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.

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NTP Report
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(CAS No. 136-35-6)

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

September 2002

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National Institutes of Health

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CONTENTS

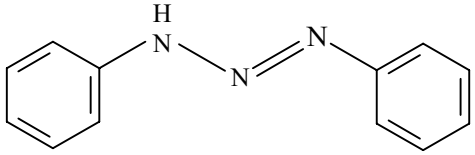
SUMMARY

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

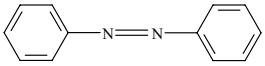
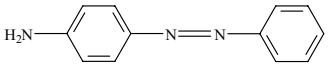
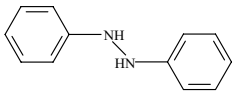
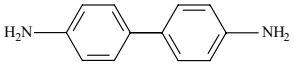
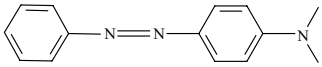
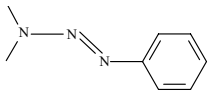
Structure	Classification	Mutagenicity Data
Azobenzene **CAS Number: 103-33-3** 	Carcinogenic in rats[a] IARC: 3[b]	*Salmonella* (+)[c] MN[d] Bone marrow (+)
***p*-Aminoazobenzene** **CAS Number: 60-09-3** 	Carcinogenic in neonate and male mice[e] IARC: 2B[b]	*Salmonella* (+)[f] MN[g] Bone marrow (+)
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.

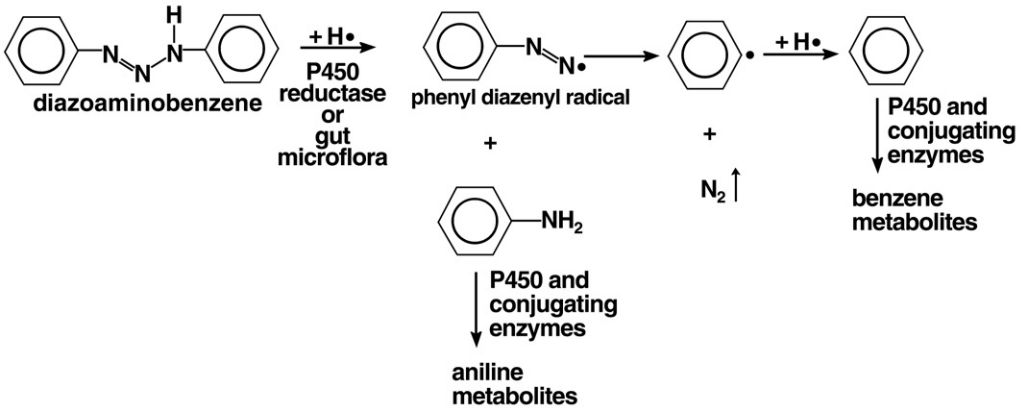


FIGURE 1
Proposed Pathway for the Metabolism of Diazoaminobenzene (Mathews and De Costa, 1999)

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

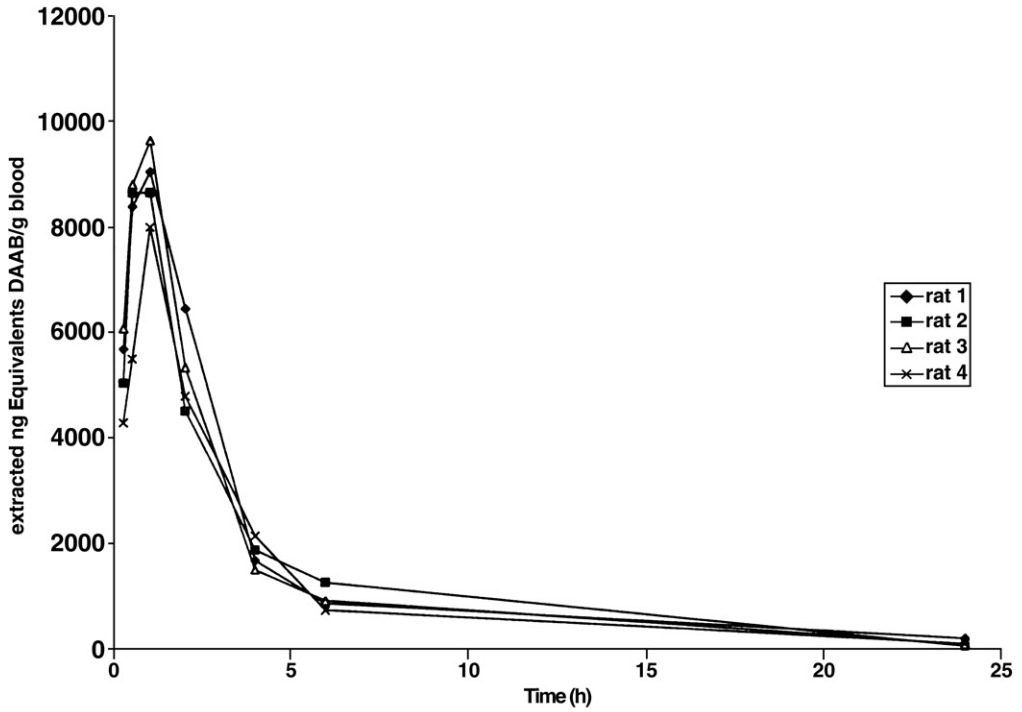
[a] Four mice were examined per dose group; data are presented as percentage of dose (mean ± standard deviation).

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

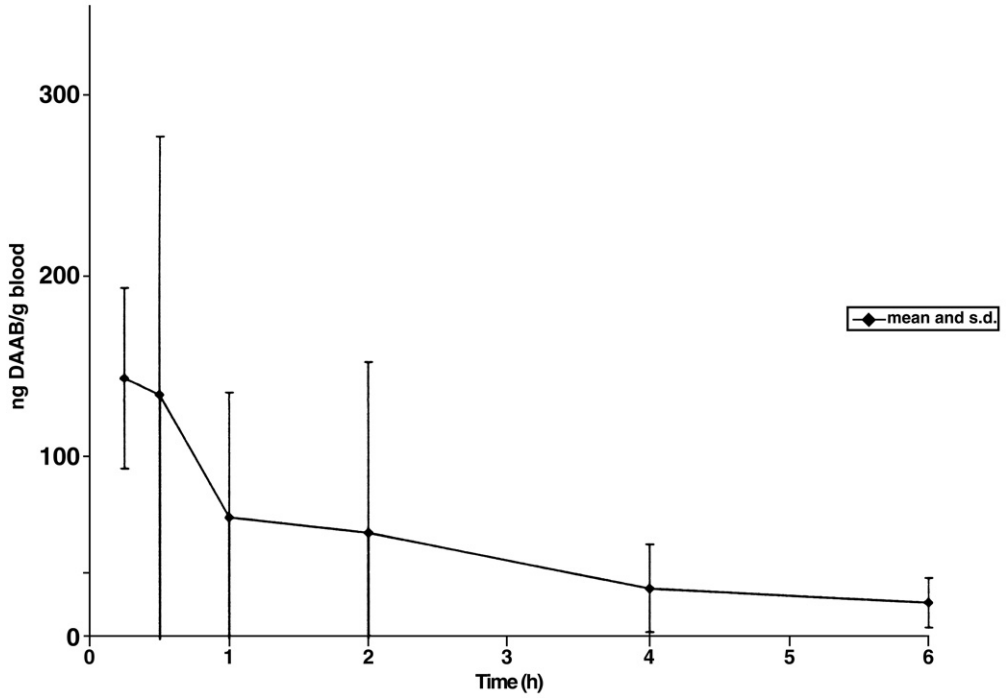
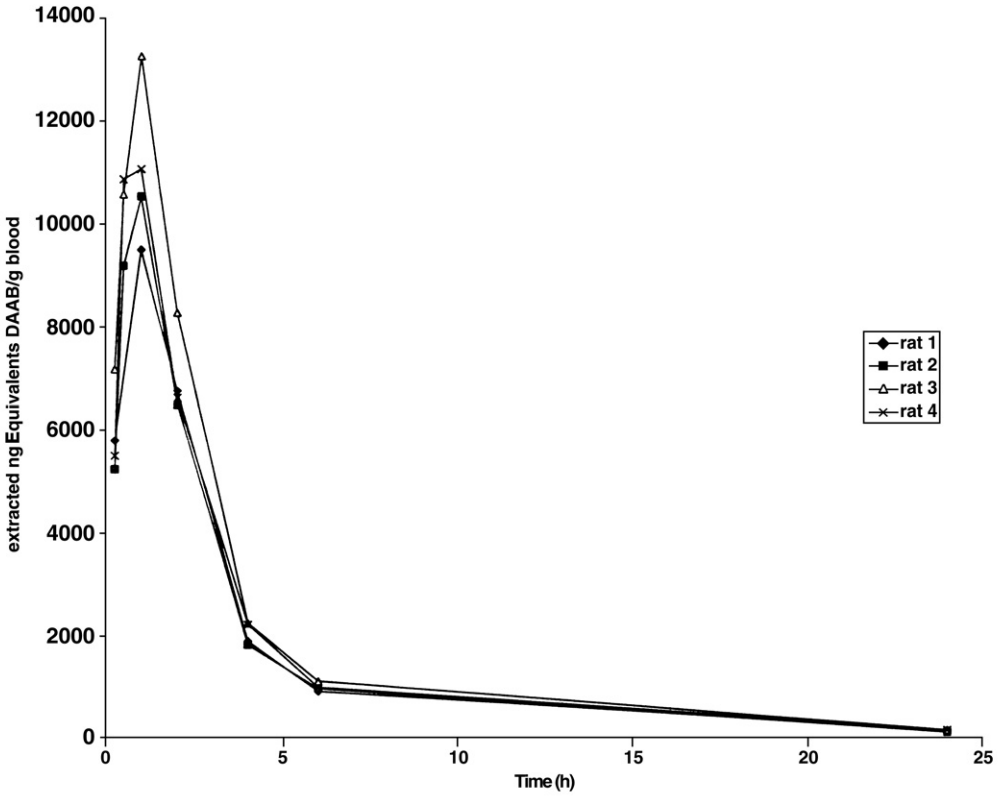


FIGURE A1
Concentration of (a) Total Diazoaminobenzene-Derived Compounds and (b) Diazoaminobenzene in the Blood of Male F344/N Rats after a Single Gavage Dose of 20 mg/kg [^{14}C]-Diazoaminobenzene

a. Total Radioactivity



b. Diazoaminobenzene

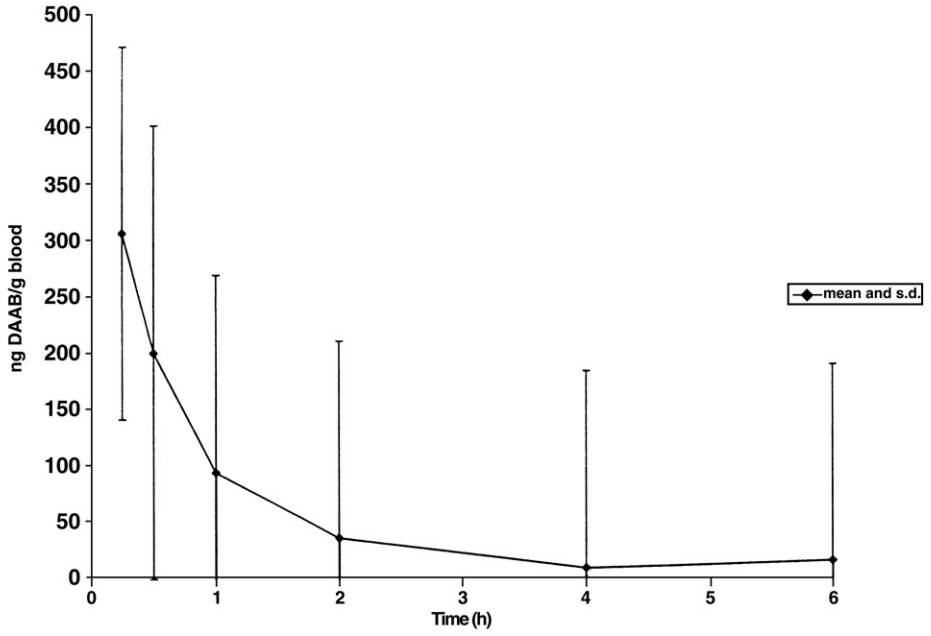
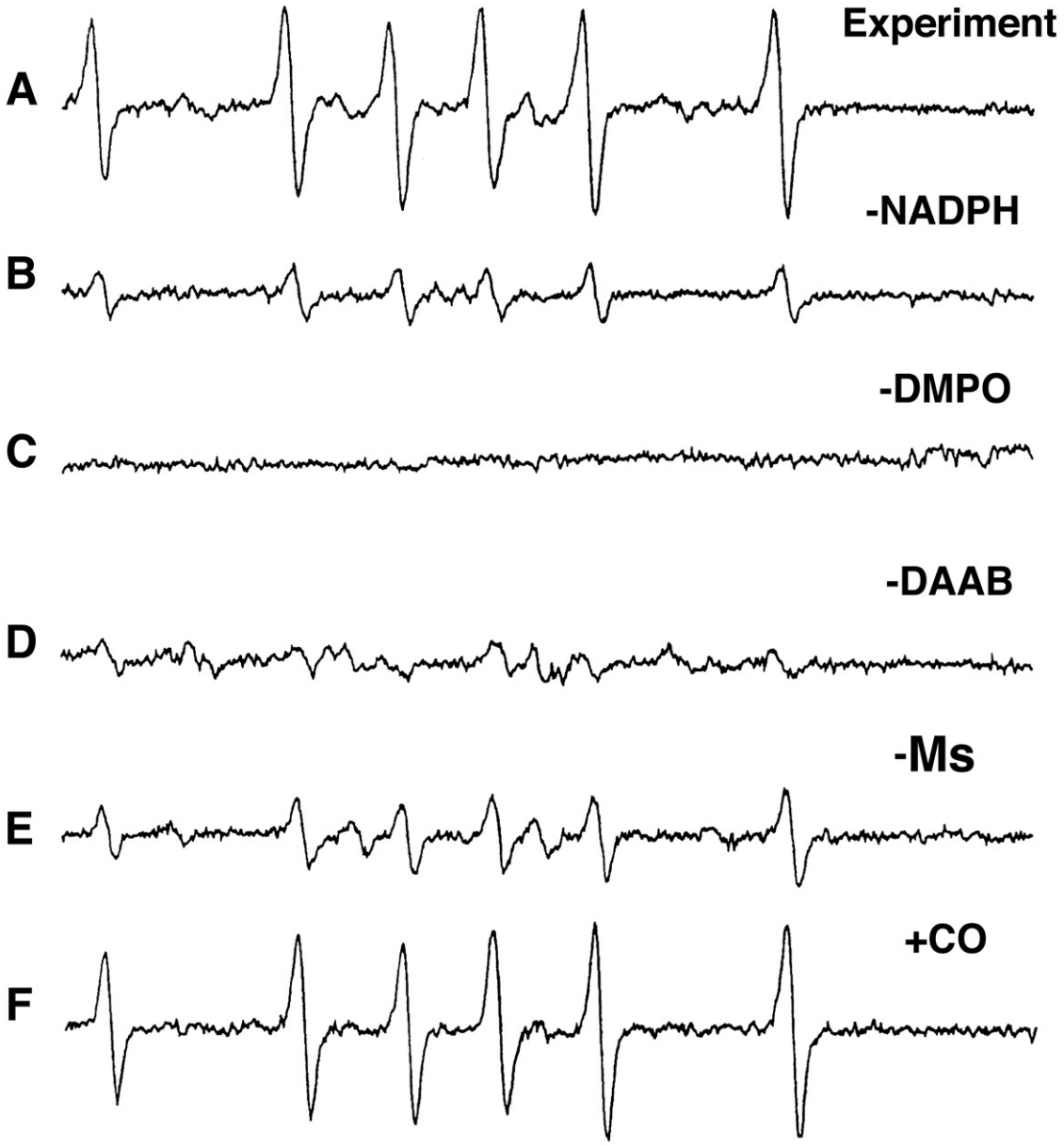
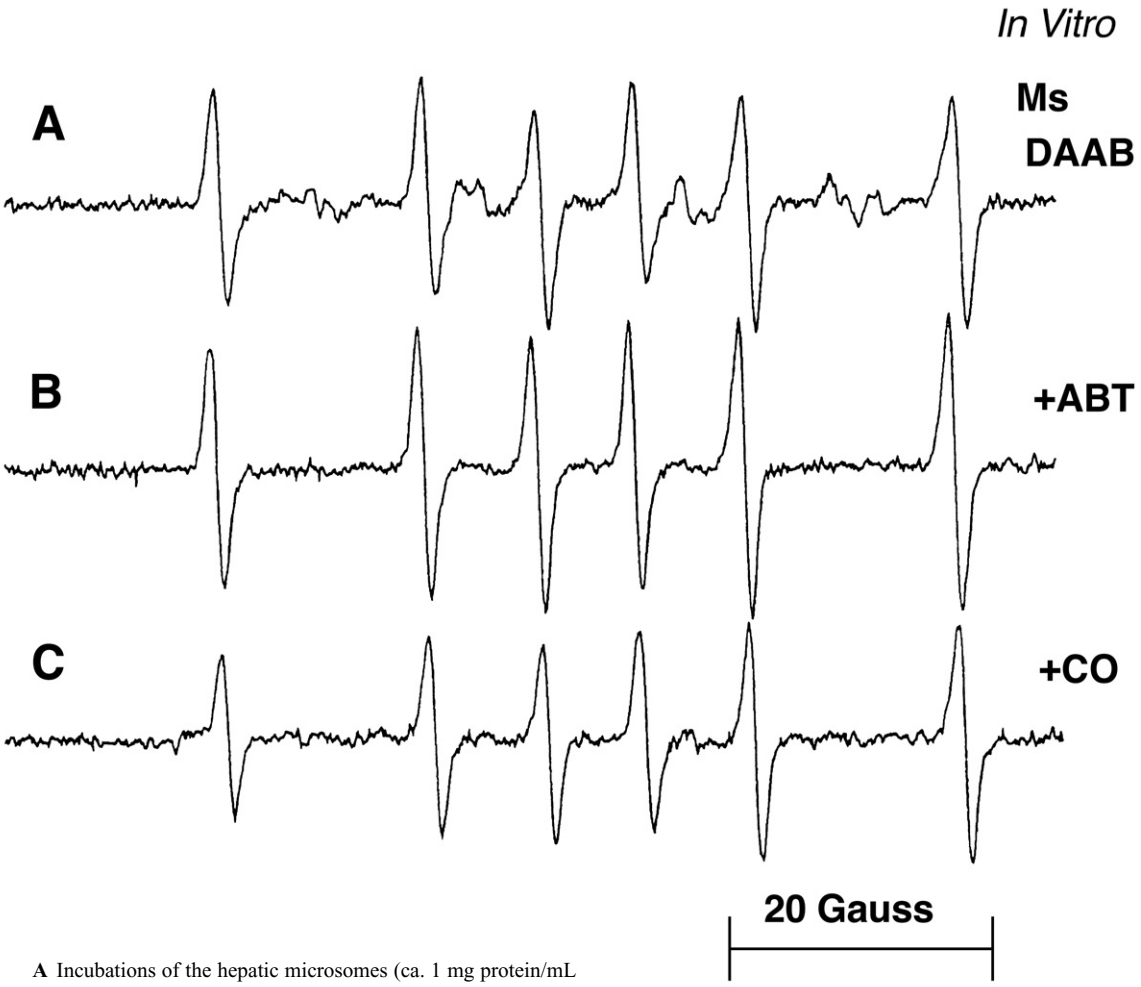


FIGURE A2
Concentration of (a) Total Diazoaminobenzene-Derived Compounds and (b) Diazoaminobenzene in the Blood of Female F344/N Rats after a Single Gavage Dose of 20 mg/kg [^{14}C]-Diazoaminobenzene



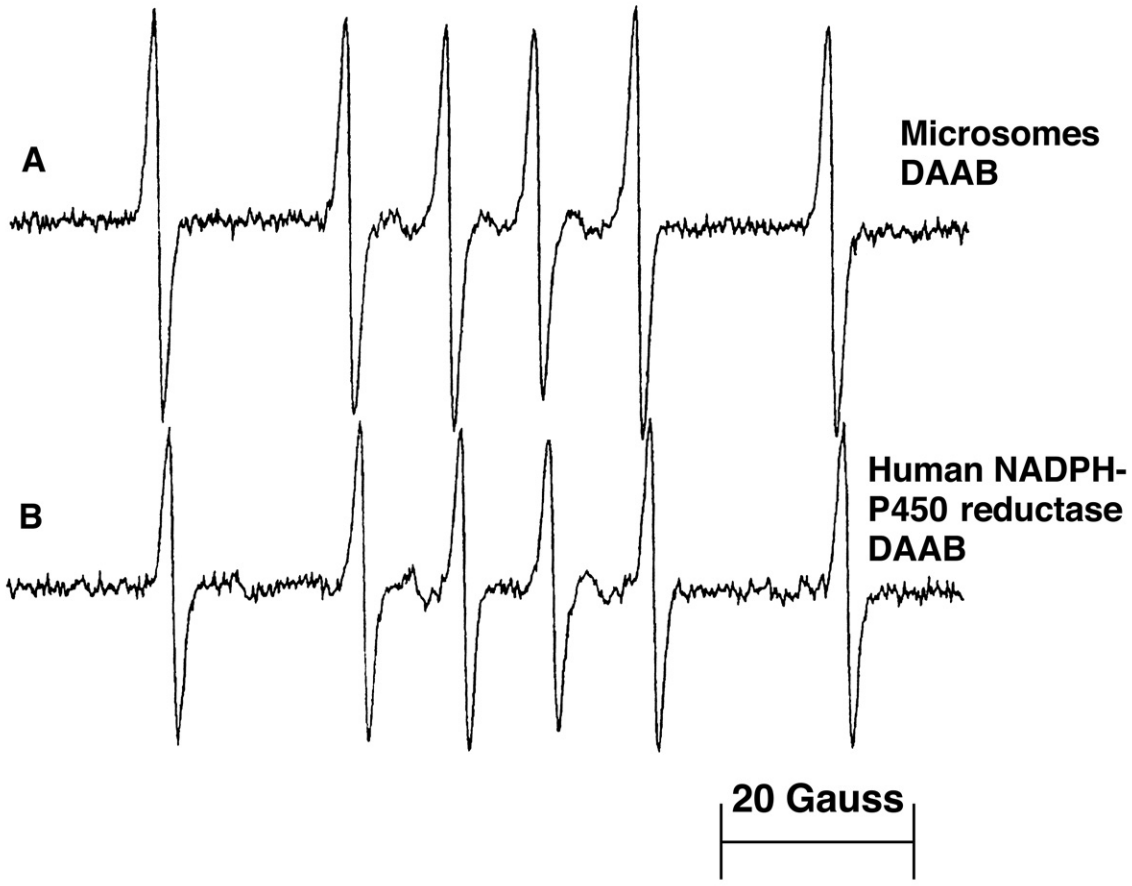
A Incubations of the hepatic microsomes (ca. 1 mg protein/mL 100 mM phosphate buffer) with diazoaminobenzene (25 mM final concentration), DMPO (200 mM), and NADPH (1 mM)
B All components of A except NADPH
C All components of A except DMPO
D All components of A except diazoaminobenzene
E All components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



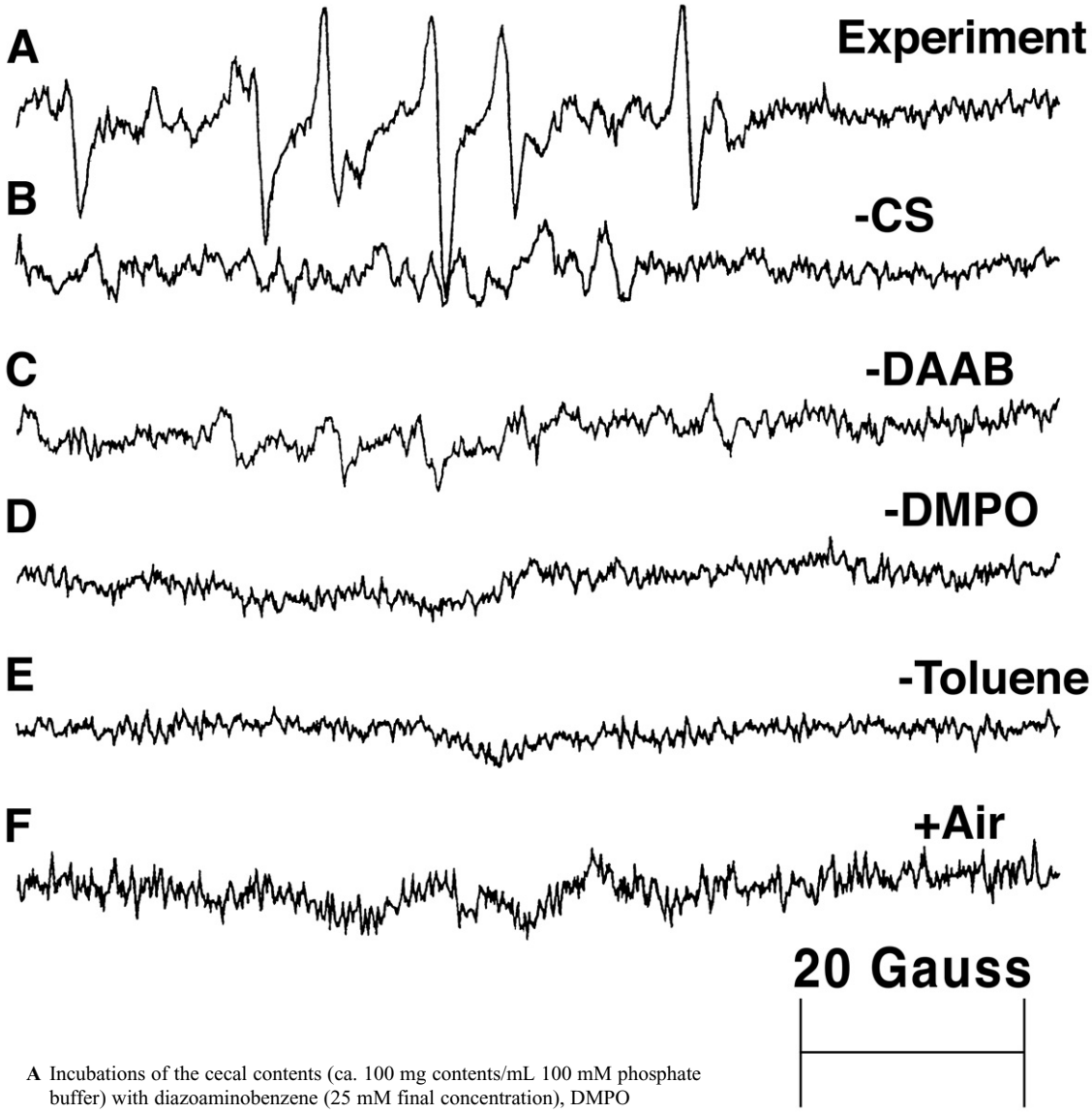
A Incubations of the hepatic microsomes (ca. 1 mg protein/mL 100 mM phosphate buffer) with diazoaminobenzene (25 mM final concentration), DMPO (200 mM), and NADPH (1 mM)
B All components of A, microsomes were preincubated for 15 minutes with NADPH and 1-aminobenzotriazole (ABT) prior to incubation with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



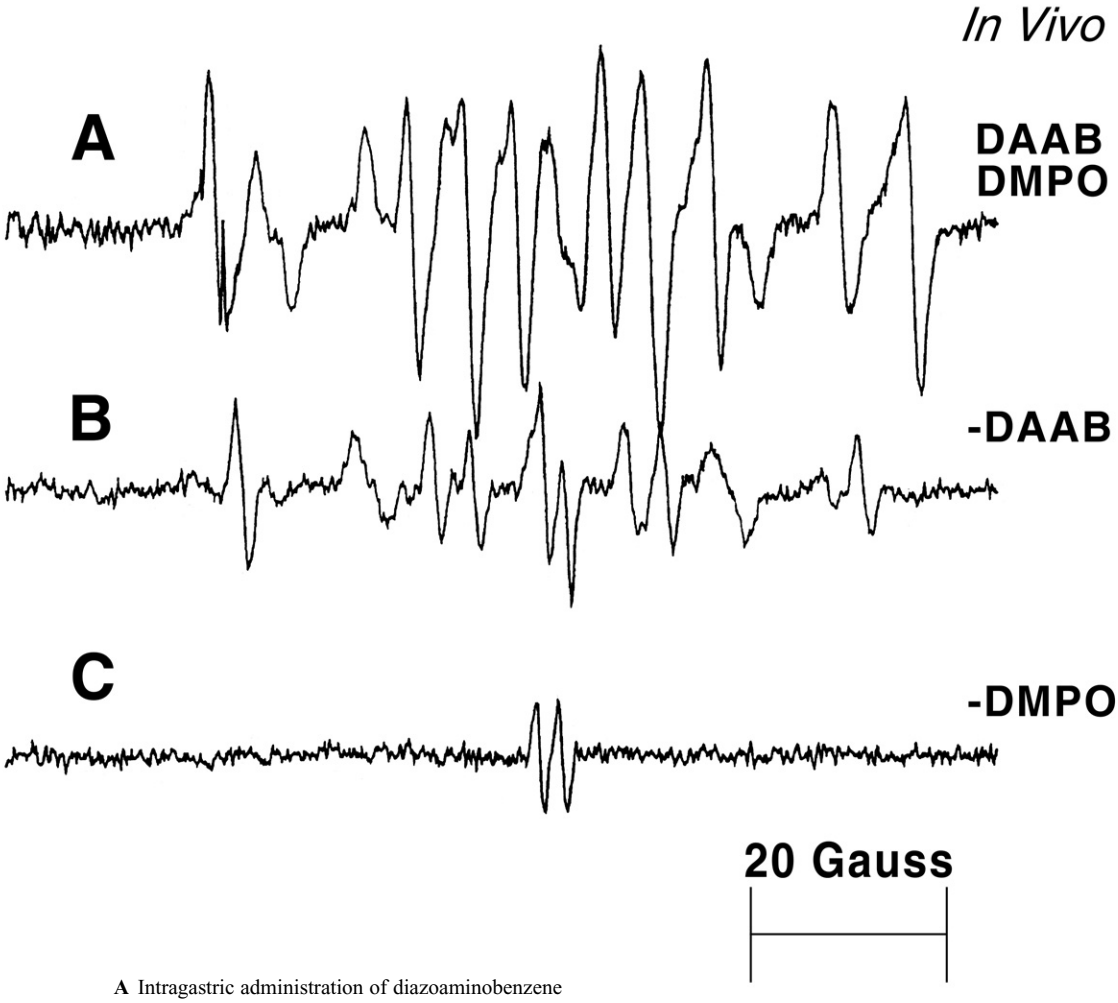
A Incubations of the hepatic microsomes (ca. 1mg protein/mL 100 mM phosphate buffer) with diazoaminobenzene (25 mM final concentration), DMPO (200 mM), and NADPH (1 mM)
B All components of **A** except human P450 reductase (2.4 pmol/mL 100 mM phosphate buffer) were substituted for hepatic microsomes.

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



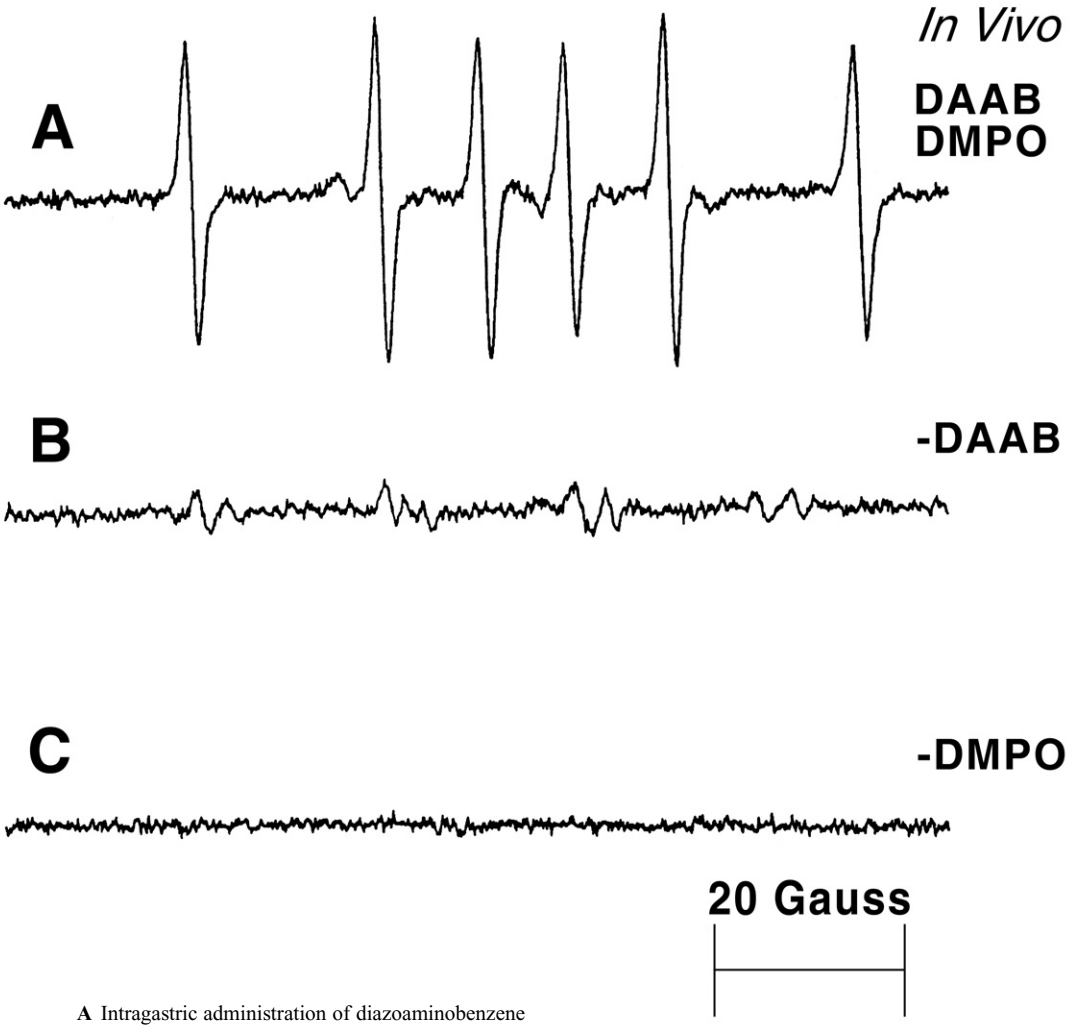
A Incubations of the cecal contents (ca. 100 mg contents/mL 100 mM phosphate buffer) with diazoaminobenzene (25 mM final concentration), DMPO (200 mM), under nitrogen gas and extraction of the incubation mixture with nitrogen-sparged toluene
B All components of A except cecal contents
C All components 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.

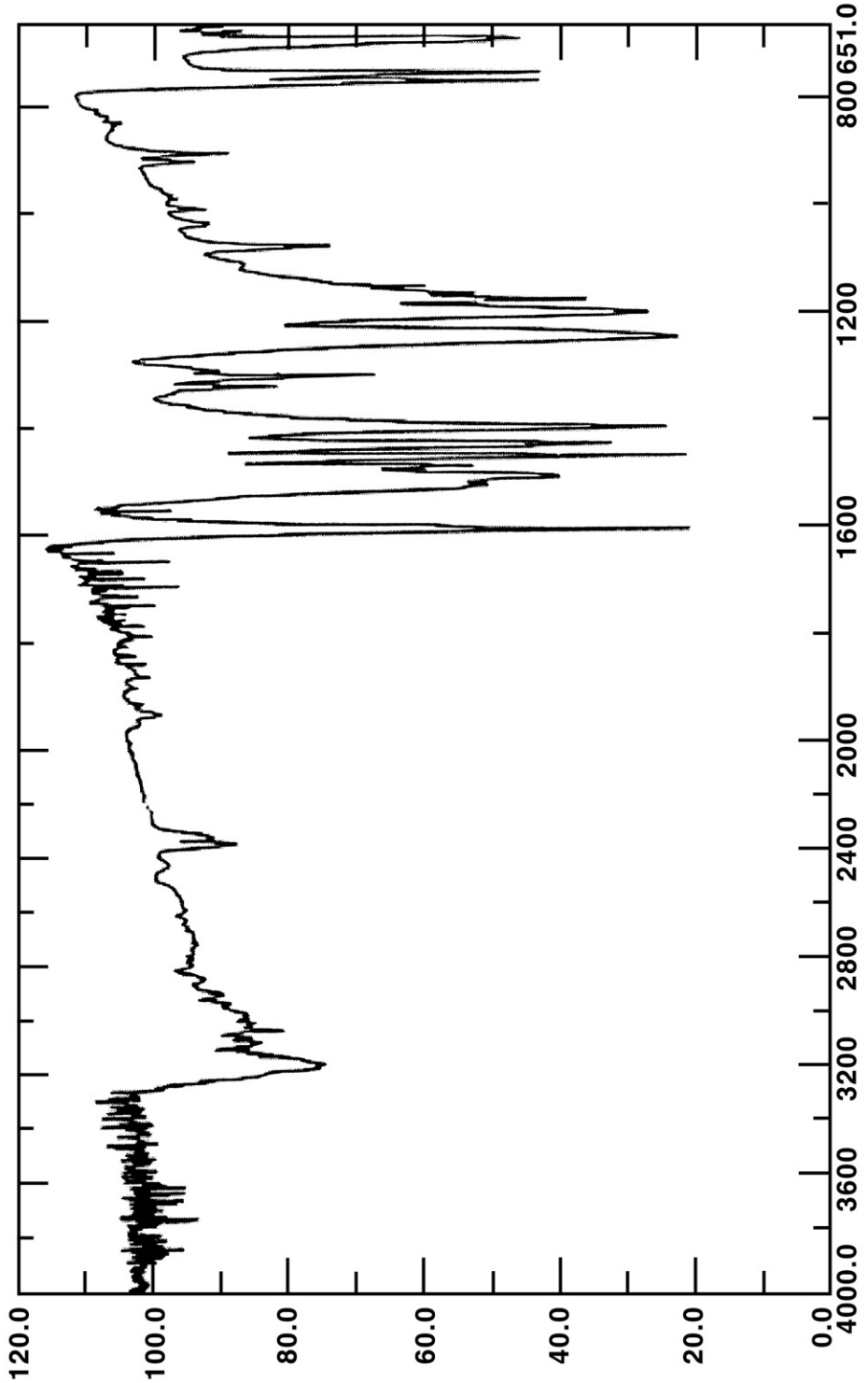


FIGURE B1
Infrared Absorption Spectrum of Diazoaminobenzene

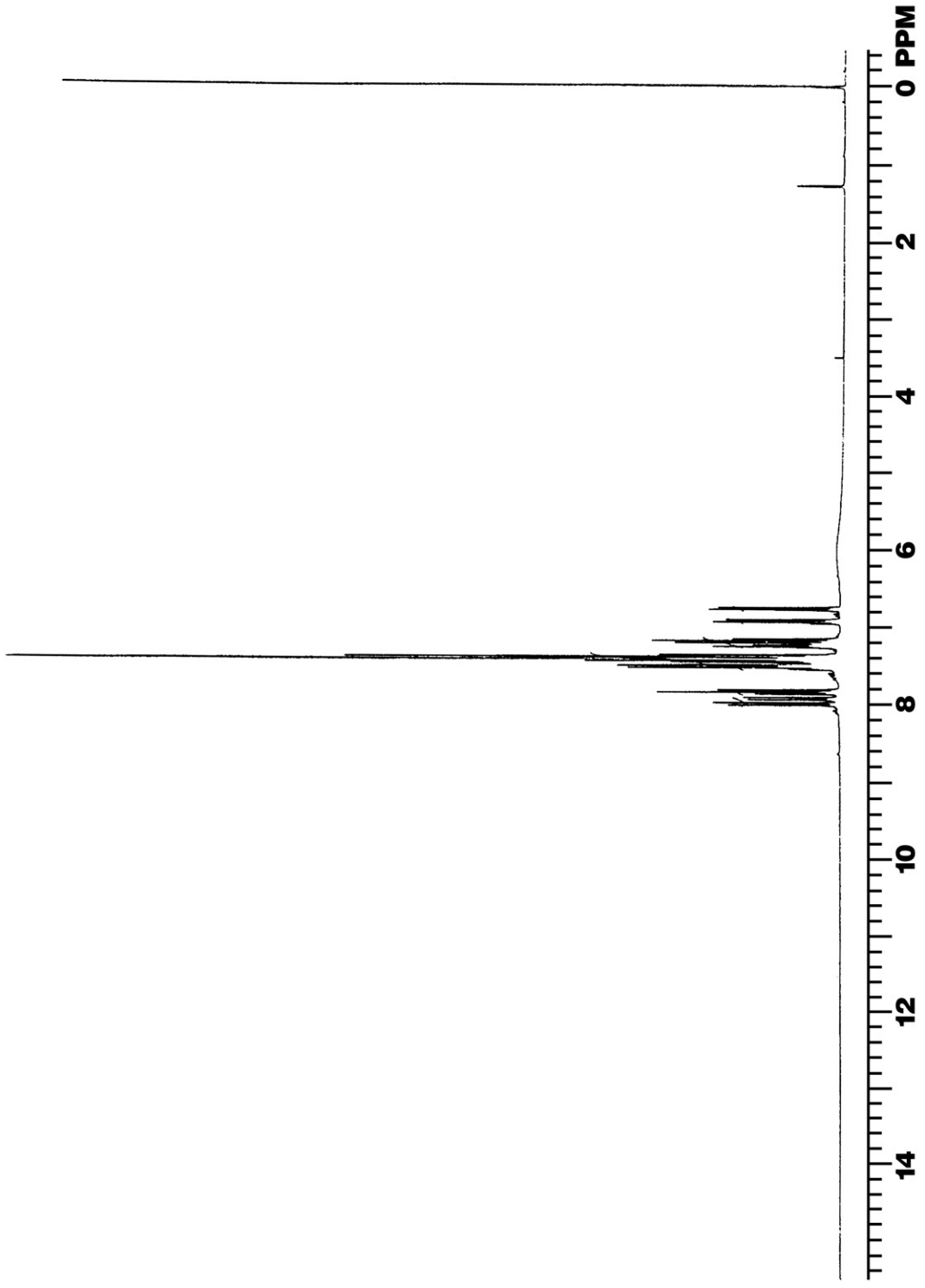


FIGURE B2
Nuclear Magnetic Resonance Spectrum of Diazoaminobenzene

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

APPENDIX C
GENETIC TOXICOLOGY

GENETIC TOXICOLOGY

SALMONELLA TYPHIMURIUM MUTAGENICITY TEST PROTOCOL

Testing was performed as reported by Zeiger *et al.* (1987). Diazoaminobenzene was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537 either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and five doses of diazoaminobenzene. The high dose was limited by toxicity. All positive trials were repeated under the conditions that elicited the positive response.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

MOUSE BONE MARROW MICRONUCLEUS TEST PROTOCOL

Preliminary range-finding studies were performed with diazoaminobenzene and doses were limited by toxicity. Benzene was tested along with diazoaminobenzene because it is a major component of the diazoaminobenzene molecule, and benzene is a known inducer of micronuclei. Thus, benzene served as a kind of standard against which the activity of diazoaminobenzene could be measured in this assay. In the first trial, benzene doses were chosen based on prior knowledge of the chemical's activity in micronucleus tests. High doses of benzene, expected to induce large numbers of micronucleated cells, were tested to demonstrate the ability of the test system to detect benzene-induced micronuclei in erythrocytes. The second trial was conducted with lower doses of benzene, equal to benzene's contribution by weight to the diazoaminobenzene molecule. Male B6C3F_1 mice, five per treatment group, were administered diazoaminobenzene or benzene twice at 0 and 24 hours by corn oil gavage. Vehicle control animals received corn oil only. The positive control animals received intraperitoneal injections of cyclophosphamide. The animals were killed 24 hours after the second treatment and blood smears were prepared from bone marrow cells obtained from the femurs. Air-dried smears were fixed and stained with acridine orange; 2,000 polychromatic erythrocytes (PCEs) were scored for the frequency of micronucleated cells in each of four or five animals per dose group. In addition, the percentage of PCEs among 200 total erythrocytes was determined as a measure of chemical-induced bone marrow toxicity.

The results were tabulated as the mean of the pooled results from all animals within a treatment group, plus or minus the standard error of the mean. The frequency of micronucleated cells among PCEs was analyzed by a statistical software package that tested for increasing trend over dose groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each dosed group and the control group (ILS, 1990). In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single dose group is less than or equal to 0.025 divided by the number of dose groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, reproducibility of any effects observed, and the magnitudes of those effects.

Evaluation Protocol

These are the basic guidelines for arriving at an overall assay result for assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple aliquots of a chemical were tested in the same assay, and different results were obtained among aliquots and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the *in vitro* assays have another variable that must be considered in arriving at an overall test result. *In vitro* assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The results presented in the Abstract of this Toxicity Report represent a scientific judgement of the overall evidence for activity of the chemical in an assay.

Results

Diazoaminobenzene, tested over a concentration range of 0.1 to 100 µg/plate, was mutagenic in *S. typhimurium* strains TA98, TA100, and TA1537 when testing occurred in the presence of induced rat or hamster liver S9 enzymes; no activity was noted in strain TA1535, with or without S9 (Table C1; Zeiger *et al.*, 1987). In the *in vivo* mouse bone marrow micronucleus assays conducted with diazoaminobenzene and benzene, diazoaminobenzene was found to be much more toxic than benzene, and therefore, the highest dose of diazoaminobenzene that could be tested for induction of micronuclei was 100 mg/kg. At that dose level, one out of five test animals died. In contrast, benzene was tested at doses up to 2,500 mg/kg with no lethality. Both diazoaminobenzene and benzene induced highly significant increases in micronucleated PCEs at all doses tested (Table C2; Ress *et al.*, 2002). The response seen with diazoaminobenzene was remarkable in that the doses tested were 10- and 20-fold lower than those of benzene in trial 1, yet the responses produced by these doses of diazoaminobenzene were highly significant. Increases in micronucleated cells of the magnitude seen with diazoaminobenzene and with benzene are produced by very few chemicals.

TABLE C1
Mutagenicity of Diazoaminobenzene in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Revertants/Plate[b] –S9	+10% hamster S9 Trial 1	+10% hamster S9 Trial 2	+10% rat S9 Trial 1	+10% rat S9 Trial 2
TA100	0	114 ± 5.8	125 ± 11.9	136 ± 10.7	122 ± 8.8	157 ± 5.3
	0.1	109 ± 8.8				
	0.3	113 ± 6.1		140 ± 4.5		146 ± 3.6
	1.0	112 ± 7.2	150 ± 20.0	193 ± 18.9	185 ± 7.5	191 ± 12.2
	3.0	109 ± 2.3	187 ± 17.0	213 ± 12.4	243 ± 15.9	262 ± 5.2
	10.0	65 ± 6.4	226 ± 6.1	276 ± 4.8	321 ± 7.6	316 ± 0.3
	33.0		259 ± 7.4	294 ± 14.8	353 ± 10.4	387 ± 13.7
	100.0		242 ± 29.4		227 ± 16.7	
Trial summary		Negative	Positive	Positive	Positive	Positive
Positive control[c]		301 ± 4.0	1,921 ± 50.9	1,346 ± 89.1	510 ± 10.1	474 ± 11.1
TA1535	0	17 ± 1.8	8 ± 0.6		9 ± 2.2	
	0.1	21 ± 2.0				
	0.3	23 ± 2.7				
	1.0	28 ± 1.7	6 ± 0.6		13 ± 1.0	
	3.0	24 ± 5.4	8 ± 0.0		12 ± 0.3	
	10.0	13 ± 3.0	10 ± 2.0		11 ± 2.3	
	33.0		7 ± 1.5		10 ± 2.6	
	100.0		8 ± 0.3		10 ± 1.8	
Trial summary		Negative	Negative		Negative	
Positive control		311 ± 14.7	388 ± 28.7		133 ± 11.7	
TA1537	0	6 ± 1.9	7 ± 0.3	5 ± 0.7	4 ± 1.2	6 ± 0.9
	0.1	7 ± 2.6				
	0.3	6 ± 0.3		13 ± 0.7		25 ± 1.9
	1.0	5 ± 0.9	24 ± 4.3	21 ± 2.0	39 ± 6.5	32 ± 3.2
	3.0	5 ± 1.2	48 ± 3.2	42 ± 4.9	97 ± 9.8	85 ± 6.1
	10.0	5 ± 0.9	76 ± 15.4	69 ± 7.5	165 ± 15.6	125 ± 14.5
	33.0		126 ± 7.6	107 ± 20.4	189 ± 9.6	124 ± 10.0
	100.0		108 ± 7.4		130 ± 14.4	
Trial summary		Negative	Positive	Positive	Positive	Positive
Positive control		201 ± 24.8	363 ± 4.0	483 ± 19.3	119 ± 10.5	180 ± 7.3
TA98	0	17 ± 1.7	24 ± 4.9	19 ± 0.3	17 ± 2.0	24 ± 2.3
	0.1	16 ± 2.0				
	0.3	16 ± 2.0		29 ± 5.5		25 ± 3.3
	1.0	13 ± 0.7	46 ± 2.2	32 ± 7.0	55 ± 0.7	45 ± 2.9
	3.0	16 ± 2.6	59 ± 5.5	73 ± 5.6	130 ± 15.1	100 ± 2.3
	10.0	12 ± 1.9	101 ± 11.0	89 ± 3.5	151 ± 1.8	133 ± 4.4
	33.0		123 ± 8.4	114 ± 4.5	158 ± 4.5	137 ± 17.2
	100.0		89 ± 8.2		92 ± 6.3	
Trial summary		Negative	Positive	Positive	Positive	Positive
Positive control		653 ± 29.5	1,089 ± 27.5	1,003 ± 30.8	365 ± 39.1	264 ± 19.6

[a] Study was performed at SRI International. The detailed protocol and these data are presented by Zeiger *et al*. (1987). 0 µg/plate was the solvent control.

[b] Revertants are presented as mean ± standard error from three plates.

[c] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA1537), and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE C2
Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Mice Treated with Diazoaminobenzene or Benzene by Gavage[a]

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	P Value[c]	PCEs (%)
Trial 1					
Corn oil[d]	0	5	0.40 ± 0.10		68.0
Diazoaminobenzene	50	5	6.30 ± 0.20	0.0000	55.4
	100	4	13.00 ± 1.14	0.0000	56.8
			P<0.001[e]		
Benzene	1,000	5	18.80 ± 1.85	0.0000	47.5
	1,500	5	23.10 ± 1.76	0.0000	47.6
	2,000	5	22.10 ± 1.45	0.0000	37.9
	2,250	5	24.20 ± 2.28	0.0000	40.9
	2,500	5	30.10 ± 2.86	0.0000	46.5
			P<0.001		
Cyclophosphamide[f]	20	5	13.30 ± 0.72	0.0000	62.7
Trial 2					
Corn oil	0	5	0.70 ± 0.12		57.0
Diazoaminobenzene	25	5	2.10 ± 0.29	0.0041	61.2
	50	5	5.00 ± 0.79	0.0000	60.4
	100	5	9.00 ± 1.11	0.0000	63.4
			P=0.000		
Benzene	10	5	2.40 ± 0.19	0.0011	60.6
	20	5	2.50 ± 0.74	0.0007	55.2
	40	5	6.30 ± 0.94	0.0000	63.9
			P=0.000		
Cyclophosphamide	20	5	17.90 ± 1.16	0.0000	57.3

[a] Study was performed at SITEK Research Laboratories, Inc. These data are presented by Ress *et al*. (2002). PCE=polychromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the vehicle control group. Dosed group values for trial 1 are significant at P≤0.013 (diazoaminobenzene) or P≤0.005 (benzene); dosed group values for trial 2 are significant at P≤0.008; positive control values are significant at P≤0.05 (ILS, 1990).
[d] Vehicle control; a single vehicle control group was used for both test chemicals.
[e] Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed Cochran-Armitage trend test; significant at P≤0.025 (ILS, 1990).
[f] Positive control; a single positive control group was used for both test chemicals.