reemay® 2016 (Snow Filtration, West Chester, OH), changed every 2 weeks

Racks
Stainless steel (Lab Products, Inc., Seaford, DE), changed every 2 weeks

Animal Room Environment
Temperature: 72° ± 3° F
Relative humidity: 50% ± 15%
Room fluorescent light: 12 hours/day
Room air changes: 10/hour

Doses
0, 12.5, 25, 50, 100, and 200 mg/kg in acetone

Type and Frequency of Observation
Observed twice daily; animals were weighed initially, on day 8, and at the end of the studies; clinical findings were recorded on dosing days.

Method of Sacrifice
Carbon dioxide asphyxiation

Necropsy
Necropsy was performed on all animals. Organs weighed were the heart, right kidney, liver, lung, spleen, right testis, and thymus.

Hematology
Blood was collected from the retroorbital sinus from all animals surviving to the end of the studies for hematology analyses: hematocrit; hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; erythrocyte morphology; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; leukocyte count and differentials; methemoglobin; and Heinz bodies.

Histopathology
Complete histopathology was performed on all vehicle control rats and mice, 200 mg/kg rats, 25 mg/kg and greater male mice, and 100 and 200 mg/kg female mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone with marrow, brain, clitoral gland, esophagus, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lungs and mainstem bronchi, lymph nodes (mandibular and mesenteric), mammary gland with adjacent skin, muscle, nasal cavity and turbinates, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate, salivary gland, skin (site of application), spleen, stomach (forestomach and glandular), testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, and uterus. Additionally, the heart, kidney, liver, mesenteric lymph node, skin (site of application), spleen, and thymus were examined in all remaining dose groups of rats and mice, the mandibular lymph node, stomach (forestomach and glandular), and testis were examined in 12.5 mg/kg male mice, and the forestomach and mandibular lymph node were examined in remaining dose groups of female mice.

Statistical Methods

Calculation and Analysis of Lesion Incidences

The incidences of lesions are presented in Tables B8 and B12 as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. The Fisher exact test, a procedure based on the overall proportion of affected animals, was used to determine significance (Gart *et al.,* 1979).

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables (Piegorsch and Bailer, 1997). Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Hematology data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1951) were examined by NTP personnel, and implausible values were eliminated from the analysis. Average severity values were analyzed for significance with the Mann-Whitney U test (Hollander and Wolfe, 1973).

RESULTS

Diazoaminobenzene was not lethal to rats at any of the concentrations tested (Table B5). In contrast, in the second week of the study, most male mice administered 50 mg/kg or greater and three female mice administered 200 mg/kg died (Table B9). Body weight gains of all dosed groups of rats were significantly less than those of the vehicle controls (Table B5). Mice administered 50 mg/kg or greater lost weight during the study (Table B9). Final mean body weights and body weight gains of female mice administered 50 mg/kg or greater were significantly less than those of the vehicle controls.

Thymus weights were significantly decreased in all dosed groups of rats and female mice, and in 25 mg/kg male mice (Tables B6 and B10). Spleen weights were increased in 100 and 200 mg/kg rats. Heart weights were significantly increased in 25 mg/kg male mice and in female mice administered 50 mg/kg or greater. Kidney weights were increased in female mice administered 50 mg/kg or greater. Relative liver weights were significantly increased in all dosed groups of male rats, female rats administered 25 mg/kg or greater, and 12.5 mg/kg mice. Other organ weight changes were likely associated with body weight changes.

Clinical pathology data indicated a chemical related methemoglobinemia and Heinz body formation (Tables B7 and B11). In rats and female mice, Heinz body formation was increased and considered to be chemically related. There was a treatment related decrease in erythroid mass evidenced by a decrease in hematocrit, hemoglobin, and erythrocyte counts suggesting a developing anemia. The erythron decrease was accompanied by an increased bone marrow response as indicated by increased reticulocytes in rats and mice and nucleated erythrocytes in rats. In mice only, the higher dose females had an increase in hemoglobin concentrations that would appear to be an inappropriate response compared to other estimates of red cell mass; this may have been a spurious result related to the increased number of Heinz bodies. Associated with the developing anemia was an increase in mean cell hemoglobin concentrations that would be consistent with the increased hemoglobin and possibly intravascular hemolyses related to Heinz body formation.

Gross observations at necropsy were limited to thickening of the skin at the site of application. Microscopically, this corresponded to hyperplasia of the epidermis and hair follicles which was evident in all dosed groups (Tables B8 and B12). Proliferation of hair follicles was a particularly prominent change of marked severity in the higher dose groups, characterized by an extensive area of the application site containing an increased density of

hair follicles. This sometimes formed a raised, plaque-like lesion with a scalloped surface due to coalescense of dilated follicles containing multiple hair shafts. In other areas the interfollicular epidermis was thickened with variable cystic or hyaline type degeneration in the stratum corneum. A slight mixed inflammatory cell infiltrate accompanied the hyperplastic change. Focal epidermal ulceration at the site of application was present in some female mice in the higher dose groups.

Various internal nonneoplastic lesions were observed and considered to be related to chemical treatment (Tables B8 and B12). Lymphoid atrophy of the thymus (a depletion of cortical lymphocytes) was a common lesion of mild to marked severity in treated rats and mice and corresponded to reduced thymus weights. A similar loss of lymphoid tissue was variably seen in the mesenteric and mandibular lymph nodes as well as in the white pulp of the spleen. Presumably, as a response to anemia, increased incidences of hematopoietic cell proliferation of generally mild severity occurred in the splenic red pulp of treated rats and mice and correlated with increased spleen weights.

Several other microscopic findings in mice were considered related to treatment, many occurring in early death animals. Atrial thrombosis of the heart was present and typically seen as a solid coagulum of proteinaceous material and embedded blood cells in the left auricle in all mice that died early. No myocardial changes were evident in either thrombotic hearts of early death animals or in survivors with increased heart weights. Renal tubule necrosis was found in early death male mice as well as in 100 mg/kg female mice that survived to study termination. Focal liver necrosis was found in most early death mice.

TABLE B5
Survival and Body Weights of Rats in the 16-Day Dermal Study of Diazoaminobenzene

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	83 ± 4	151 ± 8	69 ± 5	
12.5	5/5	82 ± 4	137 ± 5	55 ± 2**	90
25	5/5	83 ± 3	137 ± 7	54 ± 4**	90
50	5/5	82 ± 4	132 ± 6	50 ± 3**	87
100	5/5	80 ± 1	132 ± 3	52 ± 3**	87
200	5/5	83 ± 5	134 ± 6	51 ± 2**	88
Female					
0	5/5	70 ± 2	113 ± 3	44 ± 2	
12.5	5/5	69 ± 2	103 ± 4	34 ± 4**	90
25	5/5	70 ± 4	103 ± 4	33 ± 2**	91
50	5/5	70 ± 4	103 ± 5	33 ± 2**	91
100	5/5	69 ± 3	100 ± 5	31 ± 2**	88
200	5/5	69 ± 2	100 ± 3	31 ± 2**	88

** Significantly different ($P \leq 0.01$) from the vehicle control group by Williams' test
[a] Number of animals surviving at 16 days/number initially in group
[b] Weights and weight changes are given as mean ± standard error.

TABLE B6
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 16-Day Dermal Study of Diazoaminobenzene[a]

	Vehicle Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male						
n	5	5	5	5	5	4
Necropsy body wt	151 ± 8	137 ± 5	137 ± 7	132 ± 6	132 ± 3	135 ± 8
Heart						
Absolute	0.600 ± 0.019	0.614 ± 0.022	0.628 ± 0.021	0.620 ± 0.030	0.694 ± 0.031	0.649 ± 0.034
Relative	3.980 ± 0.095	4.484 ± 0.114**	4.611 ± 0.151**	4.709 ± 0.124**	5.242 ± 0.150**	4.811 ± 0.124**
R. Kidney						
Absolute	0.659 ± 0.036	0.653 ± 0.018	0.619 ± 0.023	0.620 ± 0.032	0.618 ± 0.016	0.668 ± 0.037
Relative	4.348 ± 0.056	4.774 ± 0.110*	4.539 ± 0.072*	4.700 ± 0.091*	4.680 ± 0.106*	4.948 ± 0.048**
Liver						
Absolute	7.040 ± 0.387	6.802 ± 0.220	7.079 ± 0.319	7.254 ± 0.380	7.216 ± 0.226	7.274 ± 0.304
Relative	46.477 ± 0.345	49.716 ± 1.116*	51.802 ± 0.691**	55.061 ± 1.665**	54.605 ± 1.082**	54.028 ± 1.274**
Lung						
Absolute	1.462 ± 0.162	1.435 ± 0.105	1.485 ± 0.129	1.615 ± 0.086	1.679 ± 0.084	1.616 ± 0.208
Relative	9.620 ± 0.800	10.514 ± 0.826	10.938 ± 1.030	12.328 ± 0.729*	12.750 ± 0.751*	11.835 ± 1.021
Spleen						
Absolute	0.424 ± 0.031	0.479 ± 0.016	0.491 ± 0.021	0.492 ± 0.022	0.557 ± 0.023**	0.652 ± 0.045**
Relative	2.794 ± 0.112	3.498 ± 0.086**	3.595 ± 0.099**	3.747 ± 0.144**	4.212 ± 0.136**	4.825 ± 0.133**
R. Testis						
Absolute	0.928 ± 0.059	0.931 ± 0.027	0.882 ± 0.057	0.850 ± 0.048	0.917 ± 0.026	0.861 ± 0.121
Relative	6.124 ± 0.220	6.810 ± 0.179	6.432 ± 0.126	6.445 ± 0.183	6.946 ± 0.154	6.268 ± 0.574
Thymus						
Absolute	0.416 ± 0.019	0.179 ± 0.008**	0.128 ± 0.015**	0.113 ± 0.010**	0.127 ± 0.010**	0.104 ± 0.006**
Relative	2.761 ± 0.138	1.316 ± 0.083**	0.946 ± 0.126**	0.853 ± 0.056**	0.962 ± 0.079**	0.773 ± 0.016**

TABLE B6
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 16-Day Dermal Study of Diazoaminobenzene

	Vehicle Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female						
n	5	5	5	5	5	5
Necropsy body wt	113 ± 3	103 ± 4	103 ± 4	103 ± 5	100 ± 5	100 ± 3
Heart						
Absolute	0.468 ± 0.011	0.455 ± 0.010	0.486 ± 0.018	0.473 ± 0.017	0.495 ± 0.015	0.472 ± 0.011
Relative	4.130 ± 0.036	4.456 ± 0.104*	4.724 ± 0.088**	4.598 ± 0.124**	4.955 ± 0.081**	4.734 ± 0.046**
R. Kidney						
Absolute	0.541 ± 0.022	0.496 ± 0.026	0.517 ± 0.017	0.509 ± 0.018	0.507 ± 0.020	0.500 ± 0.017
Relative	4.761 ± 0.117	4.826 ± 0.087	5.026 ± 0.166	4.943 ± 0.104	5.089 ± 0.221	5.018 ± 0.097
Liver						
Absolute	5.166 ± 0.232	4.944 ± 0.347	5.308 ± 0.170	5.377 ± 0.182	5.470 ± 0.235	5.157 ± 0.113
Relative	45.473 ± 1.177	48.013 ± 1.928	51.602 ± 1.163**	52.258 ± 0.954**	54.628 ± 0.404**	51.799 ± 0.949**
Lung						
Absolute	0.834 ± 0.047	0.829 ± 0.054	0.905 ± 0.026	0.997 ± 0.141	0.915 ± 0.033	0.856 ± 0.021
Relative	7.330 ± 0.250	8.065 ± 0.350	8.810 ± 0.305	9.619 ± 1.138*	9.157 ± 0.201*	8.612 ± 0.289*
Spleen						
Absolute	0.329 ± 0.011	0.321 ± 0.028	0.338 ± 0.009	0.380 ± 0.013	0.443 ± 0.029**	0.439 ± 0.015**
Relative	2.898 ± 0.063	3.110 ± 0.190	3.292 ± 0.081*	3.698 ± 0.100**	4.410 ± 0.088**	4.409 ± 0.132**
Thymus						
Absolute	0.358 ± 0.013	0.134 ± 0.015**	0.118 ± 0.007**	0.089 ± 0.005**	0.085 ± 0.005**	0.085 ± 0.004**
Relative	3.153 ± 0.074	1.293 ± 0.110**	1.150 ± 0.087**	0.866 ± 0.017**	0.853 ± 0.060**	0.849 ± 0.034**

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' test
** $P \leq 0.01$
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE B7
Hematology Data for Rats in the 16-Day Dermal Study of Diazoaminobenzene[a]

	Vehicle Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male						
n[b]	3	1[c]	1[c]	4	2	0
Hematocrit (%)	44.6 ± 1.4	42.3	43.6	41.3 ± 0.7*	41.5 ± 0.6	
Hemoglobin (g/dL)	15.4 ± 0.5	14.5	14.6	14.0 ± 0.2*	14.1 ± 0.1	
Erythrocytes (10^6/μL)	7.73 ± 0.23	7.51	7.77	7.26 ± 0.11	7.18 ± 0.11	
Reticulocytes (10^6/μL)	0.29 ± 0.04	0.35	0.40	0.42 ± 0.02*	0.55 ± 0.03**	
Nucleated erythrocytes/ 100 leukocytes	1.67 ± 0.67	1.00	1.00	3.75 ± 1.38	1.00 ± 0.00	
Mean cell volume (fL)	58.0 ± 0.0	56.0	56.0	57.3 ± 0.3	58.0 ± 0.0	
Mean cell hemoglobin (pg)	20.0 ± 0.0	19.3	18.8	19.4 ± 0.2*	19.7 ± 0.2	
Mean cell hemoglobin concentration (g/dL)	34.6 ± 0.1	34.3	33.5	34.0 ± 0.3	34.0 ± 0.3	
Platelets (10^3/μL)	730.3 ± 49.0	621.0	504.0	587.3 ± 18.7[d]	620.5 ± 40.5	
Leukocytes (10^3/μL)	11.00 ± 1.74	7.40	9.50	7.78 ± 0.75	8.55 ± 1.55	
Segmented neutrophils (10^3/μL)	0.98 ± 0.19	1.78	1.33	1.26 ± 0.23	1.14 ± 0.37	
Bands (10^3/μL)	0.00 ± 0.00	0.00	0.00	0.00 ± 0.00	0.00 ± 0.00	
Lymphocytes (10^3/μL)	9.60 ± 1.50	5.33	7.03	6.18 ± 0.79	6.67 ± 1.00	
Monocytes (10^3/μL)	0.39 ± 0.12	0.30	1.14	0.30 ± 0.08	0.70 ± 0.21	
Basophils (10^3/μL)	0.000 ± 0.000	0.000	0.000	0.000 ± 0.000	0.000 ± 0.000	
Eosinophils (10^3/μL)	0.03 ± 0.03	0.00	0.00	0.04 ± 0.02	0.04 ± 0.04	
Methemoglobin (% hemoglobin)	0.85 ± 0.05[e]	1.40	2.10	3.28 ± 0.09	4.00 ± 0.90	
Heinz bodies (10^3/μL)	0	0	0	2 ± 2	8 ± 8	

TABLE B7
Hematology Data for Rats in the 16-Day Dermal Study of Diazoaminobenzene

	Vehicle Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female						
n	4	5	5	4	5	5
Hematocrit (%)	45.3 ± 1.7	44.4 ± 1.7	42.8 ± 1.1	40.7 ± 1.2*	39.7 ± 1.1**	39.2 ± 0.6**
Hemoglobin (g/dL)	15.6 ± 0.6	15.2 ± 0.5	14.5 ± 0.4	13.8 ± 0.3**	13.5 ± 0.3**	13.5 ± 0.2**
Erythrocytes (10^6/μL)	7.58 ± 0.32	7.69 ± 0.32	7.51 ± 0.20	7.14 ± 0.22	6.74 ± 0.14*	6.63 ± 0.11*
Reticulocytes (10^6/μL)	0.15 ± 0.02	0.27 ± 0.06	0.21 ± 0.02	0.27 ± 0.03*	0.33 ± 0.03**	0.32 ± 0.06**
Nucleated erythrocytes/ 100 leukocytes	0.25 ± 0.25	0.80 ± 0.37	0.80 ± 0.20	1.25 ± 0.48	3.00 ± 1.41*	6.40 ± 2.54*
Mean cell volume (fL)	60.0 ± 0.4	57.8 ± 0.4	56.8 ± 0.2**	57.3 ± 0.5*	58.8 ± 0.6	59.2 ± 0.7
Mean cell hemoglobin (pg)	20.6 ± 0.3	19.8 ± 0.2	19.4 ± 0.1*	19.4 ± 0.3*	20.0 ± 0.2	20.3 ± 0.2
Mean cell hemoglobin concentration (g/dL)	34.4 ± 0.3	34.4 ± 0.3	34.0 ± 0.2	34.0 ± 0.5	34.0 ± 0.2	34.4 ± 0.3
Platelets (10^3/μL)	583.0 ± 47.6	450.6 ± 44.9	424.3 ± 28.2[f]	438.0 ± 37.3	420.0 ± 35.3	395.2 ± 26.1*
Leukocytes (10^3/μL)	12.48 ± 0.62	9.06 ± 1.00	8.72 ± 1.09*	9.38 ± 1.29	9.06 ± 0.72	11.22 ± 0.83
Segmented neutrophils (10^3/μL)	0.96 ± 0.07	1.06 ± 0.13	1.05 ± 0.19	1.02 ± 0.12	1.12 ± 0.20	1.36 ± 0.23
Bands (10^3/μL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/μL)	10.90 ± 0.94	7.76 ± 0.95	7.03 ± 0.77*	7.84 ± 1.05	7.59 ± 0.67	9.34 ± 0.64
Monocytes (10^3/μL)	0.58 ± 0.33	0.25 ± 0.08	0.64 ± 0.17	0.49 ± 0.15	0.35 ± 0.05	0.52 ± 0.09
Basophils (10^3/μL)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/μL)	0.03 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
Methemoglobin (% hemoglobin)	0.65 ± 0.13	1.44 ± 0.11*	2.26 ± 0.16**	3.55 ± 0.36**	4.56 ± 0.28**	5.08 ± 0.69**
Heinz bodies (10^3/μL)	0	0	0	2 ± 2	3 ± 2	12 ± 4**

* Significantly different ($P \leq 0.05$) from the vehicle control group by Dunn's or Shirley's test
** $P \leq 0.01$
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.
[b] Five blood samples were collected for analysis, but due to specimen clotting the number of samples was less.
[c] No standard error calculated
[d] n=3
[e] n=2
[f] n=4

TABLE B8
Incidences of Selected Nonneoplastic Lesions in Rats in the 16-Day Dermal Study of Diazoaminobenzene

	Vehicle Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male						
Skin, Site of Application[a]	5	5	5	5	5	5
Hyperplasia[b]	0	5** (1.6)[c]	5** (2.0)	5** (2.0)	5** (2.0)	5** (2.2)
Inflammation	0	5** (1.6)	5** (1.8)	3** (1.3)	5** (1.4)	5** (1.0)
Hair Follicle, Hyperplasia	0	4* (1.3)	5** (2.8)	5** (3.2)	5** (3.6)	5** (4.0)
Epidermis, Degeneration	0	0	5** (2.8)	5** (3.0)	5** (2.2)	5** (2.8)
Thymus	5	5	5	5	5	5
Atrophy	0	5** (1.0)	5** (2.6)	5** (2.4)	5** (3.0)	5** (3.0)
Lymph Node, Mesenteric	5	4	5	5	5	5
Atrophy	0	2 (1.5)	2 (2.0)	2 (2.0)	3 (1.0)	5** (2.2)
Spleen	5	5	5	5	5	5
Hematopoietic Cell Proliferation	5 (1.2)	5 (1.0)	5 (1.2)	5 (1.6)	5 (2.0)	5 (2.0)
Female						
Skin, Site of Application	5	5	5	5	5	5
Hyperplasia	0	5** (1.8)	5** (2.0)	5** (2.2)	5** (2.0)	5** (2.0)
Inflammation	0	5** (1.8)	5** (1.0)	5** (1.0)	5** (1.0)	5** (1.0)
Hair Follicle, Hyperplasia	0	5** (2.2)	5** (3.0)	5** (3.0)	5** (2.6)	5** (3.0)
Epidermis, Degeneration	0	2 (2.0)	3 (2.3)	5** (3.2)	5** (3.0)	5** (2.4)
Thymus	5	5	5	5	5	5
Atrophy	0	4* (1.0)	5** (1.2)	5** (2.6)	5** (2.8)	5** (3.0)
Lymph Node, Mesenteric	5	5	5	5	5	5
Atrophy	0	0	0	0	0	4* (1.0)
Spleen	5	5	5	5	5	5
Hematopoietic Cell Proliferation	0	4* (1.0)	5** (1.0)	5** (1.2)	5** (2.0)	5** (1.8)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Fisher exact test
** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

TABLE B9
Survival and Body Weights of Mice in the 16-Day Dermal Study of Diazoaminobenzene

Dose (mg/kg)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)
		Initial	Final	Change	
Male					
0	5/5	22.5 ± 0.5	24.6 ± 0.5	2.1 ± 0.1	
12.5	5/5	22.8 ± 0.8	25.2 ± 0.8	2.4 ± 0.1	102
25	5/5	21.0 ± 1.2	23.3 ± 0.5	2.3 ± 0.9	95
50	1/5[c]	23.2 ± 0.9	21.5	–4.0	87
100	0/5[d]	22.9 ± 0.8	—	—	—
200	0/5[e]	23.2 ± 0.6	—	—	—
Female					
0	5/5	18.9 ± 0.8	21.5 ± 0.8	2.6 ± 0.3	
12.5	5/5	19.0 ± 0.5	21.8 ± 0.4	2.8 ± 0.4	101
25	5/5	18.5 ± 0.6	21.3 ± 0.4	2.8 ± 0.3	99
50	5/5	19.4 ± 0.5	18.3 ± 0.7**	–1.1 ± 0.6**	85
100	5/5	18.6 ± 0.5	16.6 ± 1.0**	–2.0 ± 0.6**	77
200	2/5[f]	18.5 ± 0.1	17.4 ± 1.1**	–1.0 ± 1.0**	81

** Significantly different ($P \leq 0.01$) from the vehicle control group by Williams' test
[a] Number of animals surviving at 16 days/number initially in group
[b] Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study. No final mean body weights were calculated for groups with 100% mortality.
[c] Day of death: 11, 11, 11, 14
[d] Day of death: 8, 8, 9, 9, 10
[e] Day of death: 8, 8, 9, 9, 9
[f] Day of death: 8, 10, 11

TABLE B10
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 16-Day Dermal Study of Diazoaminobenzene[a]

	Vehicle Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male						
n	5	5	5	1[b]	0[c]	0[c]
Necropsy body wt	24.6 ± 0.5	25.2 ± 0.8	23.3 ± 0.5	21.5		
Heart						
Absolute	0.121 ± 0.003	0.138 ± 0.004*	0.147 ± 0.005**	0.187		
Relative	4.939 ± 0.102	5.503 ± 0.164	6.335 ± 0.294**	8.698		
R. Kidney						
Absolute	0.210 ± 0.004	0.244 ± 0.010**	0.226 ± 0.006	0.241		
Relative	8.535 ± 0.139	9.705 ± 0.330**	9.699 ± 0.060**	11.209		
Liver						
Absolute	1.371 ± 0.022	1.507 ± 0.046*	1.315 ± 0.038	1.207		
Relative	55.781 ± 0.773	59.865 ± 1.026*	56.442 ± 1.026	56.140		
Lung						
Absolute	0.204 ± 0.030	0.205 ± 0.021	0.182 ± 0.014	0.156		
Relative	8.306 ± 1.228	8.066 ± 0.607	7.801 ± 0.543	7.256		
Spleen						
Absolute	0.061 ± 0.001	0.081 ± 0.004**	0.059 ± 0.002	0.056		
Relative	2.503 ± 0.093	3.221 ± 0.083**	2.537 ± 0.048	2.605		
R. Testis						
Absolute	0.093 ± 0.003	0.100 ± 0.004	0.098 ± 0.004	0.110		
Relative	3.783 ± 0.170	3.952 ± 0.073	4.191 ± 0.150	5.116		
Thymus						
Absolute	0.049 ± 0.006	0.047 ± 0.003	0.022 ± 0.001**	0.018		
Relative	1.983 ± 0.225	1.845 ± 0.070	0.962 ± 0.057**	0.837		

TABLE B10
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 16-Day Dermal Study of Diazoaminobenzene

	Vehicle Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female						
n	5	5	5	5	5	2
Necropsy body wt	21.5 ± 0.8	21.8 ± 0.4	21.3 ± 0.4	18.3 ± 0.7**	16.6 ± 1.0**	17.4 ± 1.1**
Heart						
Absolute	0.115 ± 0.005	0.127 ± 0.006	0.129 ± 0.005	0.141 ± 0.004**	0.160 ± 0.007**	0.160 ± 0.001**
Relative	5.324 ± 0.100	5.804 ± 0.259	6.052 ± 0.198	7.714 ± 0.290**	9.727 ± 0.473**	9.202 ± 0.553**
R. Kidney						
Absolute	0.162 ± 0.004	0.180 ± 0.005	0.183 ± 0.004	0.190 ± 0.006*	0.217 ± 0.013**	0.206 ± 0.010**
Relative	7.541 ± 0.187	8.280 ± 0.218	8.604 ± 0.192	10.428 ± 0.343**	13.109 ± 0.601**	11.892 ± 1.298**
Liver						
Absolute	1.165 ± 0.040	1.345 ± 0.052	1.282 ± 0.040	1.077 ± 0.076	0.936 ± 0.75	1.021 ± 0.156
Relative	54.164 ± 0.618	61.635 ± 1.432*	60.281 ± 1.779	58.621 ± 1.959	56.296 ± 1.905	58.345 ± 5.277
Lung						
Absolute	0.220 ± 0.026	0.208 ± 0.015	0.176 ± 0.011	0.149 ± 0.009**	0.146 ± 0.006**	0.142 ± 0.009*
Relative	10.135 ± 0.880	9.522 ± 0.693	8.258 ± 0.507	8.141 ± 0.319	8.927 ± 0.548	8.134 ± 0.026
Spleen						
Absolute	0.078 ± 0.002	0.106 ± 0.003	0.093 ± 0.001	0.064 ± 0.007	0.051 ± 0.11	0.075 ± 0.34
Relative	3.620 ± 0.119	4.861 ± 0.123	4.395 ± 0.100	3.477 ± 0.231	3.036 ± 0.599	4.204 ± 1.688
Thymus						
Absolute	0.077 ± 0.004	0.060 ± 0.006*	0.051 ± 0.004**	0.024 ± 0.003**	0.014 ± 0.003**	0.026[b]
Relative	3.577 ± 0.247	2.764 ± 0.289*	2.389 ± 0.186**	1.283 ± 0.132**	0.807 ± 0.147**	1.405[b]

* Significantly different ($P \leq 0.05$) from the vehicle control group by Williams' or Dunnett's test
** $P \leq 0.01$
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] n=1; no standard error calculated
[c] No data available due to 100% mortality

TABLE B11
Hematology Data for Mice in the 16-Day Dermal Study of Diazoaminobenzene[a]

	Vehicle Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male						
n	5	4	5	1[b]	0[c]	0[c]
Hematocrit (%)	50.0 ± 1.6	47.0 ± 1.3	46.2 ± 0.6*	41.8		
Hemoglobin (g/dL)	17.0 ± 0.6	16.3 ± 0.3	16.5 ± 0.3	16.7		
Erythrocytes (10^6/μL)	10.58 ± 0.35	9.97 ± 0.26	9.80 ± 0.13	9.08		
Reticulocytes (10^6/μL)	0.16 ± 0.03	0.13 ± 0.02	0.25 ± 0.03	0.22		
Nucleated erythrocytes/ 100 leukocytes	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00		
Mean cell volume (fL)	47.2 ± 0.2	47.3 ± 0.3	47.0 ± 0.0	46.0		
Mean cell hemoglobin (pg)	16.1 ± 0.1	16.4 ± 0.3	16.8 ± 0.2*	18.4		
Mean cell hemoglobin concentration (g/dL)	34.0 ± 0.2	34.7 ± 0.7	35.7 ± 0.4*	40.0		
Platelets (10^3/μL)	742.6 ± 25.9	865.8 ± 53.6	832.4 ± 35.7	892.0		
Leukocytes (10^3/μL)	5.56 ± 0.42	5.65 ± 0.62	4.52 ± 0.28	5.60		
Segmented neutrophils (10^3/μL)	0.46 ± 0.07	0.38 ± 0.06	0.60 ± 0.10	2.18		
Bands (10^3/μL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00		
Lymphocytes (10^3/μL)	4.88 ± 0.39	5.19 ± 0.66	3.85 ± 0.27	3.19		
Monocytes (10^3/μL)	0.17 ± 0.06	0.07 ± 0.04	0.04 ± 0.02	0.17		
Basophils (10^3/μL)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000		
Eosinophils (10^3/μL)	0.05 ± 0.02	0.01 ± 0.01	0.03 ± 0.02	0.06		
Methemoglobin (% hemoglobin)	0.66 ± 0.05	2.45 ± 0.12*	5.92 ± 0.24**	10.00		
Heinz bodies (10^3/μL)	11 ± 3	27 ± 10	20 ± 3	9		

TABLE B11
Hematology Data for Mice in the 16-Day Dermal Study of Diazoaminobenzene

	Vehicle Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female						
n	4	4	5	5	5	2
Hematocrit (%)	46.5 ± 1.5	44.0 ± 0.3	43.2 ± 0.5	39.6 ± 0.8**	42.9 ± 2.5	40.9 ± 0.3
Hemoglobin (g/dL)	16.5 ± 0.4	15.5 ± 0.1	15.5 ± 0.1	16.4 ± 0.3	18.5 ± 1.1	17.5 ± 0.6
Erythrocytes (10^6/μL)	9.65 ± 0.32	9.10 ± 0.09	8.77 ± 0.08	8.15 ± 0.13**	8.94 ± 0.52	8.35 ± 0.22
Reticulocytes (10^6/μL)	0.13 ± 0.02	0.20 ± 0.02*	0.35 ± 0.05**	0.41 ± 0.07**	0.41 ± 0.07**	0.39 ± 0.05*
Nucleated erythrocytes/ 100 leukocytes	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Mean cell volume (fL)	48.5 ± 0.3	48.5 ± 0.3	49.4 ± 0.2	48.8 ± 0.5	48.0 ± 0.3	49.0 ± 2.0
Mean cell hemoglobin (pg)	17.1 ± 0.3	17.0 ± 0.1	17.7 ± 0.1	20.2 ± 0.3**	20.7 ± 0.1**	20.9 ± 0.1**
Mean cell hemoglobin concentration (g/dL)	35.5 ± 0.5	35.2 ± 0.3	36.0 ± 0.1	41.6 ± 0.7*	43.1 ± 0.2**	42.7 ± 1.6*
Platelets (10^3/μL)	724.0 ± 63.2[d]	648.3 ± 36.5	639.0 ± 26.7	879.6 ± 53.6	1,049.0 ± 113.2	1,000.5 ± 46.5
Leukocytes (10^3/μL)	7.70 ± 0.40	6.23 ± 0.16	7.46 ± 0.25	6.76 ± 0.42	11.28 ± 2.14	14.75 ± 1.55
Segmented neutrophils (10^3/μL)	0.63 ± 0.19	0.70 ± 0.19	0.63 ± 0.08	1.19 ± 0.15	3.88 ± 1.28*	2.52 ± 0.41*
Bands (10^3/μL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (10^3/μL)	6.76 ± 0.29	5.34 ± 0.06	6.61 ± 0.15	5.33 ± 0.38	7.23 ± 1.32	11.96 ± 1.40
Monocytes (10^3/μL)	0.25 ± 0.01	0.17 ± 0.04	0.18 ± 0.07	0.22 ± 0.06	0.09 ± 0.06	0.20 ± 0.20
Basophils (10^3/μL)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/μL)	0.06 ± 0.02	0.02 ± 0.02	0.03 ± 0.03	0.03 ± 0.02	0.08 ± 0.03	0.07 ± 0.07
Methemoglobin (% hemoglobin)	1.05 ± 0.59	1.55 ± 0.13	4.68 ± 0.19**	10.92 ± 0.74**	15.46 ± 0.47**	19.00 ± 3.50**
Heinz bodies (10^3/μL)	5 ± 5	14 ± 3	23 ± 7	20 ± 2	39 ± 15	17 ± 17

* Significantly different ($P \leq 0.05$) from the vehicle control group by Shirley's test
** Significantly different ($P \leq 0.01$) from the vehicle control group by Dunn's or Shirley's test
[a] Data are given as mean ± standard error. Statistical tests were performed on unrounded data.
[b] No standard error calculated
[c] No data available due to 100% mortality
[d] n=3

TABLE B12
Incidences of Selected Nonneoplastic Lesions in Mice in the 16-Day Dermal Study of Diazoaminobenzene

	Vehicle Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Male						
Skin, Site of Application[a]	5	5	5	5	5	5
Epidermis, Hyperplasia[b]	0	5** (2.0)[c]	5** (2.0)	5** (1.8)	5** (1.4)	5** (1.0)
Hair Follicle, Hyperplasia	0	5** (1.2)	5** (1.6)	5** (1.0)	5** (1.0)	2 (2.0)
Inflammation	0	5** (1.6)	5** (1.8)	5** (1.2)	5** (1.4)	5** (1.4)
Thymus	5	5	5	5	5	5
Atrophy	0	0	0	5** (3.8)	5** (4.0)	5** (4.0)
Lymph Node, Mandibular	5	5	5	5	4	5
Atrophy	0	0	0	3 (2.0)	4** (2.8)	5** (2.8)
Lymph Node, Mesenteric	4	5	4	5	5	4
Atrophy	0	0	0	2 (2.0)	3 (2.3)	4* (2.5)
Spleen	5	5	5	5	5	5
Hematopoietic Cell Proliferation	0	5** (2.0)	5** (2.0)	2 (1.5)	1 (2.0)	0
Lymphoid Follicle, Atrophy	0	0	0	1 (2.0)	2 (2.5)	5** (2.4)
Heart	5	5	5	5	5	5
Atrium, Thrombosis	0	0	0	2 (2.0)	5** (3.2)	5** (3.0)
Kidney	5	5	5	5	5	5
Bilateral, Cortex, Renal Tubule, Necrosis	0	0	0	0	5** (2.0)	5** (3.0)
Liver	5	5	5	5	5	5
Necrosis, Focal	0	0	0	0	3 (1.3)	5** (2.6)

TABLE B12
Incidences of Selected Nonneoplastic Lesions in Mice in the 16-Day Dermal Study of Diazoaminobenzene

	Vehicle Control	12.5 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
Female						
Skin, Site of Application	5	5	5	5	5	5
Epidermis, Hyperplasia	0	5** (1.2)	5** (3.0)	5** (2.6)	5** (2.4)	5** (2.0)
Hair Follicle, Hyperplasia	0	5** (1.6)	5** (3.2)	5** (3.0)	5** (3.0)	5** (3.0)
Inflammation	0	5** (2.0)	5** (1.2)	5** (2.4)	5** (2.2)	5** (2.2)
Ulcer	0	0	1 (1.0)	2 (1.5)	4* (1.3)	2 (1.0)
Thymus	5	5	5	5	5	4
Atrophy	0	0	0	4* (3.3)	5** (3.6)	3* (4.0)
Lymph Node, Mandibular	4	5	5	5	4	4
Atrophy	0	0	0	0	0	3 (2.0)
Lymph Node, Mesenteric	5	5	5	5	3	4
Atrophy	0	0	0	0	1 (2.0)	1 (3.0)
Spleen	5	5	5	5	5	5
Hematopoietic Cell Proliferation	0	5** (2.0)	5** (2.0)	5** (1.4)	2 (2.5)	2 (1.5)
Lymphoid Follicle, Atrophy	0	0	0	0	3 (2.7)	2 (2.5)
Heart	5	5	5	5	5	5
Atrium, Thrombosis	0	0	0	0	1 (1.0)	2 (2.5)
Kidney	5	5	5	5	5	5
Bilateral, Cortex, Renal Tubule, Necrosis	0	0	0	0	4* (2.5)	1 (3.0)
Bilateral, Cortex, Renal Tubule, Dilatation	0	0	0	0	0	4* (1.5)
Liver	5	5	5	5	5	5
Necrosis, Focal	0	0	0	0	0	2 (2.0)

* Significantly different ($P \leq 0.05$) from the vehicle control group by the Fisher exact test
** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked